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REVIEW ARTICLE

Comparative Metabolism of Free-living *Bodo saltans* and Parasitic Trypanosomatids

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ABSTRACT

Comparison of the genomes of free-living *Bodo saltans* and those of parasitic trypanosomatids reveals that the transition from a free-living to a parasitic life style has resulted in the loss of approximately 50% of protein-coding genes. Despite this dramatic reduction in genome size, *B. saltans* and trypanosomatids still share a significant number of common metabolic traits: glycosomes; a unique set of the pyrimidine biosynthetic pathway genes; an ATP-PFK which is homologous to the bacterial PP_i-PFKs rather than to the canonical eukaryotic ATP-PFKs; an alternative oxidase; three phosphoglycerate kinases and two GAPDH isoenzymes; a pyruvate kinase regulated by fructose-2,6-bisphosphate; trypanothione as a substitute for glutathione; synthesis of fatty acids via a unique set of elongase enzymes; and a mitochondrial acetate:succinate coenzyme A transferase. *B. saltans* has lost the capacity to synthesize ubiquinone. Among genes that are present in *B. saltans* and lost in all trypanosomatids are those involved in the degradation of mureine, tryptophan and lysine. Novel acquisitions of trypanosomatids are components of pentose sugar metabolism, pteridine reductase and bromodomain-factor proteins. In addition, only the subfamily Leishmaniinae has acquired a gene for catalase and the capacity to convert diaminopimelic acid to lysine.

KINETOPLASTIDS (Excavata, Euglenozoa, Kinetoplastea), a widespread and important group of single-celled eukaryotes, is characterized by a number of unusual features, the most prominent being the compartmentalized glycolysis, polycistronic transcription and extensive *trans*-splicing, as well as a large mitochondrial genome termed kinetoplast (k) DNA (Gunzl et al. 1997; Povelones 2014). The class Kinetoplastea is subdivided into subclass Prokinetoplastina, a small group of species with giant kDNA, and subclass Metakinetoplastina, which brings together an absolute majority of kinetoplastid flagellates (Moreira et al. 2004). The latter subclass is comprised of four orders: Eubodonida (representative genus *Bodo*), Neobodonida (*Cruzella*), Parabodonida (*Trypanoplasma*), and Trypanosomatida (*Trypanosoma*). Only trypanosomatids are exclusively parasitic, uniting medically and veterinary important *Trypanosoma* and *Leishmania* species, plant pathogens of the genus *Phytomonas*, and many insect parasites (Berriman et al. 2005; Flegontov et al. 2016; Lopes et al. 2010; Lukeš et al. 2014; Porcel et al. 2014). The former three orders, collectively called "bodonids," are free-living, commensal, or parasitic.

Importantly, eubodonids represent a bodonid clade closest to trypanosomatids (Lukeš et al. 2014), and hence may provide an insight into metabolic gains and losses that enabled the evolutionary transition to parasitism. *Bodo saltans* is a free-living bacterivorous protist found worldwide in marine and freshwater habitats (Mitchell et al. 1988). It has numerous typical kinetoplastid features, such as two flagella emanating from a specialized flagellar pocket, a corset of subpellicular microtubules, mitochondrial RNA editing, and a huge mass of kDNA in the lumen of its single mitochondrion (Blom et al. 2000; Jackson et al. 2008). However, in addition to these, bodonids exhibit a range of ultrastructural features and varied lifestyles that distinguish them from the derived obligatory parasitic trypanosomatids

found primarily in insects (Adl et al. 2012; Maslov et al. 2013; Mitchell et al. 1988). *Bodo saltans* is the closest known relative of obligatory parasitic trypanosomatids, as the early-branching *Paratrypanosoma confusum* is already a well-established parasite (Flegontov et al. 2013). Monoxenous trypanosomatids (i.e., with one host in their life cycle) are restricted to insects, where they usually reside in the intestinal tract (Podlipaev 2000). In this study they are represented by the genera *Blechomonas*, *Crithidia*, and *Leptomonas* (Flegontov et al. 2016; Jirků et al. 2012; Votýpka et al. 2013). Dixenous trypanosomatids have developed more complex life cycles, which involve a secondary host (a vertebrate for *Leishmania* and *Trypanosoma* and a vascular plant for *Phytomonas*) (Maslov et al. 2013). We argue that a comprehensive analysis of the metabolism of the monoxenous and dixenous trypanosomatids and *B. saltans* may shed light on the transition from a free-living to a parasitic lifestyle. An imperative prerequisite for such an analysis is the availability of sequenced and annotated representative genomes.

Metabolism of several human parasites belonging to the genera *Trypanosoma* and *Leishmania* has been studied extensively, following the publication of their genomes (Berriman et al. 2005; Hellemond et al. 2005; Opperdoes and Coombs 2007; Smith and Bütikofer 2010; Zíková et al. 2016). Recently, several new trypanosomatid whole genome sequences have become available, enabling us to extend metabolic analysis beyond the dixenous trypanosomatids (Table 1) (Flegontov et al. 2016; Jackson et al. 2008; Kraeva et al. 2015). The draft genome of *B. saltans* (Jackson et al. 2016) has a size of around 39.9 Mbp in 2,256 scaffolds. The number of predicted 18,963 protein-coding genes in the *B. saltans* genome is substantially higher than that in trypanosomatids (Table 1). So far, only a few individual enzymes have been described and characterized in *B. saltans* (Brown et al. 2014; Gažiová and Lukeš 2003; Jackson et al. 2008; Lawrie 1935), and the RNA editing machinery performing insertions and/or deletions of uridines is apparently present in its mitochondrion (Blom et al. 1998, 2000). However, no metabolic studies have been carried out with this omnipresent flagellate until now. The other two genomes that have recently become publicly available belong to monoxenous trypanosomatids *Leptomonas seymouri* (Kraeva et al. 2015) and *Leptomonas pyrrhocoris* (Flegontov et al. 2016). The genomes are of similar sizes, 27.3 Mbp and 30.4 Mbp, and have been assembled into 1,222 and 60 scaffolds, respectively. The number of protein-coding genes is 8,488 and 10,148 for *L. seymouri* and *L. pyrrhocoris*, respectively. Another genome, recently sequenced and annotated by us, is that of a trypanosomatid parasitizing fleas, *Blechomonas ayalai*, which belong to an early-branching clade Blechomonadinae, a sister group to subfamilies Phytomonadinae and Leishmaniinae (Fig. 1) (Jirků et al. 2012; Yurchenko et al. 2016).

In this review, the metabolic pathways of major representatives of Kinetoplastea, for which complete genome sequences are available, are compared, with special emphasis on metabolic capacities of *B. saltans*.

OBSERVATIONS AND PREDICTIONS

Peroxisomes/glycosomes

Peroxisomes are involved in a number of metabolic pathways, such as fatty acid oxidation, ether lipid formation and oxidative stress protection. In trypanosomatids, the glycolytic pathway is partly localized in peroxisome-like organelles called glycosomes (Michels et al. 2006). The peroxisomal targeting signals (PTS1 and 2) and the presence of peroxins are diagnostic of functional peroxisomes (Dodt and Gould 1996; Gould et al. 1989; Swinkels et al. 1991). The peroxins are involved in the formation of peroxisomes and import of proteins into these organelles. Orthologs of peroxins 1, 2, 4, 5, 6, 7, 10, 11, 12, 14, and 19 were detected in *B. saltans* along with putative peroxisomal proteins carrying PTSs (Opperdoes and Szikora 2006). Many of them are orthologs of the well-characterized glycosomal proteins of trypanosomatids. Moreover, the presence of numerous glycolytic genes equipped with PTS1 in the *B. saltans* genome indicates that these organelles must be very similar to the trypanosomatid glycosomes. None of the dixenous trypanosomatids possesses a gene for the peroxisomal marker enzyme catalase. In contrast, in their monoxenous relatives *Crithidia* and *Leptomonas* spp., a bacterial-type catalase has been likely obtained by lateral gene transfer (LGT). It is localized in the cytoplasm rather than in peroxisomes (Eeckhout 1970; Souto-Padron and de Souza 1982). In *B. saltans* and *B. ayalai*, no catalase gene was identified, suggesting that its acquisition via LGT was a relatively recent event (Flegontov et al. 2016; Jackson et al. 2016).

Enzymes of the glyoxylate cycle, reported to be present in the peroxisomes of some flagellates (Simon et al. 1978), and the typical peroxisomal marker enzymes, D-amino acid oxidase and 2-hydroxy-acid oxidase, are absent from the analyzed genomes. Interestingly, another peroxisomal marker, the acyl-CoA oxidase equipped with PTS1, is present in *B. saltans*, but absent from all trypanosomatids. It is likely that the activity of hydrogen peroxide-producing oxidase in glycosomes would be incompatible with the lack of catalase. Alternative mechanisms, involving Fe-superoxide dismutase and NADP-isocitrate dehydrogenase, may be in place for reactive oxygen species protection mechanisms in these organelles.

Thus, genomic analysis confirmed that *bona fide* glycosomes are present in *B. saltans*, although their biochemical properties have not been investigated yet. This is in agreement with earlier observations of glycosome-like structures in another Metakinetoplastina bodonid, *Trypanoplasma borreli* (Opperdoes et al. 1988). We conclude that glycosomes must have been already well established in the last common ancestor of bodonids and trypanosomatids.

Nutrition

Bodo saltans is a predator that lives in freshwater and marine habitats and feeds on bacteria by phagocytosis

Table 1. Genomic sequences used in the analysis

Species	Lifestyle	Host	Secondary host	Genome size, Mbp	Number of scaffolds/ chromosomes	Number of proteincoding genes	Source
Leptomonas pyrrhocoris H10	Parasitic	Insect	–	30.4	60	10,148	TrytripDB v.9
Leptomonas seymouri ATCC 30220	Parasitic	Insect	*	27.3	1,222	8,488	TrytripDB v.9
Crithidia fasciculata CfCl	Parasitic	Insect	–	40	31	9,489	TrytripDB v.9
Leishmania major Friedlin	Parasitic	Insect	Vertebrate	32.8	36	8,400	TrytripDB v.9
Blechomonas ayalai B08-376	Parasitic	Insect	–	21.6	546	8,049	Our unpublished data
Phytomonas sp.EM1	Parasitic	Insect	Plant	17.8	138	6,381	Porcel et al. 2014
Phytomonas sp. HART1	Parasitic	Insect	Plant	18.1	84	6,451	Porcel et al. 2014
Trypanosoma brucei TREU927	Parasitic	Insect	Vertebrate	35.8	131	11,567	TrytripDB v.9
Trypanosoma cruzi CL Brener	Parasitic	Insect	Vertebrate	32.5	41	10,339	TrytripDB v.9
Trypanosoma vivax Y486	Parasitic	Insect	Vertebrate	41.8	11	11,885	TrytripDB v.9
Trypanosoma congolense IL3000	Parasitic	Insect	Vertebrate	41.4	16	13,148	TrytripDB v.9
Paratrypanosoma confusum CUL13	Parasitic	Insect	–	27.7	2,114	9,304	Our unpublished data
Bodo saltans Konstanz	Free-living	–	–	39.9	2,256	18,963	Jackson et al., 2016

*Occasional infection in immunocompromised individuals.

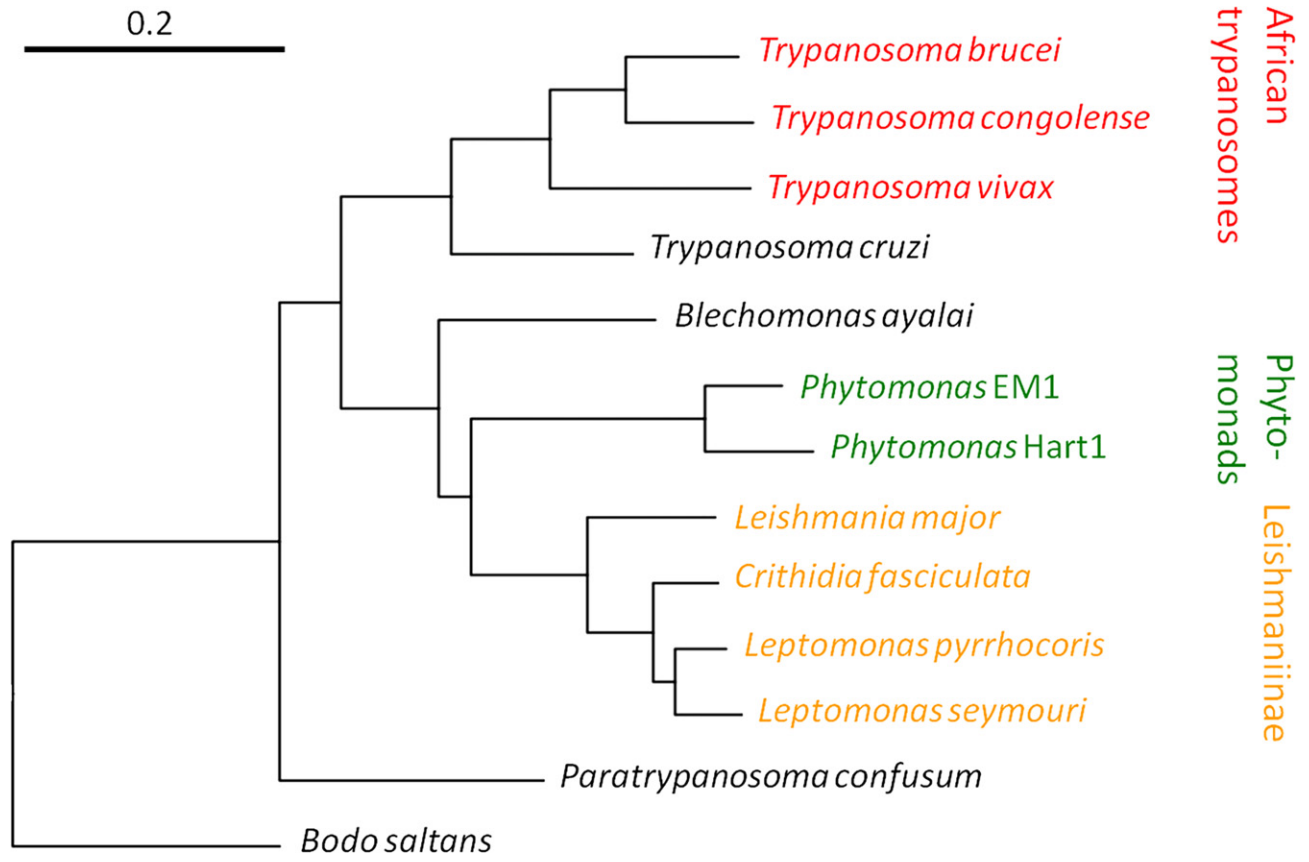

Figure 1 Phylogenetic tree of Kinetoplastea based on concatenated sequences of proteins encoded by 50 house-keeping genes. Sequences were aligned using ClustalX with default settings. The alignment of 28,384 characters long was used for phylogenetic inference with MrBayes (Ronquist et al. 2012). The Wagner phylogenetic model with gamma correction and 4 rate categories fitted best the data. The horizontal bar represents 0.2 substitutions per site. All nodes have posterior probabilities of 1.

(Mitchell et al. 1988). After having captured a bacterium, it degrades its macromolecules using acidic hydrolases within the phagolysosomal system. Accordingly, genes for several lysosomal lipases, α-N-acetyl glucosaminidase, α-glucosidase, multiple sugar hydrolases, esterases, DNAses, and RNAses were found in its genome. In addition, *B. saltans* can degrade peptidoglycans of the outer membranes of gram-negative bacteria. Chitinase and

numerous glycoside hydrolases are involved in their digestion. A bacterial-type N-acetylmuramate 6-phosphate etherase, which is required for degradation of murein, was also found. This enzyme is present in other free-living protists, such as *Naegleria, Paramecium* and *Tetrahymena* (Veiga-da-Cunha et al. 2009), but absent from parasitic trypanosomatids. Bacterial D-lactate and D-alanine are metabolized in *B. saltans* by a D-lactate dehydrogenase (D-lactate ferricytochrome oxidoreductase) and alanine racemase, respectively.

Bodo saltans is also capable of degrading storage polysaccharides, such as starch, since genes encoding α-amylase and cellobiase are present in its genome. Glycogen-like macromolecules are degraded by α- and β-glucosidases and various (gluco-) amylases. Interestingly, the vast majority of members of this hydrolase family have been lost in trypanosomatids, probably due to transition to a parasitic lifestyle. Trehalose is a natural α-linked disaccharide formed by an α,α-1,1-glucoside bond between two α-glucose units. Trehalose is used by many organisms as a protectant against desiccation, or for osmoregulation purposes (Tapia and Koshland 2014). Moreover, trehalose is the major sugar present in the hemolymph of all insects (Wyatt and Kale 1957). Genes for trehalases (enzymes that hydrolytically split trehalose) were only found in *B. saltans*, but a gene for α-trehalose phosphorylase, an enzyme that catalyzes the cleavage of the disaccharide into glucose and glucose 6-phosphate, and which was previously reported in *Phytomonas* sp. (Porcel et al. 2014) has also been found in *B. saltans* and in several trypanosomatids. The presence of this enzyme in their common ancestor may have predisposed the early trypanosomatid for an infectious relationship with the insect host and this may have resulted in the obligatory parasitic lifestyle of the entire trypanosomatid lineage.

Carbohydrate metabolism

Glycolysis and gluconeogenesis

Glycolysis has been most extensively studied in *Trypanosoma brucei*. Notably, its bloodstream stage relies exclusively on glycolysis for energy production with its mitochondrial metabolism being largely repressed (Bakker et al. 1999). The hexose sugars serve as substrates for the glycolytic pathway, the first seven enzymes of which are located inside the glycosomes (Opperdoes 1987; Opperdoes and Borst 1977). The phosphofructokinase (PFK) of *T. brucei* shares sequence similarity with the pyrophosphate-dependent class of PFKs of some bacteria and plants (Michels et al. 1997), while the 2,3-bisphosphoglycerate-independent phosphoglycerate mutase has been shown to be of plant origin (Chevalier et al. 2000).

Homologs of the *T. brucei* glycolytic pathway genes have been identified in all trypanosomatid genomes analyzed thus far. In *B. saltans*, the first enzyme of glycolysis, hexokinase (HK), is present in one copy. It lacks the typical PTS2 but this can be explained by the absence of 21 N-terminal amino acids in the predicted HK sequence. In *T. brucei* the HK gene is present in two tandemly linked copies (*TbHK1* and *TbHK2*) bearing a PTS2, while most trypanosomatids have a single copy of HK. The *T. brucei* HK phosphorylates glucose, fructose, and maltose and is an essentially unregulated enzyme (Nwagwu and Opperdoes 1982). The *TbHK1* is found in glycosomes, while *TbHK2* localizes to both glycosomes and the flagellum (Joice et al. 2012). The enzyme has a high affinity for D-glucose, yet retains broad substrate specificity. *B. saltans* and all analyzed trypanosomatids, except the African trypanosomes (*T. brucei*, *Trypanosoma vivax*, and *Trypanosoma congolense*), possess a gene for another glucose-phosphorylating enzyme, glucokinase (GCK), which is equipped with a PTS. GCKs generally have high specificity but low affinity for glucose. The presence of the two isoenzymes, HK and GCK, must have been an early trait of the Kinetoplastea.

Surprisingly, *B. saltans* possesses two genes encoding PFK isoenzymes differing by 88% of the residues at the amino acid level. One PFK gene belongs to the group II inorganic pyrophosphate-dependent PFKs (PP_i-PFKs), and is similar to its ortholog from propionibacteria and *Mastigamoeba balamuthi* (Müller et al. 2001), while the other is related to the PP_i-PFK of *Naegleria fowleri* (Mertens et al. 1993), hence an ortholog of the bacterial and plant PFKs. Phylogenetic analysis (Fig. S1) revealed that the *B. saltans* ATP-dependent PFK is most closely related to PPi-PFK of spirochetes and the trypanosomatid ATP-dependent PFKs (60–62% identity), while the PPi-PFK of *B. saltans* is most closely related to the PPi-PFK of *Naegleria gruberi* and the bacterial PPi-PFKs of the verrucomicrobium *Coraliomargarita akajimensis*, and the γ-proteobacteria *Methylomonas methanica* and *Methylomicrobium alcaliphilum* (46–49% identity).

In contrast to PFKs with high specificity for inorganic pyrophosphate, the specificity of the trypanosomatid ortholog has changed in favor of ATP (ATP-PFK) as the phosphoryl donor (Michels et al. 1997). The use of a PP_i-PFK ensues an increased ATP yield during anaerobic glycolysis (Mertens et al. 1993). Thus, *B. saltans* may survive equally well in both high and low oxygen environments, while trypanosomatids, which have lost the PP_i-dependent enzyme, are considered predominantly aerobic. The trypanosomatid ATP-PFK carries a PTS1 and has been shown to be exclusively present in glycosomes (Michels et al. 1997). The ATP-PFKs from *T. brucei* and its ortholog in *B. saltans* share 60% identity, with highly conserved residues involved in ATP binding (McNae et al. 2009). Therefore, we assume that in the last common ancestor of the free-living *B. saltans* and the parasitic trypanosomatids, this PFK isoenzyme had already changed its specificity from PP_i to ATP.

However, it remains unclear why would *B. saltans* require two PFK isoenzymes. A possible explanation, aside from oxygen deprivation, is that ATP-PFK, together with ATP-dependent pyruvate kinase (PK), are the preferred enzymes for downstream glycolysis. Both enzymes catalyze irreversible reactions, resulting in an efficient utilization of glucose as an energy substrate. Moreover, in all

trypanosomatids the activity of pyruvate kinase, the last enzyme of the glycolytic pathway, is dependent on the allosteric regulator fructose 2,6-bisphosphate. Its concentration is regulated by the bifunctional enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase2) (van Schaftingen et al. 1985). The latter is also present in *B. saltans*, suggesting that this allosteric regulation has been inherited from a common ancestor of kinetoplastids.

For the reverse pathway, called gluconeogenesis, PP_i-dependent pyruvate:phosphate dikinase, together with PP_i-PFK, would be the preferred enzyme pair, because both enzymes catalyze reversible reactions. However, *B. saltans* possesses an additional fructose-1,6-bisphosphatase (FBPase1) which, in an irreversible reaction, catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate. The simultaneous expression of both PP_i-PFK and FBPase1 could lead to futile cycling ultimately resulting in the hydrolysis of all cellular ATP. This can be prevented by differential compartmentalization of these two enzymes. Indeed, PP_i-PFK is a glycosomal protein, while FBPase1 is truncated at its C-terminus and lacks a canonical PTS1, indicating its cytosolic localization. Overall, the gluconeogenic pathway in *B. saltans* is remarkably redundant.

Another protein very recently identified in *Trypanosoma* spp., bromodomain factor 1 (BDF1), is directed to glycosomes by its PTS2 (Ritagliati et al. 2016). Bromodomain-containing proteins bind acetylated lysines in histones and other proteins regulating gene expression (Siegel et al. 2009). We speculate that such regulation may also affect glycosomal proteins. It is interesting to note that *B. saltans* lacks BDF1, while *B. ayalai*, *Phytomonas* spp. and the representatives of Leishmaniinae all have acquired it. Importantly, only the *Trypanosoma* BDF1 has a PTS2 that directs it to glycosomes. This may suggest that the complex changes of glycolytic activity observed during the *Trypanosoma* life cycle may be regulated by acetylation of one or more glycosomal proteins.

Despite the presence of all glycolytic pathway genes in *B. saltans*, the transcriptome sequence data (available from the GeneDB website at ftp://ftp.sanger.ac.uk/pub/-pathogens/Bodo/saltans/Bodo_cDNA.fasta) revealed no evidence for their expression. On the contrary, only mRNAs for several enzymes of a putative gluconeogenic pathway, such as fructose-1,6-bisphosphatase, PP_i-dependent PFK, pyruvate phosphate dikinase and NADP-dependent malic enzyme were detected. This indicates that in *B. saltans*, under conditions where it utilizes bacteria as the sole source of nutrients, the glycolytic pathway is severely suppressed in favor of the reverse, gluconeogenic pathway. It means that the free-living *B. saltans* utilizes fatty acids and amino acids, rather than sugars, to satisfy its energy and biosynthetic needs.

Other enzymes of carbohydrate metabolism

Other enzymes involved in the carbohydrate metabolism in *B. saltans* are ribokinase, galactokinase and glycerol kinase. Genes encoding ribulokinase and xylulokinase, two enzymes of pentose sugar metabolism of *Leishmania* spp. (Opperdoes and Michels 2008a), were not detected in *B. saltans*. The presence of a β-fructofuranosidase gene suggests that, similarly to *Leishmania, Phytomonas, Crithidia*, and *Leptomonas* spp., *B. saltans* can digest sucrose. It also possesses mitochondrial malate dehydrogenase (MDH), but lacks L-lactate dehydrogenase. MDH, by virtue of a single point mutation, may change its substrate specificity from malate to lactate (Wu et al. 1999). Thus, the true substrate specificity of the *B. saltans* enzyme remains to be verified experimentally.

The glucose consumption in trypanosomatids is governed by aerobic glycolysis. The reduced equivalents produced by glycosomal glyceraldehyde dehydrogenase are either re-oxidized by molecular oxygen via the glycerol-3-phosphate:dihydroxyacetone phosphate cycle, comprising NAD glycerol-3-phosphate dehydrogenase and FAD-glycerol-3-phosphate oxidase as in the bloodstream form of the African trypanosomes. Alternatively, in the procyclic stage of *T. brucei* and in most trypanosomatids, this occurs via the glycosomal succinate fermentation pathway, comprising the enzymes malate dehydrogenase, fumarate hydratase, and fumarate reductase. All components of these two pathways are also present in *B. saltans*. Moreover, *B. ayalai* and *Phytomonas* spp. possess an ortholog of alcohol dehydrogenase and are therefore predicted to also secrete ethanol.

Bodo saltans, as well as most trypanosomatids, has an enzyme called phosphoglucomutase (PGM), converting glucose 6-phosphate to glucose 1-phosphate for further use in glycosylation reactions. Only the African trypanosomes lack it, along with several related genes. In addition, the African trypanosomes do not possess genes for glucokinase, phosphomannomutase and galactokinase. PGM may be replaced by phospho-N-acetylglucosamine mutase (PAGM), which reversibly catalyzes the transfer of phosphate between the C6 and C1 hydroxyl groups of N-acetylglucosamine, mannose, glucose, and phospho-N-acetylglucosamine. Indeed, it was demonstrated that PAGM is capable of replacing PGM in *T. brucei* (Bandini et al. 2012). Galactose is essential for *T. brucei*, but the parasite not only lacks transporters for its uptake, but also a gene for galactokinase. It turned out that in *T. brucei* galactose may also be formed from glucose by the action of UDP-galactose 4′-epimerase (Urbaniak et al. 2006). *Bodo saltans* has a complete set of the enzymes catalyzing glycosylation reactions necessary for the formation of glycoproteins and oligo- and polysaccharides.

Is there a methylglyoxal bypass in trypanosomatids?

Several trypanosomatids are known to produce limited amounts of D-lactate via the methylglyoxal pathway (Darling and Blum 1988). In bacteria cultivated under phosphate starvation, the enzyme methylglyoxyl synthase converts triose-phosphates to inorganic phosphate and methylglyoxal (Huang et al. 1999). The gene encoding this enzyme was not detected in the kinetoplastid genomes analyzed so far, meaning that in these flagellates spontaneous fragmentation of triose-phosphates must occur instead. Methylglyoxal resulting from this process is then

converted into D-lactate by a thiol-dependent glyoxylase system, comprised of glyoxalases I and II. The resulting D-lactate may then be converted into pyruvate by D-lactate dehydrogenase. Trypanosomatid glyoxalases I and II use trypanothione, rather than glutathione, as an essential cofactor (Irsch and Krauth-Siegel 2004). Glyoxalase II is present in all kinetoplastids, whereas glyoxalase I and D-lactate dehydrogenase were not found in the African trypanosomes and *Phytomonas* spp. This explains why, for instance, *T. brucei gambiense* does not produce any D-lactate (Darling and Blum 1988). In the African trypanosomes the absence of glyoxalase 1 is compensated for by NADPH-dependent methylglyoxal reductase and NAD^+-dependent l-lactaldehyde dehydrogenase activities, which together allow for methylglyoxal detoxification via the methylglyoxal reductase pathway to L-lactate (Greig et al. 2009). However, genes encoding these two enzymatic activities have not yet been identified in *B. saltans*.

Spontaneous transesterification may lead to the formation of the trypanothione-thioesters which are excellent substrates for the *T. brucei* glyoxalase II. Therefore, this glyoxalase may function as a general trypanothione thioesterase instead of assisting in methylglyoxal detoxification (Wendler et al. 2009).

Alternative oxidase

Trypanosomatids possess two terminal oxidases: a classic cyanide-inhibited cytochrome *c* oxidase and a cyanide-insensitive, but salicylhydroxamate- and ascofuranone-inhibited oxidase, also known as the trypanosome alternative oxidase (TAO) (Edwards and Chance 1982; Evans and Brown 1973). Most trypanosomatids express cytochrome *c* oxidase throughout their life cycle, but the *T. brucei* bloodstream stage completely suppresses its respiratory chain in favor of TAO (Clarkson et al. 1989). Nuclear- and mitochondrial-encoded subunits of cytochrome *c* oxidase were identified in all kinetoplastids, except for *Phytomonas* spp., which possess only TAO (Porcel et al. 2014). However, *B. saltans* has two distantly related homologs of TAO. One is an ortholog of the enzyme described in the African trypanosomes and *Phytomonas* spp. (Hamilton et al. 2014; Van Hellemond et al. 1998), while the other is a distantly related TAO-like protein. It is not known whether in *B. saltans,* or in any of the trypanosomatids where this TAO-like protein is present (i.e., *Crithidia fasciculata* and *L. pyrrhocoris*), it functions as a true mitochondrial oxidase. It is worth noting that the predicted protein lacks a signal peptide required for its import into the mitochondrion.

Transcriptome profiling revealed the presence of the TAO mRNA in *B. saltans*, while no mRNA for either cytochrome *c* oxidase or cytochrome *c* was detected. We predict that in *B. saltans* fed on bacteria, respiration should be cyanide-insensitive but instead sensitive to salicylhydroxamic acid and ascofuranone, although this has to be tested experimentally. Hence, cyanide-insensitive respiration must already have been present as an ancient trait in the last common ancestor of bodonids and trypanosomatids.

Pentose-phosphate pathway

The pentose-phosphate pathway (PPP) generates NADPH for biosynthetic purposes and for protection against oxidative stress and provides ribose moieties for the synthesis of nucleic acids (Stincone et al. 2014). Three enzymes of the oxidative branch of the pathway were readily detected in all kinetoplastid genomes. However, in *B. saltans* transaldolase and transketolase, two enzymes of the non-oxidative part of PPP, were identified only in the transcriptome. To exclude the possibility of bacterial contamination, the kinetoplastid nature of the transaldolase and transketolase mRNAs was confirmed by BLAST analysis. A gene encoding ribokinase was identified in the *B. saltans* genome, but not genes for ribulokinase and xylulokinase. Thus, in *B. saltans* the PPP serves mainly the purpose of NADPH regeneration and formation of ribose 5-phosphate, while in trypanosomatids it may play a role in the utilization of pentose sugars as an alternative energy source, or as a shunt for the glycolytic pathway.

Interestingly, a transitory photosynthetic endosymbiont, which lived in an ancestral kinetoplastid, has been proposed as a possible explanation for the fact that a number of genes of plant origin were identified in *Trypanosoma* spp. (Hannaert et al. 2003). A canonical PTS1-containing sedoheptulose-1,7-bisphosphatase (SBPase), thus far only found in the genus *Trypanosoma*, was also identified in *B. saltans*. Therefore, this plant-like gene must have been already present in the common ancestor of bodonids and trypanosomatids, being eventually lost from the latter and persisting only in *Trypanosoma* spp. In photosynthetic organisms, SBPase is an essential enzyme of the Calvin cycle. Since another key enzyme of the Calvin cycle, ribulose-bisphosphate carboxylase/oxidase (RuBisCo), is absent from all kinetoplastid genomes, the function of SBPase in these protists remains unclear. However, in *B. saltans* and trypanosomes, it may presumably play a role in riboneogenesis, when the glycolytic pathway is shut down in favor of gluconeogenesis, or when the demand for ribose exceeds that for reducing power. In this metabolic situation, SBPase may establish a link between the gluconeogenic/glycolytic pathways and the pentose phosphate pathway, which results in riboneogenesis, that is, the formation of ribose 5-phosphate. This alternative pathway of riboneogenesis allows cells to uncouple the demands of redox homeostasis and biosynthesis and may be advantageous when they switch from one life cycle stage to another or change their growth rate (Clasquin et al. 2011). It is, however, not clear why other trypanosomatids have lost this seemingly beneficial capacity.

Protection against reactive oxygen species (ROS)

Oxidative stress protection in trypanosomatids is based on two principles: firstly, the presence of ROS scavenging molecules (e.g., trypanothione) and enzymes (e.g., superoxide dismutase (SOD) and catalase) and, secondly, the maintenance of a sufficiently low redox potential in the cell to keep the scavenging molecules in the reduced state (Menna-Barreto and de Castro 2014; Tomás and

Castro 2013). Trypanothione, an adduct of one molecule of spermidine and two molecules of glutathione, was considered to be unique to Trypanosomatida (Olin-Sandoval et al. 2010). We searched the *B. saltans* genome for the presence of genes involved in its synthesis and metabolism. Genes encoding trypanothione reductase, thioredoxin, tryparedoxin peroxidase, and a homolog of trypanothione synthase were identified. This indicates that trypanothione must be fully operational in this free-living bodonid.

Contrary to trypanosomatids, *B. saltans* encodes a homolog of the mammalian peroxisomal acyl-CoA oxidase, an enzyme that reduces molecular oxygen to hydrogen peroxide. Its sequence predicts a PTS, suggesting that the *B. saltans* glycosomes may contain a hydrogen peroxide-producing fatty acid β-oxidation pathway. However, it is not clear how hydrogen peroxide, formed in its glycosome, is inactivated in the absence of any catalase activity. Although many trypanosomatids seem to have a gene for an acyl-CoA oxidase as well, it does not carry a PTS. Moreover, acyl-CoA induced hydrogen peroxide production has never been detected in any trypanosomatid (FRO., unpubl. data). Thus, if such an activity was present in the common ancestor of *B. saltans* and trypanosomatids, the corresponding gene was most likely lost at the base of the trypanosomatid lineage.

Bodo saltans possesses four SOD genes, with two of them tandemly linked and differing by only 3% amino acids. Moreover, an iron/ascorbate oxidoreductase was identified in most trypanosomatids, except for *B. ayalai* and *L. seymouri*. Although monoxenous Leishmaniinae possess a bacterial-type catalase, this enzyme probably functions in the cytosol rather than in glycosomes, because it lacks targeting signals. It has presumably been acquired by a relatively recent LGT event, since this gene could not be detected in the other trypanosomatids and in *B. saltans*, and the bacterial and trypanosomatid homologs are still 70% identical. In addition, a plant-like ascorbate peroxidase was detected in *Trypanosoma cruzi* and *Leishmania* spp. (Wilkinson et al. 2002), as well as in *B. ayalai*, and *B. saltans*, but not in *Phytomonas* spp. and the African trypanosomes.

Several candidate genes encoding enzymes involved in the regeneration of cellular NADPH, essential for the maintenance of a sufficiently low intracellular redox potential, were detected in kinetoplastids. These genes encode the pentose-phosphate shunt enzymes, glucose-6-phosphate dehydrogenase and 6-phophogluconate dehydrogenase, NADP-dependent malic enzyme and NADP-dependent isocitrate dehydrogenase (Leroux et al. 2011). Malic enzyme is present in *B. saltans* in two isoforms sharing only 60% identity. One copy is presumably mitochondrial, while the other one is cytosolic. The same was noted for NADP-dependent isocitrate dehydrogenase (IDH) with one isoform being mitochondrial and the other one glycosomal. Both IDH isoenzymes have been characterized in *T. cruzi* (Leroux et al. 2011).

Serine-driven C1 (one-carbon) metabolism generates up to four molecules of NADPH per a molecule of serine consumed. Components of this metabolic pathway are all present in *B. saltans*. Within trypanosomatids, only *Trypanosoma* spp. lack this capacity because they do not have the cytosolic and mitochondrial serine hydroxymethyltransferase isoenzymes (Fig. S2).

Purine biosynthesis and salvage

Trypanosomatids are not capable of de novo purine synthesis, as only adenylosuccinate lyase, 1 of 10 enzymes required for the biosynthesis of inosine monophosphate (IMP) from phosphoribosyl pyrophosphate, was identified. Importantly, this enzyme also functions in purine salvage as a part of the purine-nucleotide cycle, where it converts IMP into AMP. The absence of the purine biosynthetic pathway is a characteristic feature of not only trypanosomatids, but many other parasites (el Kouni 2003) as well as some free-living phagotrophic protists such as *Naegleria gruberi* (Fritz-Laylin et al. 2010; Opperdoes et al. 2011). This can now be extended to the free-living *B. saltans*, which is probably able to acquire all the essential purines from bacteria.

Most of the enzymes for interconversion of the purine bases and nucleosides are present in *B. saltans*, as well as in trypanosomatids. All three enzymes of the purine-nucleotide cycle (adenylosuccinate synthetase, adenylosuccinate lyase, and AMP deaminase) are ubiquitous in kinetoplastids, as are other components of purine metabolism, such as hypoxanthine-guanine phosphoribosyltransferase (HGPRT), inosine monophosphate dehydrogenase, GMP synthase, GMP reductase, adenine phosphoribosyltransferase, ribose kinase, phosphoribosylpyrophosphate synthase, inosine-, adenosine-, guanosine-nucleoside hydrolase, and adenosine kinase. Most of them have been previously shown to be associated with glycosomes (Vertommen et al. 2008). The *T. brucei* genome sequence predicts the presence of three HGPRT isoenzymes, with two of them carrying PTS1. A related xanthine phosphoribosyltransferase (XPRT) is a unique enzyme that lacks a mammalian counterpart and is present only in *B. ayalai*, *Phytomonas* spp. and Leishmaniinae.

Pyrimidine biosynthesis

All homologs of the genes of the pyrimidine biosynthetic pathway were detected in *B. saltans*. However, dihydroorotase (PYR3) was detected in the transcriptomic data only. To exclude the possibility of bacterial contamination, the kinetoplastid nature of dihydroorotase mRNA was confirmed by BLAST analysis using the nonredundant protein database in Genbank. The dihydroorotate dehydrogenase (DHODH) of *B. saltans* is 65% identical to the cytosolic DHODH of trypanosomatids. The orotidylate decarboxylase/orotate phosphoribosyltransferase (ODC/OPRT) of *B. saltans* is an ortholog of the bifunctional protein of trypanosomatids. We conclude that this arrangement, which is rather unique for eukaryotes – namely the presence of cytosolic DHODH, rather than mitochondrial DHO oxidase

along with glycosomal ODC/OPRT – was already present in the common ancestor of free-living *B. saltans* and parasitic trypanosomatids. However, while in the latter the PYR genes cluster together in an operon-like structure (PYR1, 2, 4, and 5/6) (Nara and Aoki 2002; Opperdoes and Michels 2007), they are dispersed throughout the *B. saltans* genome.

Mitochondrion

Kinetoplastids are characterized by the presence of a single mitochondrion, which contains kDNA, presumably the most structurally complex organellar genome (Lukeš et al. 2002). In trypanosomatids, kDNA is comprised of a giant network of catenated circular DNAs of two types: maxicircles and minicircles (Jensen and Englund 2012; Povelones 2014). The maxicircles contain genes involved in the synthesis of the mitochondrial respiratory chain subunits. Most of their transcripts undergo extensive RNA editing of the uridine insertion/deletion type guided by RNAs mostly encoded by minicircles (Simpson et al. 2000; Verner et al. 2015). The structure of kDNA in bodonids is much more variable than in trypanosomatids, and is usually composed of free supercoiled circular DNAs rather than of catenated relaxed circles (d'Avila-Levy et al. 2015; Lukeš et al. 2002).

Carbohydrates, degraded via the glycolytic pathway to pyruvate, are transported to the mitochondrion. Pyruvate is oxidized to acetyl-CoA via the mitochondrial pyruvate dehydrogenase complex (PDH). In most eukaryotes, acetyl-CoA is oxidized by successive action of the tricarboxylic acid (TCA) cycle enzymes, but in kinetoplastids the canonical NAD-linked isocitrate dehydrogenase is absent and a NADP-linked enzyme is operating instead. The use of NADP implies that this enzyme, under the conditions that reign inside the mitochondrion, cannot function in the TCA cycle, but serves in replenishing the mitochondrial NADPH pool. The remaining TCA cycle enzymes serve in utilizing the end-products of amino acid and fatty acid oxidation.

Reducing equivalents (e.g., mitochondrial NADH, FADH2/ubiquinone) are oxidized via the respiratory chain consisting of several nuclear-encoded subunits of NADH dehydrogenase (=respiratory complex I), complemented with as yet unknown number of mitochondrial-encoded subunits (Opperdoes and Michels 2008b). However, in *T. brucei* complex I seems to be nonessential in both procyclic and bloodstream stages (Surve et al. 2012; Verner et al. 2011), implying the key role of another dehydrogenase (Verner et al. 2014). Other constituents of the respiratory chain identified are mitochondrial FAD-glycerol-3-phosphate dehydrogenase and several nuclear-encoded subunits of succinate dehydrogenase, several subunits of complex III (Rieske-iron sulfur protein, ubiquinol cytochrome *c* reductase and apocytochrome *c1*), as well as cytochrome *c* and numerous nuclear- and mitochondrial-encoded subunits of cytochrome *c* oxidase (complex IV).

The identification of protoheme IX farnesyltransferase in *B. saltans* is a strong indication for the presence of an *a*-type cytochrome and a functional respiratory chain. With all the genes that encode subunits of a classic respiratory chain present in its genome, *B. saltans* should be able to generate a proton gradient at the level of the respiratory complexes I, III, and IV. However, since the mRNAs for the above subunits were not detected, the respiratory chain exhibits little or no activity under the studied growth conditions. Thus, under aerobic conditions with access to prey bacteria, oxidation of NADH takes place via the upper part of the respiratory chain and TAO, and the lower part with the cytochromes is likely not functional. Interestingly, several enzymes of the ubiquinone biosynthetic pathway were not identified in *B. saltans*, while they are all present in trypanosomatids. It is not known how *B. saltans* gains access to this essential redox component (see also section 8.2).

Kinetoplastids lack mitochondrial DHODH, which has been replaced by cytosolic isofunctional enzyme of the bacterial type that uses fumarate as electron acceptor. This situation is unique among eukaryotes and has so far been encountered only in these flagellates and yeast (Annoura et al. 2005; Zameitat et al. 2007). This unique property explains why both glucose-grown yeast and some trypanosomes, when they utilize sugars as their sole source of energy, are capable of suppressing their respiratory chain without affecting their pyrimidine biosynthesis. This condition may eventually lead to partial or even complete loss of their kDNA (Lai et al. 2008). It would be interesting to test whether *B. saltans* can be induced to form viable dyskinetoplastic (=kDNA-lacking) stages.

Iron–sulfur (Fe–S) clusters form redox prosthetic groups in a wide range of proteins, many of which are localized in the mitochondrion (Lill 2009). The *B. saltans* genome encodes key enzymes required for the synthesis of these co-factors. The identification of cysteine desulfurase, which is targeted to the mitochondrion, the mitochondrial iron chaperone frataxin, likely involved in Fe–S cluster regeneration and a mitochondrial NifU-like protein suggests that the organelle is indeed involved in the generation of elemental sulfur for the use in Fe–S cluster proteins in a manner reminiscent of that described for *T. brucei* (Basu et al. 2016).

Bodo saltans possess a full set of mitochondrial solute transporters. There are specific mitochondrial carriers for both pyruvate and phosphate, some of which are homologs of the dicarboxylate carriers and tricarboxylate- and carboxylate exchangers described in trypanosomatids (Colasante et al. 2009). These carriers are probably involved in the transport of the TCA cycle intermediates, aspartate, and glutamate across the mitochondrial membrane. In addition, an ATP/ADP exchanger, a putative folate carrier and a mitochondrial ornithine carrier were identified in *B. saltans*.

Ergosterol biosynthesis and related pathways

Polyisoprenoids are widely used as farnesyl or geranylgeranyl attachments of proteins to cell membranes, for the

synthesis of dolichol, ubiquinone (UQ), heme A and sterols. They are formed through the condensation of the 5-carbon unit isopentenyl pyrophosphate (IPP) with allylic prenyl pyrophosphates to produce polyisoprenoid chains of variable length (Meganathan 2001). Prenylated proteins with short farnesyl or geranylgeranyl isoprenoid chains have been identified in *T. brucei* (Field et al. 1996). They can be synthesized by farnesyl diphosphate synthase, farnesyl transferase, and solanesyl diphosphate synthase (Buckner et al. 2002; Montalvetti et al. 2003; Opperdoes and Szikora 2006). All these genes have been identified in trypanosomatids and *B. saltans*.

The mevalonic acid pathway

Dolichol, UQ and ergosterol are all formed by the condensation of IPP units to the polyprenyl-containing compounds. In most eukaryotes, IPP is synthesized via the mevalonic acid (MVA) pathway (Fig. 2) (Meganathan 2001). The enzymes of the pathway are distributed over cytosol, glycosomes, mitochondrion, and endoplasmic

Species	Enzymes
B. saltans	1 2 3 4 5 6
P. confusum	1 2 3 4 5 6
T. cruzi	1 2 3 4 5 6
T. vivax	1 2 3 4 5 6
T. congolense	(1) 2 3 4 5 (6)
T. brucei	1 2 3 4 5 6
B. ayalai	1 2 3 4 5 6
Phytomonas sp. EM1	1 2 3 4 5 6
Phytomonas sp. HART1	1 2 3 4 5 6
L. major	1 2 3 4 5 6
C. fasciculata	1 2 3 4 5 6
L. pyrrhocoris	1 2 3 4 5 6
L. seymouri	1 2 3 4 5 6

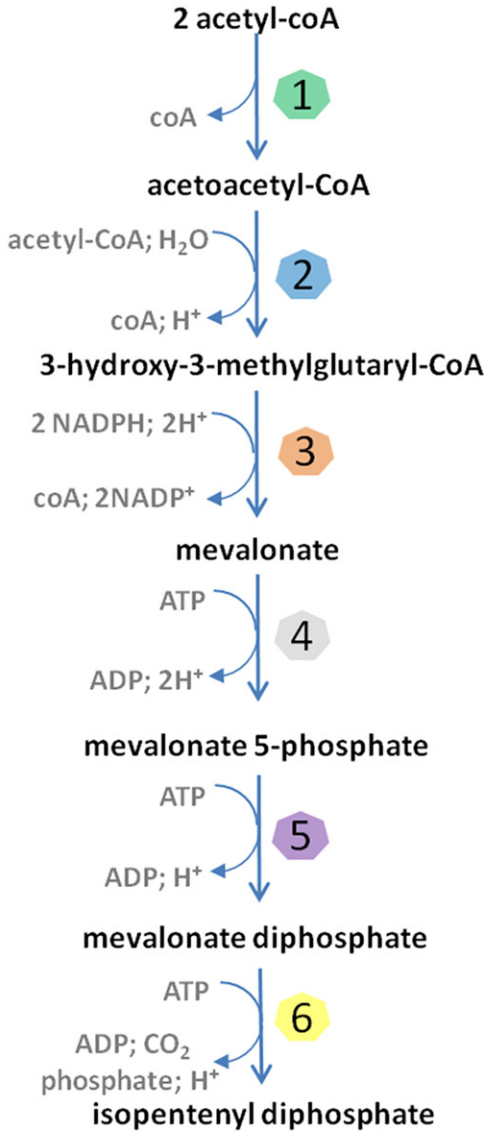

Figure 2 The mevalonic acid pathway.The enzymes catalyzing the reactions are shown as hexagons of different colors. A hexagon without color fill in the table indicates the absence of the gene encoding an enzyme in the corresponding species. A hexagon with red color fill represents the presence of a mitochondrial isoenzyme only. The first step, normally catalyzed by cytosolic acetoacetyl-CoA thiolase, is the condensation of an acetyl group from one acetyl-CoA with another acetyl-CoA resulting in the formation of acetoacetyl-CoA. In the second step, another molecule of acetyl-CoA condenses with acetoacetyl-CoA, resulting in the formation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). This step is catalyzed by a mitochondrial HMG-CoA synthase. Mitochondrial HMG-CoA is subsequently reduced to mevalonate by mitochondrial HMG-CoA reductase in a two-step reaction requiring 2 moles of NADPH. Mevalonate is converted to mevalonate diphosphate by two phosphorylation reactions mediated by glycosomal mevalonate kinase and phosphomevalonate kinase. In the next step mevalonate diphosphate undergoes dehydration-decarboxylation in an ATP-dependent reaction catalyzed by diphosphomevalonate decarboxylase. The resulting product is the C5 building block IPP.

reticulum. The first step of the pathway is usually catalyzed by acetoacetyl-CoA thiolase in the cytosol (Fig. 2). Cytosolic ortholog of this enzyme is absent in the African trypanosomes and *Phytomonas* spp., with the former having only the mitochondrial isoenzyme 3-ketoacyl-CoA thiolase (Mazet et al. 2011). The second step, catalyzed by mitochondrial hydroxy-methylglutaryl-CoA synthase (HMG-CoA synthase), was found in all kinetoplastids. Interestingly, this enzyme, which is in many trypanosomatid genomes annotated as a hypothetical protein, turns out to be more closely related to proteobacterial HMG-CoA synthases than to those from other eukaryotes (Fig. S3). However, the first and the second enzymes of the MVA pathway can be bypassed in trypanosomatids, as they are also able to form mitochondrial HMG-CoA from the breakdown of the amino acid leucine. Thus, mitochondrial HMG-CoA is directly incorporated into sterols via the isoprenoid synthetic pathway (Ginger et al. 2001). Other enzymes participating in the synthesis of IPP are present in all kinetoplastids, except that diphosphomevalonate decarboxylase was not detected in *T. congolense*. IPP isomerase catalyzes the isomerization of IPP to dimethylallyl diphosphate (DMAPP) inside glycosomes, although the *T. brucei* protein is not equipped with PTS1. Phylogenetic analysis suggests that this isomerase is of bacterial origin and was thus acquired by LGT (Opperdoes and Szikora 2006). In the next step, the enzyme geranyl diphosphate (GPP) synthase, in all kinetoplastids preceded by a mitochondrial targeting signal, catalyzes the sequential 1–4 coupling of IPP with DMAPP, resulting in the formation of C10 GPP. Putative cytosolic farnesyl diphosphate (FPP) synthase elongates GPP with an additional IPP to form C15 FPP. Enzymes synthesizing longer isoprenoid chains, the products of which are incorporated into dolichols and UQ, have been scarcely studied in trypanosomatids (Ferella et al. 2006; Lai et al. 2014).

The ubiquinone branch of the mevalonic acid pathway

Ubiquinone 9 (UQ9) plays a central role in mitochondrial respiration. Although UQs of different lengths have been found in various parasitic protists, so far only UQ9 was detected in trypanosomatids (Ranganathan and Mukkada 1995). The length of the ubiquinone prenyl side chain depends on the specificity of polyprenyl diphosphate synthase, and is constant within each organism. In *T. brucei,* the side chain of UQ9 is formed by further elongation of GPP by long-chain solanesyl diphosphate synthase. Unexpectedly, this enzyme has been localized to the mitochondrion of *T. brucei* (Lai et al. 2012), while in *T. cruzi* it is associated with the glycosomes (Ferella et al. 2006). However, it was not detected in *B. saltans* and *T. congolense*.

Few experimental studies have been done on the biosynthesis of the quinoid ring of UQ9 in trypanosomatids. Since they have no shikimate pathway, tyrosine is a likely substrate for quinoid ring synthesis in these protists. Although the necessary tyrosine aminotransferase and hydroxyphenylpyruvate reductase are both absent in *T. brucei*, the African trypanosomes are known to accumulate and excrete large amounts of aromatic ketoacids into the blood of infected animals (El Sawalhy et al. 1998). It has been hypothesized that the conversion of tyrosine to 4-hydroxyphenylpyruvate and its subsequent reduction to 4-hydroxyphenyllactate is catalyzed by cytosolic aspartate amino transferase of broad substrate specificity with high activity on aromatic amino acids (Marciano et al. 2009). This is followed by the action of malate dehydrogenase and several other enzymes leading to the formation of UQ9 (Aranda et al. 2006; Klein et al. 2013). Interestingly, none of the enzymes involved in the biosynthesis of the quinoid ring structure was found in *B. saltans*, while they are invariably present in trypanosomatids. It is not known how *B. saltans* gains access to this essential redox component, but its phagotrophic life style, during which bacterial food or bacterial endosymbionts could provide a source of ubiquinone, may have facilitated the loss of the UQ biosynthetic pathway in this flagellate.

The dolichol branch of the mevalonic acid pathway

Dolichols are α-saturated polyprenyl alcohols of variable chain length. The *T. brucei* bloodstream stage and the *Leishmania amazonensis* promastigotes contain a limited spectrum of short chain dolichols and dolichol phosphates (11 and 12 isoprene residues), while the *L. amazonensis* amastigotes synthesize mainly polyprenols of 9 isoprene units, rather than dolichol (Arruda et al. 2008; Löw et al. 1991). Little is known about dolichol synthesis in *T. brucei*, with only one possible candidate dolichol biosynthetic enzyme, dehydrodolichyl diphosphate synthase 1, identified so far. This enzyme is present in all trypanosomatids and *B. saltans*.

Ergosterol biosynthesis

In trypanosomatids, the sterol biosynthetic pathway is required for the generation of ergosterol, a major constituent of the plasma membrane (Roberts et al. 2003). In certain blood-dwelling trypanosomes, such as the bloodstream stage of *T. brucei*, the sterol biosynthesis is repressed and the external cholesterol from the host is incorporated in their plasma membranes (Coppens and Courtoy 2000). The ergosterol biosynthetic pathway has been a subject of intense investigation as a potential target for antitrypanosome drugs in *Leishmania* spp. and *T. cruzi*, organisms with an active ergosterol synthesis (Urbina 2010). Nevertheless, in the absence of heme, *Phytomonas* spp. and likely also *Leishmania* spp. are able to incorporate lanosterol instead of ergosterol into their membranes (Kořený et al. 2012). In *T. brucei*, a number of ergosterol pathway enzymes have been identified (squalene synthase, squalene monooxidase, sterol 14-alpha-demethylase, CYP51, lanosterol 14-alpha-demethylase, and sterol 24-c-methyltransferase). They are all localized to the endoplasmic reticulum and are only expressed in the procyclic stage. All these enzymes were also found in *B. saltans*, as well as in the other trypanosomatids, indicating that they all are capable of incorporating ergosterol in their plasma membranes.

Protein prenylation

Protein prenylation is a post-translational modification, which involves the transfer of either a farnesyl or a geranylgeranyl moiety to C-terminal cysteine(s) of the target protein (Zhang and Casey 1996). It mediates both protein–protein and protein–membrane interactions. Protein prenylation enzymes (α- and β-subunits of farnesyl transferase and geranylgeranyl transferases of type I and II) were identified in all kinetoplastids (Buckner et al. 2002), including *B. saltans*.

Lipid metabolism

Bodo saltans requires a full complement of lipases for the breakdown of lipids of bacterial origin. Indeed, numerous lipases such as triacylglycerol lipase, monoglyceride lipase, phospholipases A2, B, C, and D, and lysophospholipase were identified in its genome. Released glycerol and fatty acids (FA) serve as energy substrates. Free FAs are handled by β-oxidation, while glycerol is oxidized to dihydroxyacetone phosphate (DHAP), which is subsequently metabolized by the glycolytic pathway.

A functional β-oxidation pathway has been described in the extracellular promastigote (Blum 1990) and the intracellular amastigote forms of *Leishmania* (Berman et al. 1987), which use it for the synthesis of a limited number of amino acids, but not as a major carbon source for central metabolism, because of the absence of a functional glyoxylate cycle (Saunders et al. 2014). Several enzymes involved in β-oxidation have been identified enzymatically or by proteomics (Hart and Opperdoes 1984; Rosenzweig et al. 2008), but the importance of fatty acid (FA) oxidation for the trypanosomatid metabolism is not yet fully understood.

In *B. saltans* and trypanosomatids, the liberated FAs are oxidized by β-oxidation after activation to acyl-CoA by several fatty acyl-CoA synthetases with different chain-length specificity and long-chain acyl-CoA ligase (Jiang and Englund 2001). Carnitine acyltransferases together with (acyl-CoA)-carnitine transporter are translocating them through the mitochondrial membrane. Only in the glycosomes of *B. saltans* long-chain FAs are shortened by a so-called peroxisomal fatty acyl-CoA oxidase, which carries PTS, and has never been found in trypanosomatids. Short chain FAs are β-oxidized in the mitochondrion by four mitochondrial acyl-CoA dehydrogenases, each with different chain-length specificity. The second and third steps of the pathway are carried out by enoyl-CoA hydratase and hydroxyacyl-CoA dehydrogenase, representing two activities of the trifunctional enoyl-CoA hydratase/enoyl-CoA isomerase/3-hydroxyacyl dehydrogenase. However, an ortholog of this protein was not found in *B. saltans*. The fourth step is catalyzed by mitochondrial 3-ketoacyl-CoA thiolase (Mazet et al. 2011) present in all Kinetoplastea, except *T. congolense*. Aside from the African trypanosomes and *Phytomonas* spp., the kinetoplastids also have a cytosolic thiolase. As in other eukaryotes, all three trypanosomatid β-oxidation proteins carry a mitochondrial transit peptide and are therefore most likely localized in the mitochondrion. Interestingly, *Phytomonas* spp. should not be capable of any β-oxidation, as they lack all acyl-CoA dehydrogenase isoenzymes and the trifunctional protein (Porcel et al. 2014).

The odd-chain FAs are oxidized to acetyl- and propionyl-CoA. Propionyl-CoA is then converted to succinyl-CoA via the methylmalonyl pathway, which has a patchy phyletic distribution, as it is present in *B. saltans* and the Leishmaniinae, but absent in *B. ayalai*, the African trypanosomes and *Phytomonas* spp. With five acyl-CoA synthases, four acyl-CoA dehydrogenases and one peroxisomal acyl-CoA oxidase, *B. saltans* is fully equipped to oxidize various FAs, but how this oxidation is repartitioned over glycosomes and the mitochondrion has not yet been investigated.

In addition to β-oxidation, *B. saltans* is capable of FA biosynthesis, both in the cytosol and the mitochondrion. Acyl carrier protein (ACP), keto-acyl synthase, and keto-acyl reductase participate in the mitochondrial type II FA synthesis. This mitochondrial pathway is likely involved in the formation of lipoic acid (Stephens et al. 2007). In *B. saltans*, glycosomes likely participate in ether-lipid biosynthesis, as DHAP acyltransferase and alkyl-DHAP synthase carry out its first two steps. Thus, *B. saltans* seems to incorporate ether lipids in the phospholipids of its membranes. Cytosolic FA synthesis in trypanosomatids is unique. While other eukaryotes utilize cytosolic type I fatty acid synthases, trypanosomatids use a microsomal elongase pathway for the de novo synthesis of FAs (Lee et al. 2007). The presence of three tandemly linked FA elongase genes in *B. saltans* suggests that it uses the same elongation machinery as do trypanosomatids.

Contrary to the situation in *T. brucei* (Colasante et al. 2009) and *Leptomonas* spp., a mitochondrial citrate carrier is present in *B. saltans* and *T. cruzi*. Thus, in these flagellates citrate that is formed within mitochondria can be exported to the cytosol where it is used for the FA synthesis. For *T. brucei* it was suggested that cytosolic acetyl-CoA is not derived from the mitochondrially generated citrate, but directly from acetate that is produced in the mitochondrion via either of two pathways: acetate:succinate CoA-transferase (ASCT) (Rivière et al. 2009; Van Hellemond et al. 1998) and acetyl-CoA thioesterase (Millerioux et al. 2012). After export of acetate to the cytosol, it must be converted into malonyl-CoA by the concerted action of acetyl-CoA synthetase and acetyl-CoA carboxylase (Rivière et al. 2009), which are both present in *B. saltans*.

Phospholipid metabolism

The first steps of phosphatidic acid (an intermediate common to the synthesis of triacylglycerols and glycerophospholipids) synthesis take place inside glycosomes. Glycerol 3-phosphate (G3P) is formed by the reduction of DHAP with glycosomal glycerol-3-phosphate dehydrogenase (G3PDH), or by the phosphorylation of glycerol with glycosomal glycerol kinase. Either DHAP or G3P undergo first acylation from a fatty acyl-CoA molecule (Heise and Opperdoes 1997). Multiple DHAP acyltransferase genes and a single G3P acyltransferase gene were identified in *B. saltans*. Ether-lipid biosynthesis is associated with

glycosomes as well (Heise and Opperdoes 1997; Opperdoes 1987). After formation of acyl-DHAP and the reduction of FA by a yet unidentified acyl-DHAP reductase, alkyl-DHAP is formed by alkyl-DHAP synthase. Genes encoding acyl-DHAP transferase and alkyl-DHAP synthase were found in all kinetoplastids. The second acyltransferase reaction of phosphatidic acid formation is catalyzed by 1-acyl-sn-glycerol-3-phosphate acyltransferase, which is invariably present in kinetoplastids. Since the enzyme lacks PTS, subsequent reactions of both phospholipid and ether-lipid biosynthesis most likely take place in the cytosol and endoplasmic reticulum.

The formation of triacylglycerols and neutral phospholipids from phosphatidic acid begins with a dephosphorylation, catalyzed by phosphatidate phosphatase. The resulting diacylglycerol is directly acetylated to form triacylglycerol by phospholipid:diacylglycerol acyltransferase, or can react with CDP-choline or CDP-ethanolamine to form phosphatidylcholine, or phosphatidylethanolamine, respectively. The homologs of ethanolamine-phosphate cytidylyltransferase and cholinephosphate cytidylyltransferase were found in both *B. saltans* and trypanosomatids. However, no candidate gene for the formation of phosphatidylcholine from phosphatidyl-ethanolamine by methylation was detected in trypanosomes and *B. ayalai*.

Finally, acidic phospholipids can be formed from phosphatidic acid after its reaction with CTP to form CDP-diacylglycerol. Both phosphatidylserine and phosphatidylinositol can be produced from the latter compounds by phosphatidylinositol synthase and phosphatidylserine synthase, with the latter enzyme being absent only from *T. cruzi*. Phosphatidylserine can be converted to phosphatidylethanolamine by phosphatidylserine decarboxylase. The formation of the carbon-phosphorus bond in naturally occurring phosphonates is catalyzed by phosphoenolpyruvate mutase (PEPM) (Sarkar et al. 2003). Its product, phosphonopyruvate, is subsequently converted into 2-aminoethylphosphonate, which is incorporated into phosphonolipids and proteoglycans. Apparently, this pathway is specific only to *B. saltans* and *T. cruzi,* because PEPM was not detected in any other kinetoplastid analyzed so far. The second enzyme of the pathway, aminotransferase was, however, found in all kinetoplastids except for the African trypanosomes and *Phytomonas* spp. Moreover, 2-aminoethylphosphonate was detected in *T. cruzi*, but not in *T. brucei* (Ferguson et al. 1982).

Out of phospholipases required for the degradation of phospholipids, phospholipase A1 was detected only in trypanosomatids, while phospholipase A2 homolog, lysophospholipase, phospholipase C, myo-inositol-monophosphatase, and phosphatidic-acid phosphatase, but not phospholipase D, were found in all kinetoplastids. A homolog of the *T. brucei* glycosylphosphatidylinositol-specific phospholipase C is absent only from *Phytomonas* spp.

Amino acid metabolism

Amino acids of bacterial origin are a major source of energy, carbon and nitrogen for *B. saltans*. Indeed, a particularly high number of genes involved in the breakdown of polypeptides, with annotations such as protease, peptidase, cathepsin, calpain, etc., were detected in the *B. saltans* genome. Some amino acids can be catabolized into the TCA cycle intermediates in the mitochondrion. The nonessential amino acids along with threonine and methionine can be synthesized by kinetoplastids.

L-Ala can be formed from D-Ala (enzyme #1 in Table 2) and then converted to pyruvate (by enzyme #2). D-Ala is an amino acid of the peptidoglycan cell wall of some bacteria and is likely to be present in the insect midgut. Asp and Asn can be synthesized from oxaloacetate (by #3 and #4, respectively). Asp may also be produced from the Asn by asparaginase (#5) and subsequently converted to fumarate via the purine-nucleotide cycle (#6). Another nonessential amino acid, Arg is an important intermediate of the urea cycle (see below) and is also a predecessor of polyamines. Only *C. fasciculata* and *Leptomonas* spp. have the capacity to synthesize Arg from citrulline and Asp. Cys can be produced from homocysteine via *trans*-sulfurilation reaction (by #7 and #8) or by de novo synthesis from Ser (by #9 and #10). It is further metabolized to acetyl-CoA via mercaptopyruvate. Although a specific Cys transaminase (#11) has not been detected in kinetoplastids, they can convert cyanide and mercaptopyruvate into pyruvate and thiocyanate (by #12) (Williams et al. 2003).

Glu and Pro, very abundant amino acids of the insect midgut, are used by trypanosomatids as a major source of carbon and ammonia (Bringaud et al. 2012). In *B. saltans* and trypanosomatids, Glu and Gln are formed from 2-ketoglutarate (by #3 and #13, respectively). Glutaminase (#14), catalyzing the conversion of Gln to Glu, is absent. In turn, Pro is oxidized to glutamic acid (by #15 and #16), which is then oxidatively deaminated to a TCA cycle intermediate 2-oxoglutarate by the mitochondrial NAD-dependent glutamate dehydrogenase (#17). *T. cruzi* and Leishmaniinae possess an additional gene encoding the cytosolic NADP-dependent isoenzyme (#18), closely related to that of γ-proteobacteria. Hence, it must have been acquired by LGT at the basal node of trypanosomatids. However, the true function of this enzyme in trypanosomatids is not clear (Barderi et al. 1998). Only Leishmaniinae are able to synthesize Pro from Glu (by #19 and #20) since other trypanosomatids lack one or both enzymes. However, in *B. saltans*, Pro synthesis is possible from ornithine (by #21 and #22). Interestingly, *B. saltans* is the only kinetoplastid flagellate capable of formation of L-glutamate 5-semialdehyde, an intermediate in the Pro biosynthesis (by #21).

Gly is formed from Ser by serine hydroxymethyl transferase (#23). It is split into carbon dioxide and formic acid by the glycine-cleavage system (#24). Met can be either synthesized de novo from homoserine or salvaged from homocysteine by two isofunctional enzymes (#25 and #26). As most microorganisms, *B. saltans* and Leishmaniinae have genes encoding both enzymes, while *Phytomonas* spp. and *T. cruzi* have lost them. Met can also be regenerated via the methionine salvage cycle (Fig. 3). Since eight of nine genes involved in the cycle were

Table 2. Enzymes of the amino acid metabolism in kinetoplastids

#	Enzyme	Amino acid	Bsal	Pcon	Tcru	Tviv	Tcon	Tbru	Baya	PEM	PHA	Lmaj	Cfas	Lpyr	Lsey
1	alanine racemase	Ala													
2	alanine aminotransferase	Ala													
3	aspartate aminotransferase, mitochondrial	Asn, Asp													
4	asparagine synthase	Asn, Asp													
5	asparaginase	Asn, Asp													
6	purine-nucleotide cycle enzymes*	Asn, Asp													
7	cystathionine β-synthase	Cys													
8	cystathionie γ-lyase	Cys													
9	serine acetyltransferase	Cys, Ser													
10	cysteine synthase	Cys													
11	cysteine transaminase	Cys													
12	mercatopyruvate sulfurtransferase	Cys													
13	glutamine synthetase	Gln													
14	glutaminase	Gln, Glu													
15	proline oxidase	Pro													
16	P5CDH	Pro													
17	glutamate dehydrogenase (NAD)	Pro													
18	glutamate dehydrogenase (NADP)	Pro													
19	γ-glutamyl kinase	Pro													
20	γ-glutamylphosphate reductase	Pro													
21	ornithine aminotransferase	Orn													
22	proline oxidase	Pro													
23	serine hydroxymethyltransferase**	Gly, Ser													
24	glycine-cleavage system***	Gly													
25	methionine synthase (B12)	Met													
26	methionine synthase	Met													
27	branched chain aminotransferase	Ile, Val													
28	ACADSB	Ile, Val													
29	methylmalonyl-CoA epimerase	Ile, Val, Met													
30	methylmalonyl-CoA mutase	Ile, Val, Met													
31	HMG-CoA synthase/lyase	Leu													
32	phenylalanine-4-hydroxylase	Phe													
33	TDO	Trp													
34	kynurenine 3-monooxygenase	Trp													
35	kynureninase	Trp													
36	HAD	Trp													
37	ACMSD	Trp													
38	3-PGDH	Ser													
39	phosphoserine phosphatase	Ser													
40	threonine dehydratase	Ser, Thr			ps										
41	histidine-ammonia lyase	His													
42	urocanate hydratase	His													
43	imidazolonepropionase	His													
44	formiminoglutamase	His													
45	lysine ketoglutarate reductase	Lys													
46	saccharopine dehydrogenase	Lys													
47	aldehyde dehydrogenase	Lys													
48	AADAT	Lys													
49	ornithine decarboxylase	Orn													
50	homoserine kinase	Thr													
51	threonine synthase	Thr													
52	threonine dehydrogenase	Thr				ps									

*Enzymes of this cycle, that is, adenylosuccinate synthase, adenylosuccinate lyase, and AMP deaminase are all present. **has cytosolic and mitochondrial isoenzymes. ***the subunits (E, H, and T) of this enzyme complex were detected in all kinetoplastids. Only P subunit was not detected in *Phytomonas*. ps, pseudogene; P5CDH, delta-1-pyrroline-5-carboxylate dehydrogenase; ACADSB, short/branched chain acyl-CoA dehydrogenase; TDO, tryptophan-dioxygenase oxidoreductase; HAD, 3-hydroxyanthranilate-dioxygenase; ACMSD, 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase; 3-PGDH, 3-Phosphoglycerate dehydrogenase; AADAT, kynurine/2-aminoadipate aminotransferase; Bsal, *B. saltans,* Pcon, *P. confusum,* Tcru, *T. cruzi,* Tviv, *T. vivax,* Tcon, *T. congolense*, Tbru, *T. brucei*; Baya, *B. ayalai*, PEM, *Phytomonas* EM1; PHA, *Phytomonas* HART1; Lmaj, *L. major*; Cfas, *C. fasciculata*; Lpyr, *L. pyrrhocoris*; Lsey, *L. seymouri.*

identified in *B. saltans*, this cycle was already present in its common ancestor with trypanosomatids. It is functional in all trypanosomatids, except for *T. cruzi* and the African trypanosomes, which lost one and two genes, respectively.

Contrary to other eukaryotes, the Tyr aminotransferase reaction is catalyzed by a homolog of the cytosolic Asp aminotransferase with unusually broad substrate specificity (Vernal et al. 1998). Met is utilized for methylation reactions and for the formation of polyamines (see below). In Leishmaniinae and *B. saltans*, it can also be converted to 2-ketobutyrate in the cytosol and subsequently oxidized in the mitochondrion to succinyl-CoA, while in other kinetoplastids, Met cannot be oxidized beyond the stage of propionyl-CoA.

Neither the aromatic amino acids (Phe, Tyr, or Trp), nor any of the branched amino acids (Leu, Ile, and Val) can be synthesized by kinetoplastids, although multiple genes encoding enzymes for their degradation are present. In *B. saltans* and Leishmaniinae, Ile and Val are transaminated in the cytosol to ketocarboxylic acids (by #27). They are further oxidized in the mitochondrion (by #28) to propionyl-CoA and the TCA cycle intermediates. However, because of the absence of genes encoding two important enzymes (#29 and #30), *B. ayalai*, *Trypanosoma* spp., and *Phytomonas* spp. lack the capacity to oxidize propionyl-CoA any further. Succinyl-CoA and acetyl-CoA are also intermediates of the mitochondrial ASCT cycle. Leu is converted to HMG-CoA, which is either cleaved into acetyl-CoA and acetoacetate (by #31) or directly incorporated into sterols via the isoprenoid synthetic pathway (Ginger et al. 2001).

The enzymes of the classic aerobic pathway of aromatic amino acid oxidation are missing in kinetoplastids. Although in Leishmaniinae, *B. saltans* and *B. ayalai* Phe can be converted to Tyr (by #32), the other enzymes necessary for the conversion of the latter metabolite to end-products of the aerobic aromatic amino acid metabolism are missing. However, the first three enzymes of the anaerobic degradation pathway were identified in *B. saltans*. This suggests that phenylacetate can be formed from Phe, a situation similar to what has been reported in trypanosomatids (Berriman et al. 2005; El Sawalhy et al. 1998). The pathway of Trp degradation is operational in *B. saltans* (#33–37), while the entire pathway has been lost in trypanosomatids. This is probably a consequence of the parasitic lifestyle: the need for carbon from amino acids is largely compensated for by an abundance of sugars from the host.

Ser and His cannot be synthesized de novo by trypanosomatids and *B. saltans*. The first enzyme of the Ser synthesis (#38) is present in several species (Table 2), but the second enzyme (#39) was not detected. Since Thr dehydratase (#40), which acts upon Ser as well, was not found in *B. saltans*, it is likely that this gene was acquired after the separation of *B. saltans* and trypanosomatids. Through the action of Ser acetyl transferase (#9) and Cys synthase (#10), Ser is converted to Cys and then by Cys desulfurase to pyruvate (this pathway is shared by most kinetoplastids, except for the African trypanosomes and *Phytomonas* spp.). In the Ser-driven C1 metabolism, Ser is converted to Gly (Fig. S2), which is then converted to ammonia and carbon dioxide by the mitochondrial Gly cleavage system. The degradation pathway from His to Glu is present in *B. saltans* and *T. cruzi* (#41–44). Only *B. saltans, Leptomonas* spp. and *C. fasciculata* are capable of Lys synthesis from bacterial diaminopimelate. While trypanosomatids have also lost the Lys degradation pathway, *B. saltans* carries all but one of the enzymes of this pathway (#45–48). Ornithine is decarboxylated (by #49) to putrescine and used for the polyamine biosynthesis. Ornithine decarboxylase is absent from *B. ayalai* and *T. cruzi*. The latter species has been shown to be a diamine auxotroph (Ariyanayagam and Fairlamb 1997).

In kinetoplastids, Thr is formed from homoserine rather than from Asp (by #50 and #51), and Thr can be degraded in two different ways (Opperdoes and Michels 2008a). In the African trypanosomes, Thr dehydrogenase (#52) is responsible for its conversion into Gly and acetyl-CoA via an intermediate L-2-amino-3-oxobutanoate (Cross et al. 1975). Alternatively, Thr is catabolized by Thr dehydratase (#40) to ammonia and 2-ketobutyrate, which is irreversibly converted to propanoyl-CoA and formate. The first pathway appears to be ancestral, since Thr dehydrogenase is present in *B. saltans* and all *Trypanosoma* spp. The common ancestor of trypanosomes and *B. ayalai* must have acquired the Thr dehydratase gene, which was retained by *Phytomonas* spp. and Leishmaniinae, which then lost the dehydrogenase gene. It is also intriguing that *B. ayalai* retained both genes, which may indicate that the Thr pathway enzymes are either differentially expressed or their activity is precisely regulated.

The urea cycle and related reactions

The classic urea cycle consists of five reactions, two mitochondrial and three cytosolic (Fig. S4). The cycle is fed by mitochondrial carbamoylphosphate (CP), formed by ammonia-dependent CP synthase (CPS). Organisms that can easily remove an excess of ammonia by simple diffusion usually lack a functional urea cycle and have only a cytosolic glutamine-dependent CPS, but not an ammonia-dependent mitochondrial isoenzyme.

The urea cycle was retained only partly and has undergone many gene gains and losses. Three distantly related arginases were identified in kinetoplastids. *B. saltans* has genes encoding two widely different arginase isoenzymes, with one of them also present in *T. cruzi*, but lost in all other trypanosomatids. The latter enzyme is a formimidoglutamase rather than an arginase. The other arginase gene has been lost by all trypanosomatids, while a new arginase gene appears again in the Leishmaniinae clade (Gaur et al. 2007; Opperdoes and Michels 2008a). Both *B. saltans* and Leishmaniinae arginases are glycosomal. The proper compartmentalization of the *L. amazonensis* arginase has been shown to be important for its activity and for retaining wild type infectivity (da Silva et al. 2012). Phylogenetic reconstruction revealed that the

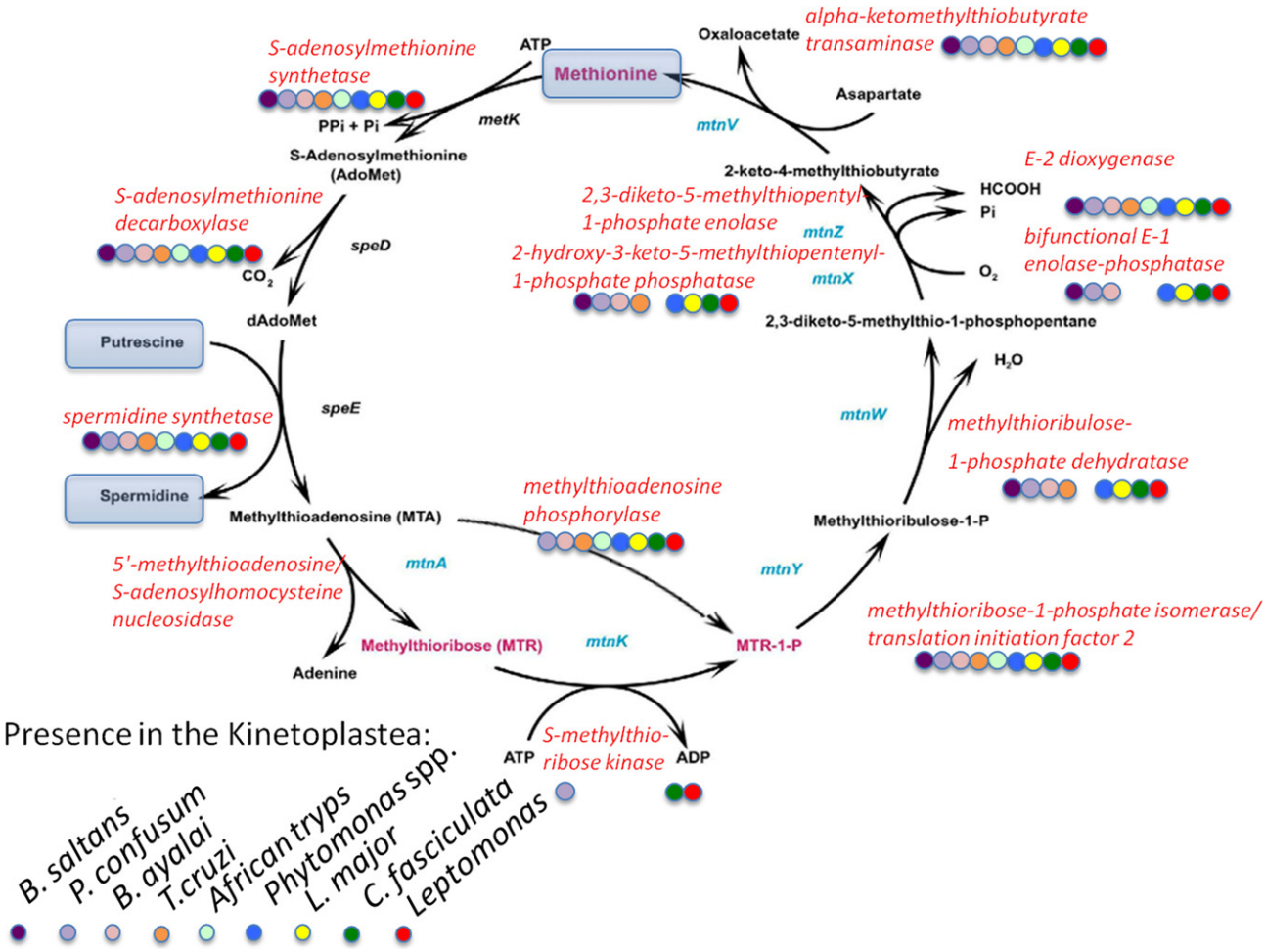

Figure 3 Methionine salvage cycle in kinetoplastids. The cycle functions to regenerate methionine for use as an aminopropyl donor in polyamine synthesis.

Leishmaniinae gene has been acquired by LGT from fungi. Similarly, the argininosuccinate synthase and argininosuccinate lyase genes of Leishmaniinae must have been acquired by LGT. These two genes are closely related to their respective homologs from a *Clostridium*-like bacterium, where the two genes are juxtaposed. Moreover, in *L. pyrrhocoris* and *C. fasciculata* these genes are still tandemly linked. In *Leishmania* spp. the lyase was lost, while in *L. seymouri* the synthase was lost and the lyase was mutated into a pseudogene. The simultaneous presence of the synthase and lyase in *C. fasciculata* and *L. pyrrhocoris* predicts that they must be able to utilize exogenous citrulline, an amino acid abundant in certain fruits.

Simultaneous acquisition of three urea cycle enzymes by a predecessor of *Leishmania* may have predisposed it for survival inside the phagolysosomal compartment of macrophages. The flagellate's arginase might have lowered the host cell's intra-lysosomal Arg, which is required for the formation of nitric oxide and peroxinitrate inside the lysosome, while citrulline, produced as a by-product, might have served as a substrate for parasite's polyamine synthesis.

Thus, the urea cycle is not operational in trypanosomatids, but some of the enzymes have been retained. Gains and losses of the urea cycle genes (Fig. S4) are in agreement with the reported widely different ureotelic nature of trypanosomatids, which can excrete urea, ammonia, or both. *Leishmania, Leptomonas* spp. and *C. fasciculata* are ureotelic, but can be, same as *Trypanosoma* spp. ammonotelic, depending on the composition of growth media (Yoshida and Camargo 1978).

Polyamine biosynthesis

Polyamines are positively charged di-cations that neutralize charges on nucleic acids and stabilize membranes and certain enzymes (Lindemose et al. 2005). They are essential for normal cell growth. In *B. saltans*, arginase converts Arg to ornithine and urea (Fig. S4), with putrescine being then formed from ornithine by ornithine decarboxylase. In the following reaction, spermidine synthase converts

putrescine to spermidine. Therefore, *B. saltans* synthesizes polyamines from ornithine in a way similar to trypanosomes. In addition, Leishmaniinae (except for *L. seymouri*) possess a gene for Lys decarboxylase producing cadaverine. In some organisms Arg may serve as the substrate for polyamine biosynthesis via the alternative agmatine pathway (Janowitz et al. 2003; Nakada and Itoh 2003). However, Arg decarboxylase, required for the conversion of Arg to agmatine, is absent in kinetoplastids. Trypanosomatids lost arginase and acquired an agmatinase-like protein, a distant arginase homolog with unknown function (Hai et al. 2015). It may be responsible for the conversion of Arg to polyamines via the classic ornithine pathway. However, although Leishmaniinae acquired an additional arginase of fungal origin (see above), they all retained the agmatinase gene, which suggests that the latter does have a redundant arginase function. *T. cruzi* lost the ornithine decarboxylase gene, became a polyamine auxotroph, and shares with *Leptomonas* a gene for a polyamine transporter. A similar gene is reported to be absent in *T. brucei* (Hasne and Ullman 2011).

Vitamins and cofactors

The available genomic information indicates that kinetoplastids are auxotrophic for a number of exogenous cofactors and vitamins. They can synthesize neither biotin (vitamin B7), nor cobalamin (vitamin B12). Although these flagellates possess several cobalamine-biosynthesis proteins, they are all related to the same bacterial *CobW* gene. *B. saltans* and some trypanosomatids encode cobalamine adenosyltransferase catalyzing the conversion of cobalamin into one of its coenzyme forms, adenosylcobalamine (coenzyme B12). Nevertheless, they are not capable of forming the complex corrin ring structure of cobalamin.

Unlike trypanosomatids, *B. saltans* can synthesize its own thiamine (vitamin B1), since both thiamine-phosphate diphosphorylase and a thiamine biosynthesis-like protein are encoded in its genome. *B. saltans* also converts nicotinamide to NAD and NADP, and coenzyme A can be formed from pantothenic acid. In *B. saltans* and trypanosomatids, pyridoxin (vitamin B6) is converted to pyridoxal phosphate and riboflavin (vitamin B2) to flavin mononucleotide. *B. saltans* possesses a gene for ascorbate-dependent peroxidase and can thus synthesize ascorbic acid (vitamin C). It also contains gulonolactone oxidase, the last enzyme of the ascorbate biosynthetic pathway, which in trypanosomatids functions as D-arabinono-lactone oxidase (Logan et al. 2007). The presence of several copies of arabinose dehydrogenase suggests that the yeast pathway for the synthesis of erythroascorbate, which is operational in trypanosomatids, may also function in *B. saltans*.

Heme is an essential growth factor for trypanosomatids and is required for many heme-containing proteins, such as cytochromes and catalase (Tripodi et al. 2011). It is synthesized from succinyl-CoA produced by the TCA cycle. However, trypanosomatids lack the genes of the heme biosynthetic pathway and are thus heme auxotrophs, acquiring heme from their hosts via a dedicated haptoglobin-hemoglobin receptor (Vanhollebeke et al. 2008). In Leishmaniinae, the last three enzymes of the pathway (coproporphyrinogen III oxidase, protoporphyrinogen oxidase, and ferrochelatase) have been acquired from bacteria by LGT (Kořený et al. 2010). It is not clear what purpose they serve for. *Phytomonas* spp. and *B. ayalai* possess only the ferrochelatase gene. Except for *Phytomonas* spp., all trypanosomatids also encode protoheme IX farnesyltransferase that converts heme *b* into heme *a*. This gene is indicative of the presence of a classic respiratory chain with the cyanide-sensitive cytochrome *c* oxidase containing heme aa3. The absence of such a gene in *Phytomonas* spp. is thus in agreement with the fact that these plant parasites lack all cytochromes.

Folate metabolism

Kinetoplastids are not capable of synthesizing folates, such as folate and biopterin, which must be imported from exogenous sources. However, in contrast to trypanosomatids, which have multiple copies of the folate/pteridine transporter gene, *B. saltans* has just a single copy.

The enzymatic reduction of folate to tetrahydrofolate (THF), a coenzyme required for C1 transfer reactions (Fig. S2), is catalyzed by bifunctional dihydrofolate reductase–thymidylate synthase (DHFR-TS). In trypanosomatids a similar reduction is also carried out by pteridine reductase (PTR1), but a gene for this enzyme was not found in the *B. saltans* genome. PTR1 is closely related to aldoketo reductases from proteobacteria and has no obvious eukaryotic homolog. It is plausible that the acquisition of the PTR1 gene via LGT was an adaptation of an ancestral trypanosomatid to the parasitic life style. PTR1, in addition to pteridine reductase activity, may also act as a quinonoid dihydropteridine reductase (QDPR) and this wide substrate specificity of PTR1 may explain the why the QDPR gene is now missing in *T. brucei* (Ong et al. 2011). The QDPR gene has been found in *B. ayalai, T. cruzi, Leishmania* spp. (7 copies), *C. fasciculata*, and *Leptomonas* spp. (2 copies).

The activation of C1 units by conjugating with THF is carried out by either Ser hydroxymethyl transferase (SHMT) or the glycine-cleavage system (GCS). In kinetoplastids other than the genus *Trypanosoma*, two SHMT isoenzymes, one cytosolic and one mitochondrial, were identified. The activated C1 units are used in the synthesis of thymidylate by the thymidylate-synthase domain of DHFR-TS or for the formation of Met from Cys by methionine synthase. Formyl-C1 units are also used for the formation of formyl-Met, which is essential for the initiation of mitochondrial protein synthesis. However, other THF-dependent formyl transferases such as those involved in the biosynthesis of either purines or His, are all absent, and this correlates with the absence of these

biosynthetic pathways in trypanosomatids. Interestingly, formate-THF ligase, methylene-THF-cyclohydrolase and methylene-THF dehydrogenase reactions, which in vertebrates are carried out by trifunctional enzyme C1-THF synthase, are present in kinetoplastids as a separate ligase and a bifunctional cyclohydrolase–dehydrogenase enzyme (Opperdoes and Coombs 2007).

CONCLUSIONS

Arguably, the apicomplexan and kinetoplastid protists developed the most successful parasitic strategies. Recently, the genomes of *Chromera* and *Vitrella*, two protists that constitute a free-living sister group of obligatory parasitic apicomplexans, have been analyzed in order to shed light on the emergence of parasitism (Janouškovec and Keeling 2016; Woo et al. 2015). Indeed, the intention of presented comparative analysis of the *B. saltans* genome with several trypanosomatid genomes was to bring novel insight into the emergence of parasitism from a free-living lifestyle.

A general observation is that abundant LGT of genes of bacterial origin is encountered in both bodonid and trypanosomatid lineages. Genes coding for enzymes of glycolytic, pentose phosphate and pyrimidine biosynthetic pathways, as well as genes for trypanothione reductase and folate/(bio)pterin transporters, were already acquired by the common ancestor of bodonids and trypanosomatids. The subsequent adoption of a parasitic life style led to the loss of complete metabolic pathways, such as lysine and histidine catabolism and aromatic amino acid degradation. Also the presence of a gene encoding a functional trehalose-splitting enzyme would have provided an enormous evolutionary benefit to an ancient symbiont or opportunistic parasite by means of a direct access to one of the insect's major energy sources. It would have been essential for survival in, and adaptation to, an accidental insect host. In two separate trypanosomatid clades specialized in glucose metabolism, the African trypanosomes and *Phytomonas* spp., this specialization led a striking convergent evolution, where similar sets of genes were lost. On the other hand, the acquisition of novel genes involved in pteridine reduction, threonine dehydration, the urea cycle, protection against ROS, and diaminopimelate metabolism by LGT, may have increased the metabolic flexibility and the possibility to adapt to new hosts, making trypanosomatids one of the most successful group of parasites on Earth.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Comparison of the molecular properties of two phosphofructokisases (PFKs) of *B. saltans* and phylogenetic tree of PPi and ATP-dependent PFKs.
Figure S2. Serine-driven C1 metabolism in Kinetoplastea.
Figure S3. Phylogenetic tree of HMG-CoA synthases of kinetoplastid origin, from selected and other eukaryotes and of the delta-proteobacterium *E. salina.*
Figure S4. Urea cycle in Kinetoplastea.