Phosphatidylcholine synthesis in *Crithidia deanei*: the influence of the endosymbiont

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Abstract
In this study, the role of phospholipid biosynthetic pathways was investigated in the establishment of the mutualistic relationship between the trypanosomatid protozoan *Crithidia deanei* and its symbiotic bacterium. Although the endosymbiont displays two unit membranes, it lacks a typical Gram-negative cell wall. As in other intracellular bacteria, phosphatidylcholine is a major component of the symbiont envelope. Here, it was shown that symbiont-bearing *C. deanei* incorporates more than two-fold $^{32}$Pi into phospholipids as compared with the aposymbiotic strain. The major phospholipid synthesized by both strains was phosphatidylcholine, followed by phosphatidylethanolamine and phosphatidylinositol. Cellular fractioning indicated that $^{32}$Pi-phosphatidylcholine is the major phospholipid component of the isolated symbionts, as well as of mitochondria. Although the data indicated that isolated symbionts synthesized phospholipids independently of the trypanosomatid host, a key finding was that the isolated bacteria synthesized mostly phosphatidylethanolamine, rather than phosphatidylcholine. These results indicate that phosphatidylcholine production by the symbiont depends on metabolic exchanges with the host protozoan. Insight about the mechanisms underlying lipid biosynthesis in symbiont-bearing *C. deanei* might help to understand how the prokaryote/trypanosomatid relation has evolved in the establishment of symbiosis.

Introduction

*Crithidia deanei* is a trypanosomatid that coevolves with a symbiotic bacterium through a mutualistic relationship, constituting a valuable model for the investigation of cell evolution. In the past decade, the authors’ group had investigated the metabolic exchanges that promote the symbiotic relationship between the insect trypanosomatid *C. deanei* and its endosymbiont (De Souza & Motta, 1999). Antibiotic treatment of the endosymbiont-bearing strain of *C. deanei* led to the obtention of aposymbiotic cells, thus enabling the analysis of the nutritional roles of the endosymbiont bacterium (Mundim et al., 1974). As expected, the slow-growing aposymbiotic strain has more stringent nutritional requirements as compared with the endosymbiont-bearing strain of *C. deanei* (Mundim & Roitman, 1977). Analysis of the growth requirements of aposymbiotic strains indicated that the endosymbiont supplies the trypanosomatid host with essential nutrients as amino acids, vitamins and hemin (De Souza & Motta, 1999). Furthermore, the symbiont promotes alterations in surface charge (Oda et al., 1984) and in carbohydrate composition of host cell plasma membranes (Esteves et al., 1982), all of which are accompanied by ultrastructural modifications of the host protozoan (Freymuller & Camargo, 1981). Conversely, there is evidence that the symbiotic bacterium may exploit the metabolic pathways of trypanosomatids, such as those providing ATP through the activity of host glycosomes (Motta et al., 1997b).

The endosymbiont is enclosed by two unit membranes, but the origin of its outer membrane is still controversial; while early work suggested that the outer membrane derives from the host trypanosomatids (Chang, 1974), there are evidences that it presents prokaryotic characteristics (Soares & De Souza, 1988; Motta et al., 1991). Phosphatidylcholine is the major membrane-forming phospholipid in most
eukaryotes, being usually absent in prokaryotes, with the notable exception of bacteria involved in symbiotic or pathogenic interactions with plant or animal hosts (López-Lara & Geiger, 2001; Wilderman et al., 2002; Comerci et al., 2006; Wessel et al., 2006). Recently, the authors’ group showed that phosphatidylcholine is a major component of the C. deanei endosymbiont envelope (Palmié-Peixoto et al., 2006). In eukaryotes, phosphatidylcholine is synthesized through two alternative biosynthetic pathways: (1) the CDP-choline pathway, known as the Kennedy pathway, in which free choline is converted to phosphatidylcholine via choline-phosphate intermediates; and (2) the methylation pathway, also known as the Greenberg pathway, which requires three successive methylations of phosphatidylethanolamine in order to produce phosphatidylcholine (Kennedy, 1989). However, in lower eukaryotes, the methylation pathway is the principal route for phosphatidylcholine biosynthesis, especially in cells growing in medium without choline supplementation (Kanipes & Henry, 1997). It has been proposed that phosphatidylcholine biosynthesis by prokaryotes occurs exclusively through the methylation pathway (Rock et al., 1996); however, studies on plant-associated bacteria led to the description of a novel pathway for phosphatidylcholine biosynthesis, where choline is directly condensed with CDP-diacylglycerol by the action of the enzyme phosphatidylcholine synthase (Pcs) (de Rudder et al., 1999; López-Lara & Geiger, 2001). Many bacteria, especially those closely associated with eukaryotic cells, are capable of engaging both the methylation and the Pcs pathways of phosphatidylcholine production. Consistent with this, mutant cells deficient in the methylation pathway have a stringent dependency of exogenous choline provided by the host, to form phosphatidylcholine through the Pcs pathway (de Rudder et al., 1999; Comerci et al., 2006). It was also demonstrated that the phosphatidylcholine synthesis occurs exclusively via Pcs pathway in Pseudomonas aeruginosa (Wilderman et al., 2002) and in Brucella abortus (Comerci et al., 2006). A double mutant, for N-methyltransferase and Pcs enzymes, in Pseudomonas aeruginosa was unable to produce phosphatidylcholine and showed reduction in cell viability (Wilderman et al., 2002). As phosphatidylcholine is only present in a restricted group of bacteria, one may predict that phosphatidylcholine is probably involved in some specialized functions. Accordingly, mutant bacteria defective in phosphatidylcholine synthesis have lost important biological functions, such as cell growth (de Rudder et al., 2000), maintenance of symbiotic relationship (Minder et al., 2001), establishment of host–parasite interaction (López-Lara & Geiger, 2001) and virulence capacity (Wilderman et al., 2002; Comerci et al., 2006; Wessel et al., 2006).

Recently, it was reported that the treatment of C. deanei with azasterol promoted alterations in the phospholipid composition of both the protozoan and its symbiotic bacterium (Palmié-Peixoto et al., 2006). The decrease of the phosphatidylcholine production and the increase of phosphatidylethanolamine indicated that N-methyltransferase activity was reduced, suggesting that the Greenberg pathway was targeted by this drug, as described previously in other trypanosomatids (Contreras et al., 1997; Rodrigues et al., 2002).

In the present work, it was shown that the symbiotic bacterium modulates phospholipid production in C. deanei, with strong impact in phosphatidylcholine formation. Analysis of 32P-labeled phospholipids revealed that phosphatidylcholine is the main component of mitochondrion membranes and of the endosymbiont envelope. Interestingly, the freshly isolated symbiont was able to synthesize phospholipids in increasing amounts, but the main component was phosphatidylethanolamine, rather than phosphatidylcholine. Furthermore, the data emerging from analysis of 32P-labeled phospholipids composition of mitochondrion vs. endosymbiotic bacterium provided new clues to understand how prokaryotes and primitive eukaryotes have coevolved during establishment of symbiosis.

### Materials and methods

#### Cell growth

Normal and apsymbiotic strains of C. deanei were grown at 28 °C for 24 h in Warren culture medium (Warren, 1960) supplemented with 10% fetal calf serum. Cells were grown in medium containing the tracer (10 μCi mL⁻¹ [32P]-orthophosphate) for 5 or 10 h. It is worth mentioning that due to differences in cell growth between the symbiont-bearing and the apsymbiotic strains, cells were quantified before each experiment in order to use equal amounts of protozoa (10⁶ cell mL⁻¹).

#### Endosymbiont and mitochondrion fractions

A modified version of the procedure described by Alfieri & Camargo (1982) was used. Briefly, cells were grown to log phase for 24 h in Warren medium. Protozoa were centrifuged at 4000 g for 10 min and washed twice in phosphate-buffered saline (PBS), pH 7.2. The pellet was resuspended in cold distilled water and left for 45 min on ice. Cells were then centrifuged at 4000 g, resuspended in 12 mL of 20 mM Tris-HCl, 0.25 M sucrose and sonicated using a W-380 Ultrasonic disruptor (three 15 s pulses at 10% amplitude). The volume was adjusted to 20 mL with 20 mM Tris-HCl, 0.25 M sucrose, 2 mM CaCl₂, 10 mM MgCl₂ and 25 μg mL⁻¹ DNase type I (Sigma). Cells were incubated with this solution at 25 °C for 30 min and the volume was increased to 30 mL with 20 mM Tris-HCl, 0.25 M sucrose and 2 mM EDTA. The homogenate was centrifuged at 5000 g for
20 min and the pellet obtained was resuspended in Tris-HCl/sucrose/EDTA buffer containing 0.5 mg mL\(^{-1}\) of Pronase (protease type XIV from Streptomyces griseus – Sigma). The homogenate was then centrifuged at 4000 g for 20 min. The pellet was resuspended in 10 mL of Tris-HCl/sucrose/EDTA buffer and 2.5 mL aliquots were layered over 2.5 mL of 0.5 M sucrose. After centrifugation at 550 g for 10 min, the upper layer was collected. The upper layer was centrifuged at 4000 g for 10 min. The obtained supernatant was centrifuged at 10 000 g for 10 min originating a pellet, which corresponds to the mitochondrial fraction, whereas the pellet obtained after the upper layer centrifugation was resuspended in 6 mL of 20 mM Tris-HCl, 0.25 M sucrose. The resuspended material was layered on the top of six tubes (13 mm × 51 mm), each containing a two-step sucrose gradient consisting of 0.44 M (2 mL) and 0.88 M (1 mL) sucrose. After centrifugation at 1740 g for 30 min, the endosymbiont-containing pellet was collected.

Transmission electron microscopy (TEM)

For routine TEM, protozoa were fixed in 2.5% glutaraldehyde, pH 7.2. Then, cells were postfixed in 1% OsO\(_4\), 0.8% K\(_2\)Fe(CN)\(_6\), 5 mM CaCl\(_2\) in 0.1 M cacodylate buffer. Then, cells were washed, dehydrated in acetone and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate before observation.

\[\text{[32P]-orthophosphate label of the phospholipids}\]

The \textit{C. deanei} phospholipids were metabolically labeled as follows: protozoa were grown to log phase at 28°C. Then, 10 mCi mL\(^{-1}\) \[\text{[32P]-orthophosphate}\] were added to the culture medium for 5 or 10 h. Protozoa were then washed with PBS and the cell pellet was used for lipid extraction as described below. Isolated mitochondria and symbionts were also incubated with the tracer. Thus, after obtaining these fractions, either mitochondria or symbionts were resuspended in cell growth medium containing 2 mCi mL\(^{-1}\) \[\text{[32P]-orthophosphate}\] for 1 and 3 h at 28°C. After incubation, mitochondria or symbionts were washed with PBS and lipid extraction was performed as follows.

\[\text{Lipid extraction}\]

The method described by Horwitz & Perlman (1987) was followed for lipid extraction. Briefly, the biological material (cell pellet, mitochondria or symbionts fractions) was solubilized with 4 mL of CHCl\(_3\):CH\(_2\)OH: HCl (200:100:0.75 v/v) in conical glass tubes, vortexed and added to 0.8 mL of 0.6 N HCl. After vigorous agitation of the solution with the use of a Pasteur pipette, the phases were separated by centrifugation at 800 g for 10 min at room temperature. The organic (lower) phase was transferred to a new tube and washed twice with 2.0 mL of a mixture containing CHCl\(_3\):CH\(_2\)OH: HCl 0.6 N (3 : 48 : 47 v/v). At the end of this procedure, the organic phase was adjusted to pH 7.0 with 0.2 N NH\(_4\)OH in methanol. The solvent was evaporated under N\(_2\) and the lipids were stored at –20°C until use.

\[\text{Separation and identification of phospholipids}\]

Lipid samples were dissolved in 90\,\mu L of a mixture of CHCl\(_3\):CH\(_2\)OH:H\(_2\)O (75 : 25 : 0.2 v/v) and applied (equal amounts) to silica gel plates along with phospholipid standards for thin layer chromatography (TLC). The plates were activated at 110°C for 10 min. The chromatograms were developed in CHCl\(_3\):CH\(_2\)COCH\(_3\):CH\(_2\)OH:CH\(_3\)COOH:H\(_2\)O (120 : 45 : 36 : 24 v:v) for 80 min. The plates were dried in a fume hood and the lipid spots were visualized by exposure to I\(_2\) vapor. Autoradiography of the TLC plates was carried out using X-ray film (Kodak T-Mat). The cassette was stored at –70°C for 3–5 days and the film developed following the manufacturer’s specifications. For quantification of radiolabeled spots, they were localized and scraped off the plate into scintillation vials. Radioactivity was measured using liquid scintillation in a Beckman counter.

\[\text{Results}\]

\[\text{Phospholipid biosynthesis in symbiont-containing and aposymbiotic strains of C. deanei}\]

In order to compare the phospholipid composition on symbiont-bearing and aposymbiotic strains of \textit{C. deanei}, \(^{32}\)Pi was added to the culture medium after 24 h of cultivation, this corresponds to the log phase of growth. It is worth mentioning that cell counts were performed before each experiment in order to adjust the number of protozoa, which makes results comparable for both strains. Phospholipid analysis performed after 5 or 10 h of incubation showed that phosphatidylcholine was the major phospholipid produced (Fig. 1a), followed by phosphatidylethanolamine (Fig. 1b) and phosphatidylinositol (Fig. 1c). It is important to point out that both strains of \textit{C. deanei} presented more than double the content of phosphatidylcholine and phosphatidylethanolamine when protozoa were grown for 10 h, instead of 5 h, in the presence of \(^{32}\)Pi. Furthermore, the endosymbiont-bearing strain presented more than two-fold of radiolabeled phospholipids (400 × 10\(^{4}\) c.p.m. for phosphatidylcholine, 100 × 10\(^{4}\) c.p.m. for phosphatidylethanolamine and 23 × 10\(^{4}\) c.p.m.) when compared with the aposymbiotic strain (160 × 10\(^{4}\) c.p.m. for phosphatidylcholine, 42 × 10\(^{4}\) c.p.m. for phosphatidylethanolamine and 7.5 × 10\(^{4}\) c.p.m.) – (Fig. 1a–c).
Phospholipid composition of symbiont and mitochondrion fractions after C. deanei cell fractioning

The cell fractioning assays were performed with protozoa cultivated for 10 h in the presence of 32Pi, because the production of radiolabeled phospholipids was much higher than the cell growth after 5 h in the presence of the tracer. Biochemical analysis revealed that phosphatidylcholine was the main radiolabeled phospholipid detected either in symbionts (7.5 \times 10^4 \text{ c.p.m.}) or in mitochondria (58 \times 10^4 \text{ c.p.m.}) fractions. Similar amounts of phosphatidylethanolamine and phosphatidylinositol (\approx 4 \times 10^4 \text{ c.p.m.}) were found in the isolated endosymbionts (Fig. 2a), while in the mitochondrial fraction the amount of phosphatidylethanolamine was two-fold higher (48 \times 10^4 \text{ c.p.m.}) when compared with phosphatidylinositol (24 \times 10^4 \text{ c.p.m.}) (Fig. 2b). It is worth mentioning that the mitochondrial fraction presented increased amounts of radiolabeled phospholipids when compared with the symbiont fraction: the phosphatidylinositol content was more than fivefold higher, while phosphatidylcholine and phosphatidylethanolamine were eight- or 10-fold higher, respectively (Fig. 2a and b). The large amount of phospholipids in the mitochondrion may reflect its higher relative volume in trypanosomatids, when compared with other structures in these protozoa.

Symbiotic bacterium is able to synthesize phospholipids once isolated from the host cell

Isolated endosymbionts and mitochondria, obtained after C. deanei cell fractioning, were maintained for 1 and 3 h in culture medium containing 32Pi. Longer periods of incubation could not be tested, considering that the symbiotic bacterium presents low osmotic stability after isolation from the host protozoan. The isolated symbionts were able to synthesize increased amounts of radiolabeled phospholipids after 3 h of incubation in medium containing 32Pi (Fig. 3a and b). Conversely, the isolated mitochondria were not able to incorporate 32Pi, and radiolabeled phospholipids in this fraction were not observed, even after 3 h of incubation in medium containing the tracer (Fig. 3a). It is interesting to note that the main radiolabeled phospholipid identified in the symbiont fraction incubated for 1 h in medium containing 32Pi was phosphatidylcholine, followed by phosphatidylethanolamine and phosphatidylinositol. However, after longer periods in the presence of 32Pi, higher amounts of radiolabeled phosphatidylethanolamine were found in the isolated bacteria, followed by phosphatidylinositol and phosphatidylcholine (Fig. 3b). In order to check the symbiont and the mitochondrion ultrastructure after cell fractioning, samples submitted to 3 h of incubation with medium containing 32Pi were compared with the same structures present in the whole cell of C. deanei (Fig. 4a). Data obtained by TEM showed that isolated endosymbionts presented an integral envelope, which was constituted by the two unit membranes. In some bacteria, the inner and outer membranes were slightly separated and it is probably related to osmotic shock, because reduced cell walls are present in the symbiont of trypanosomatid protozoa (Fig. 4b). The

![Graphs showing phospholipid biosynthesis](image1.png)

**Fig. 1.** 32Pi incorporation and phospholipid biosynthesis by endosymbiont-containing (+) and symbiont-free (−) strains of Crithidia deanei after 5 or 10 h of incubation with the tracer. (a) Phosphatidylcholine production, (b) phosphatidylethanolamine production and (c) phosphatidylinositol production. Results are representative of three independent assays.
isolated mitochondria were seen fragmented, as expected and composed by two unit membranes (Fig. 4c).

**Discussion**

*Crithidia deanei* is a trypanosomatid, which maintains a mutualistic relationship with an endosymbiotic bacterium. The envelope composition of the endosymbiont of *C. deanei* presents phosphatidylcholine, as recently described (Palmié-Peixoto et al., 2006). This phospholipid is found in restricted groups of bacteria, specially the ones living in close association with eukaryotes (López-Lara & Geiger, 2001). Membrane phospholipids are regarded as essential to maintain the selective cell permeability and to support membrane-associated proteins (Raetz & Dowhan, 1990). In eukaryotes, phosphatidylcholine is the major structural component of cell membranes, also playing important roles in signal transduction (Exton, 1994). In prokaryotes it has been suggested that phosphatidylcholine not only serves as an important structural component but also contributes to normal growth (de Rudder et al., 2000), to symbiotic interactions (Goldfine, 1982; Minder et al., 2001), and to prokaryote virulence (Wilderman et al., 2002; Comerci et al., 2006; Wessel et al., 2006).

Here, it is shown for the first time that the endosymbiont-bearing strain of *C. deanei* was able to uptake higher amounts of $^{32}$Pi from the culture medium than the aposymbiotic strain. Such data are related to the fact that symbiont-harbouring strains present high rates of proliferation, when compared with the aposymbiotic ones (Mundim et al., 2007).

![Fig. 2.](image1.png) **Fig. 2.** The radiolabeled phospholipid content in symbiont (a) and mitochondrion (b) fractions, which were obtained after the cell fractioning of *Crithidia deanei* grown in the presence of $^{32}$Pi for 10 h. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Results are representative of three independent assays.

![Fig. 3.](image2.png) **Fig. 3.** (a) $^{32}$Pi-phospholipids synthesized by isolated symbionts (S) after 1 and 3 h of incubation with the tracer. Note that the isolated mitochondria (M) were not able to produce radiolabeled phospholipids. (b) Quantification of $^{32}$Pi-phospholipids produced by isolated symbionts after 1 and 3 h of incubation with the tracer. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; O, origin. Results are representative of three independent assays.
Roitman, 1977). Recently, the authors’ group demonstrated that the symbiont of *C. deanei* produces a proteinic factor that enhances the protozoan ornithine decarboxylase (ODC) activity. This enzyme is involved in DNA replication and cellular growth, and its high activity in endosymbiont-containing trypanosomatids may in part explain the elevated cell proliferation observed in these protozoa (Frossard et al., 2006).

Endosymbionts and mitochondria obtained after cell fractioning presented phosphatidylcholine as the major phospholipid, followed by phosphatidylethanolamine and phosphatidylinositol. These results are in accordance with those previously obtained and emphasize the presence of phosphatidylcholine in the endosymbiont envelope (Palmié-Peixoto et al., 2006). The mitochondrial phospholipid content, especially phosphatidylcholine and phosphatidylethanolamine, is far larger than that found in the endosymbiont and is related to the highest volume occupied by this organelle in *C. deanei* (Motta et al., 1997b). Interestingly, in this work isolated symbionts were shown to be able to produce phospholipids independently from the host cell. Novak et al. (1988) also showed the autonomy of endosymbionts in synthesizing proteins, after 2 h of isolation. The symbiont of trypanosomatids presents a degenerated cell wall and this may justify the low osmotic stability of this prokaryote once purified from the host (Motta et al., 1997a).

Furthermore, recent data showed that some genes pertaining to the division and cell wall cluster were lost by the symbiont, explaining in part its inability to divide outside the host (Yim et al., 2005).

Endosymbionts and mitochondria were not able to synthesize phospholipids independent of the host cell. Biochemical data showed that the mitochondrial fraction presents succinate cytochrome c-reductase activity, indicating that the organelle maintained an energetic respiratory chain, even after its fragmentation (Motta et al., 1997b). Recently, the authors’ group also observed that the mitochondrial fraction consumes high rates of O$_2$ (data not shown), which suggests that although viable after the cell fractioning, there was no phospholipid synthesis after mitochondrion isolation. During the evolutionary process, the mitochondrion lost, or transferred to the host cell nucleus, most of its DNA content (Timmis et al., 2004). As a consequence, this organelle imports the majority of its proteins and lipids from the cytoplasm, which could explain its incapacity to synthesize phospholipids once isolated from the trypanosomatid.

Considering the phospholipid content in the endosymbiont envelope and the mutualistic relationship with the host trypanosomatid, some important questions must still be answered: how does the symbiont obtain the phosphatidylcholine that integrates its membranes? Which biosynthetic pathways are used to produce phosphatidylcholine in endosymbiont-bearing trypanosomatids? The authors’ previous work evidenced an active Greenberg pathway in *C. deanei*, as the protozoan treatment with azasterol inhibited the phosphatidylcholine production and enhanced the phosphatidylethanolamine content of the whole cell and also of the bacterial fraction (Palmié-Peixoto et al., 2006). Symbionts were able to synthesize phospholipids independent of the host protozoan 3 h after purification, but in this case phosphatidylethanolamine was the major phospholipid produced, while phosphatidylcholine was found in minor amounts. Taken together, these results suggest that the symbiotic bacterium may obtain part of its phosphatidylcholine, or even phosphatidylcholine precursors, from the
host trypanosomatid. Prokaryotes that contain phosphatidylcholine usually have both the methylation (Greenberg pathway) and the Pcs pathways for phosphatidylcholine formation. However, pathways involved in phosphatidylcholine production in prokaryotes are more complex than originally expected. There are for example some bacteria, such as *Pseudomonas aeruginosa* (Wilderman et al., 2002) and *Brucella abortus* (Comerci et al., 2006), where phosphatidylcholine biosynthesis occurs exclusively via the Pcs pathway. In such cases, the phosphatidylcholine synthesis depends on the choline provided by the host. Similarly, a *Sinorhizobium meliloti* mutant deficient in enzymes of the methylation pathway requires the supply of choline from the host partner to form phosphatidylcholine through the Pcs pathway (de Rudder et al., 1999). The strategy to synthesize phosphatidylcholine using host precursors was maintained in the course of evolution, as some mammal parasite protozoa, such as *Leishmania major* (Zufferey & Mamoun, 2002), *Plasmodium falciparum* (de Rudder et al., 1991; Pessi et al., 2004), and *Babesia bovis* (Florin-Christensen et al., 2000), also require transport of choline from the host to synthesize phosphatidylcholine.

Further studies are necessary to identify the metabolic pathways involved in the phosphatidylcholine biosynthesis of endosymbiont-bearing trypanosomatids. The full-genome sequencing of the symbiotic bacterium, as well as the use of specific inhibitors to phosphatidylcholine biosynthesis enzymes might thus clarify new aspects of symbiosis in trypanosomatids.

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