

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 rRNA 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 archaea 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 unable to survive and replicate once isolated from the host, whereas aposymbiotic trypanosomatids are incapable of colonizing insects [2, 3].

The endosymbiont of trypanosomatids was first described as diploosomes in *Blastocrithidia culicis*, a species isolated from the hemipteron *Triatoma infestans*, each flagellate usually harboring a single diplosome [4]. Fifty years later, a similar structure was described in *Crithidia oncopelti*

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 diploosomes 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 *Ornidia 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 loses the bacterium, thus generating a cured or aposymbiotic 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 aposymbiotic strains, as well as biochemical

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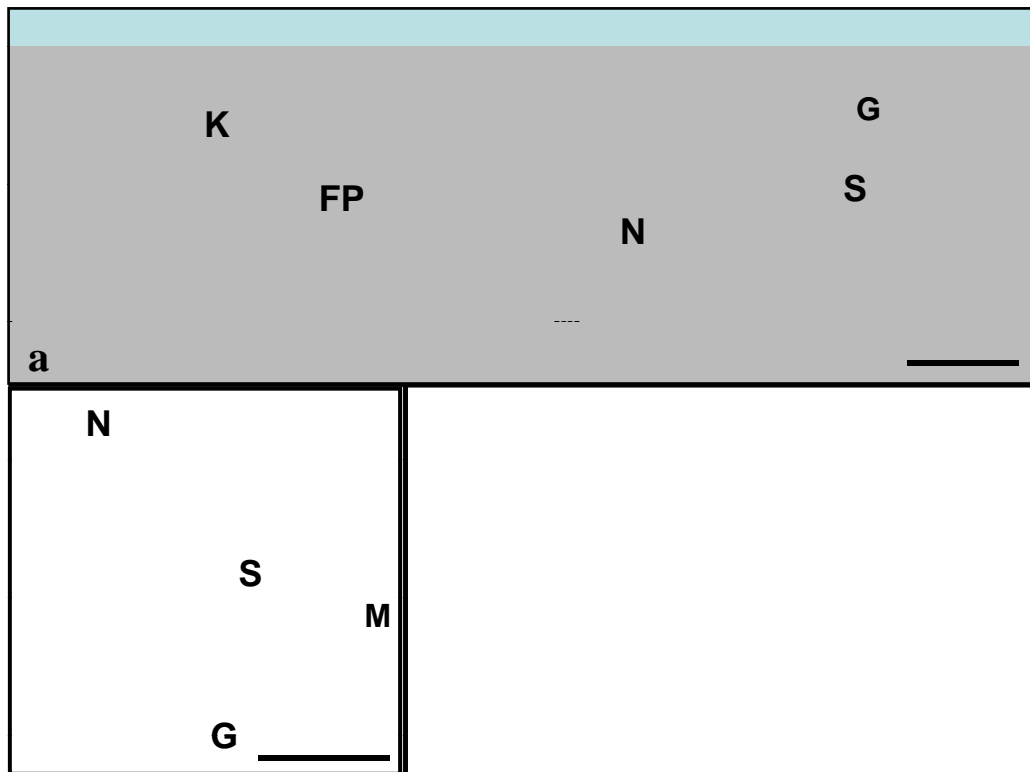


Fig. (3). (a-b) Ultrastructure of *Crithidia deanei* obtained by transmission electron microscopy, showing the kinetoplast (K), flagellar pocket (FP), glycosome (G), portions of the mitochondrion (M) located at the periphery of the protozoan, nucleus (N) and symbiont (S). Note that the symbiont is enclosed by two unit membranes (b) – Bars: 1μm, micrographs by Cristina Motta. Three-dimensional reconstruction of 12 planes of *C. deanei*, note the close association between the dividing endosymbiont (magenta) with the nucleus (green) and glycosomes (yellow), as also observed in figures a and b. Cell membranes are in blue color. Three-dimensional reconstruction by Marcia Attias, Cristina Motta and National Center for microscopy and image research at San Diego, CA/USA.

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 haemocoel [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 parasitize 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

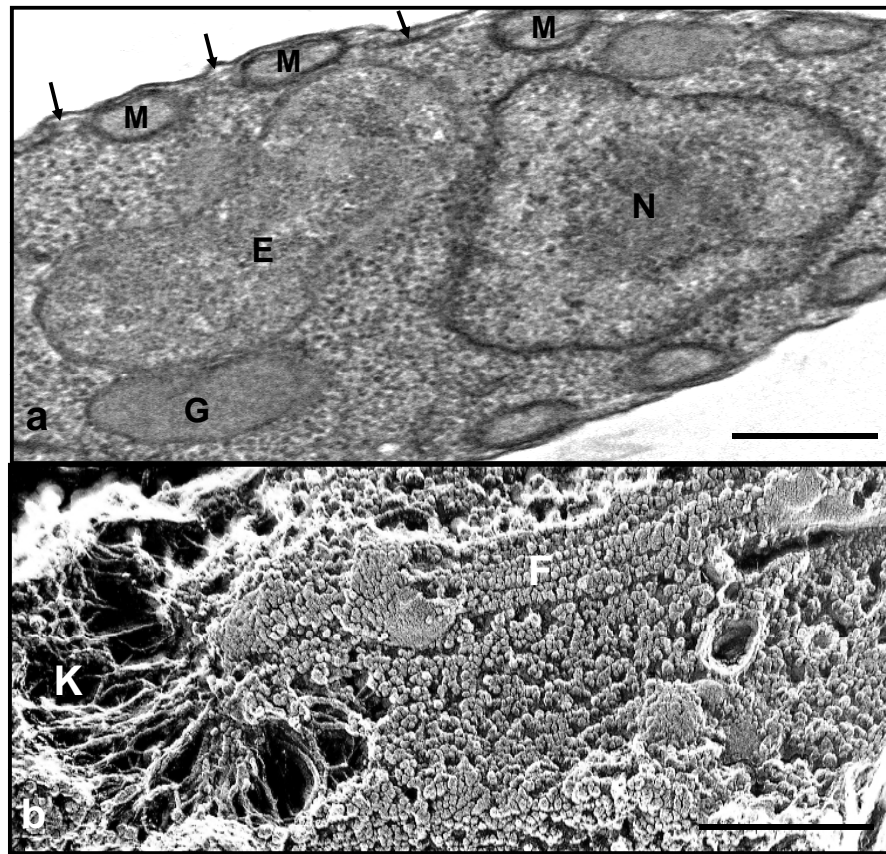


Fig. (4). Ultrastructure of *C. deanei* obtained by transmission electron microscopy (a) and by the freeze fracture followed by the deep-etching technique (b). Note that in this endosymbiont-bearing trypanosomatid the microtubules (arrows) are absent in sites where the mitochondrial branches (M) are juxtaposed to the plasma membrane. Other ultrastructural alterations associated to these cells are the presence of a round kinetoplast (K) presenting a looser arrangement of the kDNA fibers and a typical flagellum (F) containing a reduced paraflagellar rod (b). Bars: 1 μ m. Micrographs by Cristina Motta. E- endosymbiont, G – glycosome, N – nucleus.

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 Malpighian 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 metalloproteinase 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, sialyllactose 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-

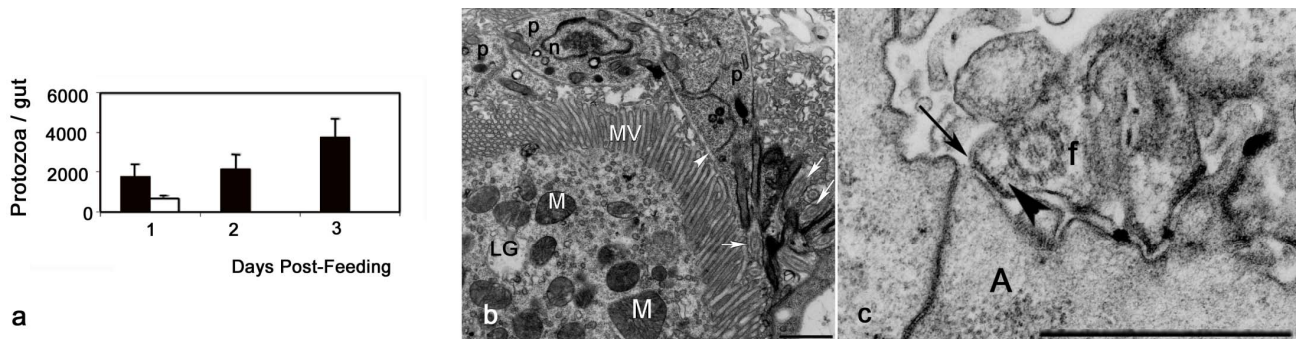


Fig. (5). (a) Outcome of infections in *A. aegypti* after artificial feeding on cultures of *B. culicis* endosymbiont-harboring (solid bar) and cured (open bar) strains. Histograms represent the number of protozoa in mosquito guts (mean \pm SEM) of two experiments in which 7–10 insects were examined per time-point. (b-c) Transmission electron microscopy of the interaction between *B. culicis* and explanted guts of *Lutzomyia longipalpis* (b) and *Aedes albopictus* (c). (b) Protozoa (p) interact with gut microvilli (MV) via cell body (arrowhead) and via flagella, which can be seen inserted between microvilli (arrows). (c) Longitudinal section showing the attachment of *B. culicis* flagellum to *A. albopictus* epithelium gut (AG). Note the presence of dense hemidesmosome plaque along the flagellar membrane attached to the epithelium (arrow). The flagellum (f) is greatly expanded and presents filaments connecting the axoneme to the attached flagellar membrane (arrowhead). Mitochondrion (M), nucleus (n). Bars: 1 μ m. Reprinted from Int J Parasitol 2003, 33: 1019–1026, Fampa *et al.* “Interaction of insect trypanosomatids with mosquitoes, sand fly and the respective insect cell lines”, with permission from Elsevier.

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 ENDOSYMBIONT 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 en-

dosymbiont-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 grown 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. Trypanosomatid 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 aminoacids, 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 aminoacids, 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 aminoacids. In such protozoa the enzyme arginase converts arginine into ornithine and the citrulline can be splitted into ornithine, ammonia and carbon dioxide by citrulline hydrolase. Differently, in symbiont-harboring species of *Crithidia* the urea cycle is complete and such aminoacids 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 trypanosomatids hydrolyze ATP obtained from the host glycosomes to generate a proton motive force that would maintain the

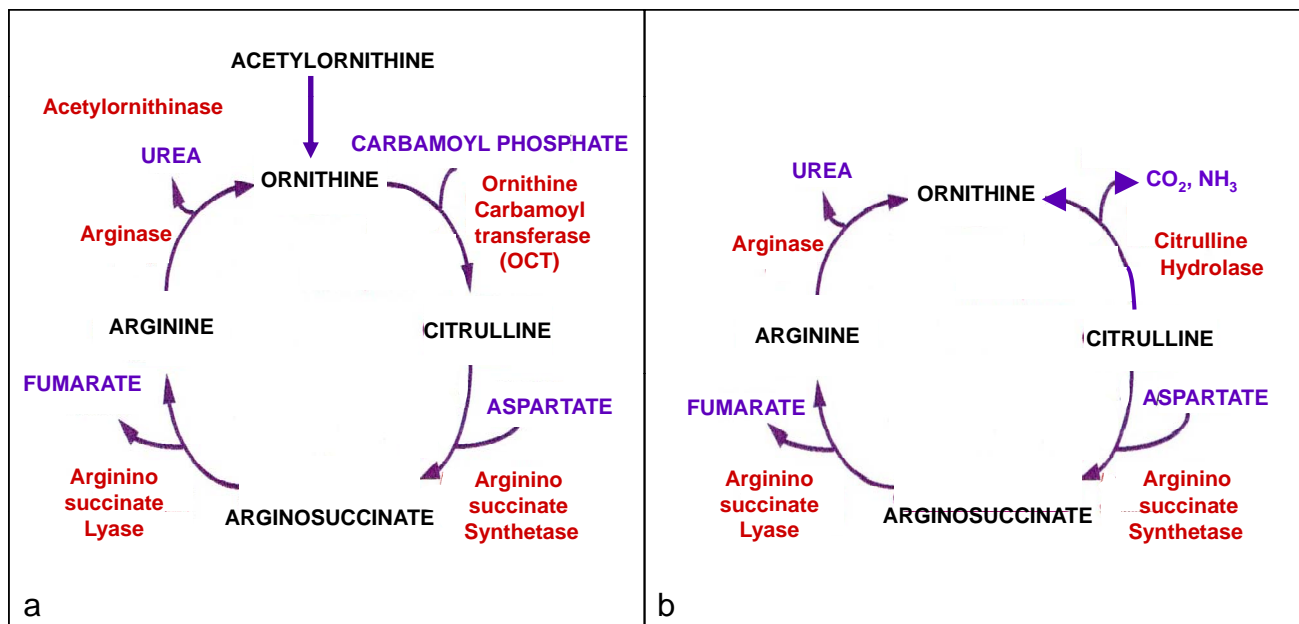


Fig. (6). Urea cycle in endosymbiont-containing (a) and endosymbiont-free (b) *Crithidia* species. Note that the enzyme ornithine carbamoyltransferase is only present in endosymbiont-bearing strains, closing the urea cycle in these protozoa. Conversely, the citrulline hydrolase is only found in endosymbiont-free species that need exogenous arginine or citrulline in culture medium, but not ornithine, which does not substitute for either aminoacids.

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.

ACKNOWLEDGEMENTS

I would like to thank Dr. Wanderley de Souza for the critical revision of the manuscript and Allan de Azevedo Martins for assistance with the figures.

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Received: October 12, 2009

Revised: April 06, 2010

Accepted: April 08, 2010

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