Endosymbiosis in Trypanosomatids as a Model to Study Cell Evolution

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Abstract: Some trypanosomatids harbor a symbiotic bacterium, which maintains a close association with the host, constituting an excellent model to study organelle origin and cellular evolution. Molecular data show that all endosymbiont-harboring trypanosomatids are grouped together in a single phylogenetic branch. According to RNA sequences, symbionts of different species are similar, being classified in the β division of Proteobacteria, thus suggesting that a single evolutionary event gave rise to the symbiosis in the Trypanosomatidae family. The bacterium is enclosed by two unit membranes and presents a reduced peptidoglycan layer, which is essential for cell division and morphological maintenance. Regarding the protein composition, the number of intramembrane particles in the endosymbiont envelope is similar to that described for Gram-negative bacteria. Lipid analyses of purified endosymbionts show absence of sterols and indicate phosphatidylcholine as a major component of the envelope, as described in other intracellular bacteria. The presence of the endosymbiont is associated with distinct ultrastructural and physico-chemical alterations in the trypanosomatid and influences the protozoan interaction with the insect host and mammalian cells. Symbiont-containing trypanosomatids are able to infect and to replicate inside fibroblasts and macrophages, whose microbicidal activity was deactivated by HIV-1 infection. The symbiosis in trypanosomatids is characterized by intensive metabolic exchanges; the bacterium expresses enzymes and metabolic precursors that complete essential biosynthetic pathways of the protozoan. Conversely, the symbiont is capable of obtaining part of the required energetic molecules from the host glycosomes. Taken together data suggest that endosymbiosis in trypanosomatids represents an interesting model to study cell evolution.

Keywords: Trypanosomatidae, Proteobacteria, Endosymbiont, Organelle origin, Cellular evolution.

INTRODUCTION

There are numerous hypotheses to explain the origin of eukaryotes, all of them consider a co-evolution by endosymbiosis between microorganisms, as archaean and bacteria [1]. Trypanosomatids are valuable models to understand, at least in part, the origin of organelles in the eukaryotic cell, as well as the colonization and survival of symbionts and parasites within host cells. This group of flagellated protozoa is exclusively composed by parasites that live in association with plants and animals; some are agents of human illnesses, as Chagas disease and leishmaniasis, but the majority presents a single invertebrate host during all its life cycle, thus named monoxenous protozoa. Five species of insect trypanosomatids harbor an obligate intracellular bacterium. A mutualistic relationship maintains both organisms together, since the endosymbiont is associated with distinct ultrastructural and physico-chemical alterations in the trypanosomatid and influences the protozoan interaction with the insect host and mammalian cells. Symbiont-containing trypanosomatids are able to infect and to replicate inside fibroblasts and macrophages, whose microbicidal activity was deactivated by HIV-1 infection. The symbiosis in trypanosomatids is characterized by intensive metabolic exchanges; the bacterium expresses enzymes and metabolic precursors that complete essential biosynthetic pathways of the protozoan. Conversely, the symbiont is capable of obtaining part of the required energetic molecules from the host glycosomes. Taken together data suggest that endosymbiosis in trypanosomatids represents an interesting model to study cell evolution.

as bipolar bodies, a basophilic rod-like organelle, capable of self-reproduction and largely composed by ribonucleoproteins [5]. Thereupon, it was suggested that C. oncopelti contained a symbiotic bacterium, which provided the host trypanosomatid with biosynthetic capabilities [6]. Bipolar bodies also were reported on Crithidia deanei, a trypanosomatid isolated from the hemipteron Zelus leucogrammus. Such protozoan presented limited requirements for growth, reinforcing the idea that the symbiont supplied the host with essential nutrients [7]. According to ultrastructural analysis of antibiotic treated trypanosomatids, it was concluded that diplosomes and bipolar bodies corresponded to the same structure, thus called endosymbionts [8]. Later on, two new species of trypanosomatids containing endosymbiont were isolated from the fly Ornithia obesa: Crithidia desouzai and Herpetomonas roitmani [9, 10] (Table 1). Ultrastructural and immunocytochemical data revealed that C. desouzai also contains virus-like particles (VLPs) in the cytoplasm. Such particles, which present a hexagonal array, are usually observed close to the protozoan nucleus and are composed by RNA [11, 12].

Once treated with penicillin or chloramphenicol the host trypanosomatid looses the bacterium, thus generating a cured or apysymbiotic strain [5, 6, 13, 14]. Furthermore, it is possible to obtain a pure fraction of endosymbionts after cell fractioning of the host cell using sonication or immunolysis, followed by differential centrifugation in sucrose or Percoll gradients [5, 15-17]. The comparative study between symbiont-bearing and apysymbiotic strains, as well as biochemical

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assays with isolated bacteria have opened up new possibilities to study the endosymbiosis in trypanosomatids and to better understand the appearance of bacterial origin organelles in eukaryotic cells.

THE ENDOSYMBIOSIS IN TRYPANOSOMATIDS REPRESENTS A SINGLE EVENT IN EVOLUTION

Data obtained by isoenzyme eletrophoresis and also after sequencing the 16S ribosomal RNA gene revealed that endosymbiont-harboring trypanosomatids are grouped together in a monophyletic cluster, when compared to other protozoa of the same genus. Thus, *Blastocritidia*, *Crithidia* and *Herpetomonas* species containing the symbiotic bacterium compose an independent phylogenetic branch in relation to other protozoa of the same genera [10, 18-21] (Fig. 1). For this reason, a new taxonomic classification was proposed to cho-

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<th>Trypanosomatid Species</th>
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<tr>
<td><em>Blastocritidia culicis</em></td>
<td>Triatoma infestans</td>
<td>[4]</td>
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<tr>
<td><em>Crithidia deanei</em></td>
<td>Zelus leucogrammus</td>
<td>[7]</td>
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<td><em>Crithidia desouzai</em></td>
<td>Ornidia obesa</td>
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<td><em>Crithidia oncopelti</em></td>
<td>Oncopeltus fasciatus</td>
<td>[5]</td>
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<td><em>Herpetomonas roitmani</em></td>
<td>Ornidia obesa</td>
<td>[9, 10]</td>
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Table 1. Endosymbiont-Bearing Trypanosomatids Isolated from Insects

Fig. (1). Majority consensus likelihood SSU rRNA tree of Trypanosomatidae. Search options with fastDNAm program included empirical base frequencies, randomized sequence addition order and global arrangements. Ln-likelihood value of the maximum likelihood tree is – 9092.81296. Scale bar corresponding to 0.1 substitutions per site is shown under tree. Members of the polyphyletic genera *Herpetomonas* and *Crithidia* are shown in boldface. Reprinted from J Euk Microbiol 1998, 45: 293-297, Hollar et al. “Monophyly of endosymbiont containing trypanosomatids: phylogeny versus taxonomy”, with permission from Wiley-Blackwell, Oxford, UK.
anomastigote-shaped trypanosomatids that harbor the symbiont, with the inclusion of the genus Angomonas [22, 23].

Regarding the origin of the endosymbiont in trypanosomatid protozoa, molecular analysis of small-subunit ribosomal DNA showed that B. culicis and Crithidia symbionts share high identity, suggesting that they descend from a common ancestor, a β-Proteobacteria of the genus Bordetella [19, 24] (Fig. 2). The first study with the endosymbiont DNA showed a G+C content of 32%, which is similar to that described in most bacteria [25]. Taken together data suggest that the endosymbiosis in trypanosomatids represents a unique event, which involved the acquisition of a Bordetella Proteobacterium by an ancestral host, followed by the co-evolution of both partners into different species.

ULTRASTRUCTURAL AND BIOCHEMICAL CHARACTERISTICS OF THE SYMBIOTIC BACTERIUM

The endosymbiont in trypanosomatids is enclosed by two unit membranes, the inner one corresponds to the plasma membrane and is in contact with the bacterial matrix, while the outer membrane faces the host protozoan cytoplasm. The origin of the outer membrane is controversial; it could represent part of the cell envelope of a Gram-negative bacterium, but conversely it could be derived from the host protozoan [26, 27]. Data obtained by the freeze-fracture technique showed that the number and pattern of distribution of intramembranous particles (IMPs) on both faces of the symbiont outer membrane are significantly different from those of the host protozoan plasma membrane. However, such distribution of membrane particles is similar to that observed in Gram-negative bacteria [28, 29].

The intermembrane space in Gram-negative bacteria is designated as periplasmic space and is usually occupied by a peptidoglycan layer, also referred to as cell wall. Such structure is not observed in the symbiont of trypanosomatids, which differ from bacteria but similar to most mitochondrion lacking the septum and also the Z ring, structures which play essential roles in bacterial division [28-30]. However, when endosymbiont-harboring trypanosomatids are grown in medium containing β-lactam antibiotics, which blocks the peptidoglycan layer synthesis, the bacterium presents morphological alterations and is incapable of division, culminating in its cell lysis. These data indicate that a reduced cell wall may exist in the endosymbiont, playing important physiological roles as in shape maintenance and in bacterial division [17].

According to ultrastructural data, the symbiont matrix presents a granular electron-dense area containing ribosomes and a filamentous electron-lucid region with the genetic material [12, 28, 29]. The bacterium matrix also contains structural proteins, enzymes and metabolic intermediates that complement essential biosynthetic pathways of the host protozoa, as will be discussed later on.

Biochemical studies showed that similar to most prokaryotes, the endosymbiont envelope does not contain any type of sterol. Regarding the phospholipid composition, cardiolipin is the major constituent of symbiont membranes, followed by similar amounts of phosphatidylcholine (PC) and phosphatidylethanolamine and a minor quantity of phosphatidylinositol [31]. It is important to point out that PC, the major structural component in most eukaryotic cell membranes, is usually absent in prokaryotes, except in species closely associated with plant or animal hosts [32]. In such cases, PC is essential to maintain the symbiotic and pathogenic interactions, as well as the prokaryote virulence [33, 34].

The endosymbiont phospholipid content is significantly altered when the host protozoan is growth in the presence of lipid biosynthesis inhibitors, such as 22,26-azasterol, resulting in a decrease of the PC level in the bacterium [31]. When the host trypanosomatid is cultivated in medium containing 32Pi and then is submitted to cell fractioning, PC was the main radiolabeled phospholipid detected in the symbiont fraction. Interestingly, once isolated from the host cell and incubated in culture medium containing 32Pi, the endosymbiont is capable of synthesizing phospholipids independently from the protozoan, however the PC content is highly reduced [35]. Taken together, we suggest that the endosymbiont takes, at least in part, PC or PC precursors from the host cell, reinforcing the idea that this phospholipid contributes to the establishment of the symbiosis in trypanosomatids. Isolated symbionts are also able to synthesize proteins [36], indicating that although it maintains a close association with the host cell, it is still autonomous to produce essential molecules. Probably, the endosymbiont in trypanosomatids represents an intermediate evolutionary step between bacteria and organelles.

THE PRESENCE OF THE ENDSYMBIONT IS ASSOCIATED TO ULTRASTRUCTURAL AND BIOCHEMICAL ALTERATIONS IN THE HOST TRYPANOSOMATID

As with other protozoa from the Trypanosomatidae family, symbiont-containing species present: a single flagellum that emerges from a flagellar pocket, a unique and branched
mitochondrion whose DNA is contained in the kinetoplast, glycosomes a special type of peroxisome that contain the glycolytic pathway, and a cytoskeleton formed by subpellicular microtubules organized in a cage array [37]. The three-dimensional reconstruction of symbiont-harboring trypanosomatids indicates that the bacterium is in close association with the host cell nucleus and is usually surrounded by glycosomes [38, 39] (Fig. 3). The synchrony in cellular division is another remarkable feature of this symbiotic relationship: the endosymbiont divides in coordination with other cellular structures, thus each daughter cell carries just a single bacterium [2].

The presence of the symbiotic bacterium is related to ultrastructural modifications on the host trypanosomatid. In such case, the subpellicular microtubules are absent in sites where the mitochondrial branches are juxtaposed to the plasma membrane and the kinetoplast presents a typical round shape with a more relaxed arrangement of the kDNA fibers [40, 41]. Furthermore, the paraflagellar rod, which is involved in adhesion and cell motility, is greatly reduced in symbiont-harboring trypanosomatids [42-44] (Fig. 4). Surprisingly, such protozoa are able not only to attach to the insect midgut [3], but also to disrupt the tight junctions of epithelial cells to reach the haemocoele [45]. These results suggest that the paraflagellar rod is not essential to cellular motility and insect colonization, at least in symbiont-containing trypanosomatids. It is worth mentioning that removal of the symbiotic bacterium by antibiotic treatment did not reverse the protozoan characteristics [40].

The symbiotic association also promotes physicochemical alterations in the host trypanosomatids. Thus, the presence of the bacterium greatly influences the polysaccharide and glycoprotein composition, as well as the general carbohydrate exposition on the protozoan plasma membrane [46-50]. Measurements of cellular electrophoretic mobility revealed that the aposymbiotic strain of C. deanei presents a high negative surface charge, when compared to symbiont-harboring cells [51]. Furthermore, the absence of the symbiotic bacterium enhances de differentiation process in Herpetomonas roitmani. Thus, the ophistomastigote form predominates in cured cells as compared with the symbiont-harboring strain, in aging cell cultures [49].

**THE INTERACTION OF TRYPANOSOMATIDS WITH THE INSECT HOST AND MAMMALIAN CELLS IS INFLUENCED BY THE SYMBIOTIC BACTERIUM**

As previously assumed, monoxenous trypanosomatids parasite invertebrates, specially insects belonging to Diptera and Hemiptera orders [52]. The colonization of insects by trypanosomatids is directly dependent on the protozoa interaction with host cells and organs. The complete life cycle of
monoxenic trypanosomatids is not well established, but the insect midgut has been described as the preferential colonization site. However, such protozoa are also found within Malphigian tubules, as well as in the hemolymph and the haemocel [45, 52, 53].

Studies of cellular interaction showed that endosymbiont-bearing cells interact better with insect cell lines and explanted guts, when compared with their aposymbiotic counterpart strains. Such data revealed that Blastocrithidia culicis is the species which best associates with Diptera, in special with Aedes mosquitoes, thus constituting an attractive model to study the monoxenous protozoan-insect interaction. Furthermore, cured cells are unable to colonize insects, revealing that the symbiont is essential for the trypanosomatid existence in nature, which characterizes an obligate and mutualist association between both partners [3] (Fig. 5).

Several factors influence the interaction of endosymbiont-containing species with the insect midgut. Proteolytic enzymes play essential roles in host-parasite relationship, which includes cell attachment, survival and pathogenesis [54]. The metallopeptidase gp63, a major glycoprotein found in the leishmanial cell surface is also present in C. deanei. This protein is anchored to the plasma membrane via the glycosylphosphatidylinositol (GPI) and mediates the adhesive process of the protozoan to Aedes aegypti explanted guts, as demonstrated using anti-gp63 antibodies and phospholipase C (PLC). The higher expression of gp63-like molecules on the surface of symbiont-bearing trypanosomatids, when compared to the aposymbiotic cells, may be correlated with a more efficient interaction of the wild strain with insect guts [55].

Glycoconjugates are essential for a specific association between protozoa and host cells, which can be mediated by the interaction of carbohydrate-binding proteins, also known as lectin-like molecules [56-58]. Symbiont-containing and cured strains present different patterns of glycoconjugates, which influences the protozoa interaction with explanted guts from A. aegypti. The treatment of both strains of B. culicis and C. deanei with sialidase, reduces in at least 90%, the interaction between such trypanosomatids and explanted guts. Furthermore, the interaction of symbiont-harboring cells with insect guts was inhibited in the presence of mucin, fetuin, siaallylactose and methylmannosidase, thus indicating that sialomolecules and mannose-rich glycoconjugates are involved in monoxenous-host invertebrate interaction [50].

While considered nonpathogenic, infections caused by monoxenic trypanosomatids have been described in different vertebrate hosts [59, 60]. The ability of symbiont-harboring trypanosomatids to interact, to invade and to multiply inside host cells is related to the protozoan cell surface composi-
tion, which is influenced by the presence of the bacterium, as previously mentioned. It has been reported that once inside the host cell, the symbiont-harboring strain of *C. deanei* is more resistant to the macrophage killing mechanisms [61], also promoting increased cell death in infected fibroblasts [62], when compared to the aposymbiotic strain. *In vitro* assays demonstrated that symbiont-harboring trypanosomatids, but not the symbiont-free trypanosomatids are able to infect and to survive for 48h inside dermal mouse fibroblasts [63]. Since these cells are numerous in the skin, they can represent a potential target for early infection and spreading [64]. Recently, it was shown that the symbiont-harboring strain of *C. deanei* presents a higher interaction rate with mammalian fibroblasts at the very beginning of the interaction process (1-2h). However, as the assay proceeds (24-96h), the association index is similar in both strains, indicating that the protozoan ability to survive and replicate inside the host cell is independent of the presence of the endosymbiont. Such interaction is mediated by leishmanolysin, a surface metallopeptidase which promotes association between *Leishmania* and macrophages, which is highly expressed in symbiont-harboring *C. deanei* [62].

Interestingly, monoxenous trypanosomatids were isolated from HIV-positive patients, promoting visceral or cutaneous leishmaniasis-like lesions [65, 66]. Recently, it was demonstrated that *B. culicis* not only survive, but also replicates, in human macrophages whose microbicidal activity was deactivated by HIV-1 infection [67]. Taking together these data indicate that monoxenous trypanosomatids are opportunistic pathogens in immunocompromised patients [68].

**METABOLIC EXCHANGES BETWEEN THE ENDO-SYMBIONT AND ITS HOST TRYpanosomatID**

Mutualism is a symbiotic relationship between different organisms in which both partners gain benefits from the association. This concept usually assumes that individuals rely on one another for nutrients and other life functions. In endosymbiont-harboring trypanosomatids intense metabolic changes occur between the host cell and the bacterium: the prokaryote complement important biosynthetic pathways of the protozoa and in exchange receives growth factors, suitable physical conditions and energy supply.

Lwoff [69] was the first to report that *Crithidia oncopelti* presented low nutritional requirements and that differently from other trypanosomatids, this symbiont-containing species was able to grow in culture medium without any source of hemin. Later on, it was established that the bacterium-harboring species could grow in chemically defined medium containing few vitamins and aminoacids, as methionine and tyrosine, and usually with no source of hemin or purin. Such culture medium can not support the growth of naturally symbiont-free species or even the cultivation of aposymbiotic strains obtained after antibiotic treatment, suggesting that the endosymbiont benefits the growth of their hosts by supplying essential factors [7, 9, 49, 70-73]. According to this idea, endosymbiont-harboring trypanosomatids show a reduced secretion of extracellular proteinases, which presents a proteolytic digestive role, when compared to cured strains [72].

A special nutritional feature of flagellates is their requirements for heme compounds, usually supplied as hemin, hematin or hemoglobin. Trypanosomatids are able to catalyze the insertion of iron into protoporphyrin IX, indicating that it contains ferrochelatase, the terminal enzyme in the heme biosynthesis [73]. However, only symbiont-bearing protozoa dispense hemin for growth and this is related to the ability of the bacterium to complement the host pathway for heme biosynthesis with enzymes and metabolic precursors. It was demonstrated that the symbiotic bacterium contains uroporphyrinogen I synthase, thus enhancing the very limited heme biosynthetic capability of the host cell [73], as well as enzymes involved in porphyrin synthesis [74]. Taken together, data suggest that both partners, the symbiont and the
host trypanosomatid, do complementary and essential contributions to complete the heme biosynthetic pathway.

Regarding the metabolism of amino acids, differently from other trypanosomatids, symbiont-harboring protozoa are capable of isoleucine synthesis, since the first enzyme of this biosynthetic pathway is present in the symbiotic bacterium. Furthermore, species containing the endosymbiont do not require the metabolically related amino acids, valine and leucine [16]. Species bearing the symbiont are also autonomous for lysine production, due to the presence of diamino-pimelic decarboxylase, the last enzyme of this pathway, in the bacterium [6, 75].

Studies of the urea cycle in trypanosomatids have shown that symbiont-free species of *Crithidia* need exogenous arginine or citrulline in culture medium, but not ornithine, which does not substitute for either amino acids. In such protozoa the enzyme arginase converts arginine into ornithine and carbon dioxide by citrulline hydrolase. Differently, in symbiont-harboring protozoa the enzyme arginase converts arginine into ornithine, ammonia and carbon dioxide by citrulline hydrolase. Differently, in symbiont-harboring species of *Crithidia* the urea cycle is complete and such amino acids are not required for cellular growth. This is related to the presence of enzymes in the symbiotic bacterium, as acetylornithinase, which converts acetylornithine in ornithine, and ornithine carbamoyltransferase (OCT), which transforms ornithine in citrulline, as demonstrated by biochemical and cytochemical assays [76-78] (Fig. 6).

In trypanosomatids, the production of ornithine in urea cycle can lead to the simultaneous production of carbamoyl phosphate, used in the synthesis of pyrimidines, as well as to the synthesis of proline and putrescine, a type of polyamine [79-81]. Usually trypanosomatids do not require proline, a relevant metabolic factor, in the culture medium [82, 83]. In *C. deanei*, a single type of proline transporter is present in symbiont-harboring or symbiont-free strains, whose activity is up-regulated by the presence of the symbiotic bacterium [84]. The ornithine production is directly associated to the polyamine biosynthesis, which is catalyzed by the enzyme ornithine decarboxylase (ODC), which converts ornithine into putrescine [85, 86]. Endosymbiont-harboring trypanosomatids present higher ODC activity when compared to the aposymbiotic cells and this is related to the production of protein factors by the symbiotic bacterium, thus increasing the host polyamine metabolism [87]. Since polyamines are essential for cell growth and differentiation, the increased production of these polycations in symbiont-harboring trypanosomatids, explains in part why these protozoa grow faster in culture medium, when compared to symbiont-free trypanosomatids [26, 49].

There are scanty data indicating that the symbiont receives metabolites and nutritional factors from the host trypanosomatid. However, its inability to survive once isolated from the protozoan, strongly suggest that the bacterium obtains essential nutrients and metabolic precursors produced by the host cell. As previously mentioned the bacterium seems to obtain part of the phosphatidylcholine, a component of its envelope, from the host cell [35]. Other studies suggest that the symbiotic bacterium may obtain part of the energetic molecules required for its metabolism from host protozoan glycosomes, which are involved in ATP biosynthesis by the glycolytic pathway. This idea is reinforced by the low enzymatic activity of respiratory chain enzymes and by the presence of an ecto-ATPase in the symbiont surface, as also observed for other obligate intracellular bacteria. Taken together the data suggest that the endosymbiont in trypanosomatid strain hydrolyze ATP obtained from the host glycosomes to generate a proton motive force that would maintain the

![Fig. (6). Urea cycle in endosymbiont-containing (a) and endosymbiont-free (b) *Crithidia* species. Note that the enzyme ornithine carbamoyltransferase is only present in endosymbiont-bearing strains, closing the urea cycle in these protozoa. Conversely, the citrulline hydrodase is only found in endosymbiont-free species that need exogenous arginine or citrulline in culture medium, but not ornithine, which does not substitute for either amino acids.](image-url)
transport of compounds from the protozoan cytoplasm [38]. The investigation of nutritional aspects in symbiont-harboring trypanosomatids not only elucidates the bacterium-protozoan metabolic relationship, but also brings some light on regulation and biogenesis of organelles in the eukaryotic cell.

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