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When bacteria meet mitochondria: the strange case of the tick symbiont *Midichloria mitochondrii*

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Abstract

Mitochondria are key eukaryotic organelles, that perform several essential functions. Not surprisingly, many intracellular bacteria directly or indirectly target mitochondria, interfering with innate immunity, energy production or apoptosis, to make the host cell a more hospitable niche for bacterial replication. The alphaproteobacterium *Midichloria mitochondrii* has taken this behavior to another level by physically colonizing mitochondria, as shown by transmission electron micrographs of bacteria residing in the mitochondrial intermembrane space. This unique localization provokes a number of questions around the mechanisms allowing and reasons driving intramitochondrial tropism. We suggest possible scenarios that could lead to this peculiar localization and hypothesize potential costs and benefits of mitochondrial colonization for the bacterium and its host.

Introduction

Symbiosis, the tight interaction of organisms of different species, is ubiquitous in nature, across all branches of the tree of life (Brucker and Bordenstein, 2012). Intracellular symbioses are among the most fascinating, as they clearly require constant and complex interaction between the symbiont and its host (Wernegreen, 2012). All symbiotic relationships can have important impact on both partners, but intracellular symbioses can play key roles in the evolution of hosts and symbionts (Archibald, 2015; Brucker and Bordenstein, 2012). The archetype of intracellular symbiosis is the evolution of mitochondria from a bacterial ancestor, an event that was instrumental in the evolution of eukaryotic cells and multicellularity (Gray et al., 2001; Roger et al., 2017).

Despite their prevalence and foundational role in shaping eukaryotic evolution, our knowledge of intracellular symbiosis between bacteria and eukaryotic cells remains sparse. Recent studies have started to unveil the molecular basis of some specific cases of symbiotic bacteria-cell interactions (e.g (Douglas, 2014)), but advances are limited by the paucity of genetically amenable systems and culturable symbionts. Arthropods are among the most studied symbiotic hosts, due to their extreme species-richness, broad distribution and importance as pathogen vectors, but also due to their strong propensity to harbour symbionts (Gil and Latorre, 2019). Many intracellular mutualists of arthropods play fundamental nutritional roles, supplementing the diets of hosts that feed on unbalanced substrates such as phloem sap or blood (Moran et al., 2008). Ticks are obligate hematophagous arthropods and appear to be a true “receptacle” for a wide variety of both pathogenic and non-pathogenic bacteria (Duron et al., 2017; Kernif et al., 2016). A portion of these tick-borne bacteria are maternally inherited, obligate intracellular bacteria and are thought to be nutritional symbionts (Bonnet et al., 2017). Here, we will focus on *Candidatus* Midichloria mitochondrii (hereafter *M. mitochondrii*), a maternally inherited symbiont that can colonize the oocyte mitochondria of the tick *Ixodes ricinus*, one of the most widespread ticks in Europe.

Mitochondria, an emerging target for intracellular bacteria

Mitochondria originated when an ancestral proteobacterium entered the ancestor of eukaryotic cells, a unique and crucial event for the evolution of eukaryotic life. Determining the nature of the bacterial lineage giving rise to the ancestor of mitochondria is a highly debated topic, and among other hypotheses (Martijn et al., 2018), one indicates intracellular alphaproteobacteria of the *Rickettsiales* order as possible candidates, based on analyses indicating them as the closest extant relatives of the organelle (Andersson et al., 1998; Ferla et al., 2013; Fitzpatrick et al., 2006; Sassera et al., 2011; Wang and Wu, 2015; Williams et al., 2007). Mitochondria are essential eukaryotic organelles responsible for vital functions including energy production, biogenesis of iron-sulphur clusters, porphyrin and lipids, anaplerotic reactions, amino acid metabolism and programmed cell death (intrinsic apoptosis (Green et al., 2014).

Most of our knowledge on mitochondria stems from model organisms, essentially yeast or mammalian cells, but most key mitochondrial functions and molecular machineries appear largely conserved (Pernas and Scorrano, 2016; Westermann, 2010). Due to their central role as metabolic and signalling hubs, mitochondria are a prime target for intracellular bacteria, which can obtain nutrients from them or manipulate intrinsic apoptosis. Indeed, several intracellular bacteria, associated with a range of eukaryotic hosts, can affect mitochondria (reviewed in (Spier et al., 2019). The bacterial effects on mitochondria include morphological and functional changes, as exemplified by the facultative intracellular bacterium *Listeria monocytogenes*, which induces fragmentation of the mitochondrial network while leading to a collapse of the mitochondrial membrane potential (Stavru et al., 2011). Several bacteria target mitochondria through secreted proteins, that act directly on the organelles. For example, *Helicobacter pylori* secretes the toxin VacA, which is inserted in the mitochondrial inner membrane (Foo et al., 2010), while the *Listeria* toxin listeriolysin O inserts into the plasma membrane, causing a calcium influx and thus indirectly leading to mitochondrial fission (Stavru et al., 2011). Some bacteria even interact physically with mitochondria, establishing close contacts. Stable contacts have been detected between mitochondria and the avian pathogen *Chlamydia psittaci* , increasing concomitantly to bacterial replication. This led the authors to suggest that mitochondria might provide ATP for bacterial replication via the C. psittaci – encoded ATP-ADP transporter (Matsumoto et al., 1991). In contrast, the human pathogen *Legionella pneumophila* was shown to establish dynamic contacts with mitochondria that were lost at late timepoints of infection. In addition, these contacts were also observed upon infection with avirulent *L. pneumophila* (deficient in the type four secretion system), leading the authors to propose that transient mitochondrial association with bacteria-containing phagosomes might represent a general, virulence-independent host response to infection (Escoll et al., 2017). The recently described alphaproteobacterium *Midichloria mitochondrii* (Sassera et al., 2006) provides one of the most extreme examples of a physical interaction between an intracellular bacterium and mitochondria.

Midichloria mitochondrii

Midichloria is a genus of obligate intracellular bacteria belonging to the order Rickettsiales, a group of alphaproteobacteria composed by intracellular bacteria (with one exception (Castelli et al., 2019), which includes human pathogens (e.g many Rickettsia species (Parola et al.,

2013; Weinert et al., 2009), mutualists (Hosokawa et al., 2010; Landmann, 2019; Taylor et al., 2005), reproductive parasites (Werren et al., 2008), and a number of less-studied lineages with unknown interactions with their host (Castelli et al., 2016). *Midichloria* have been found in several tick species, including *Ixodes ricinus* (Beninati et al., 2004), *Rhipicephalus bursa* (Epis et al., 2008), *Amblyomma maculatum* (Budachetri et al., 2018), among others (Cafiso et al., 2016; Epis et al., 2008). These bacteria are among the most abundant members of the microbiome in multiple tick species (Di Lecce et al., 2018; Duron et al., 2017; Hernández-Jarguín et al., 2018), suggesting a potential mutualistic relationship (Lo et al., 2006) and mechanisms to avoid immune recognition by the host.

Among *Midichloria*, the most studied is *Candidatus* Midichloria mitochondrii (hereafter *M. mitochondrii*). *M. mitochondrii* is present at high prevalence and abundance in females and immature *I. ricinus* ticks (Lo et al., 2006; Sassera et al., 2008). In this species, most characteristics of the host-symbiont relationship are those common to nutritional mutualisms: high prevalence, widespread distribution, transovarial transmission, low symbiont genetic diversity, and symbiont genome reduction while retaining B vitamin synthesis genes (Al-Khafaji et al., 2019; Lo et al., 2006; Sassera et al., 2011). *Ixodes ricinus* is a competent vector for a range of viral, parasitic and bacterial diseases of high medical impact (Lejal et al., 2019; Moutailler et al., 2016; Reis et al., 2011). Diseases transmitted by *I. ricinus* include the highly virulent tick-borne encephalitis caused by the homonymous virus, rickettsioses, and Lyme disease caused by multiple *Borrelia* species (Boulanger et al., 2019).

Although it is largely unknown whether *M. mitochondrii* plays a role in pathogen transmission, or is itself productively transmitted through blood feeding (Bazzocchi et al., 2013; Cafiso et al., 2019; Mariconti et al., 2012a; Serra et al., 2019), one study found a positive correlation between levels of the *Midichloria* symbiont of a different tick species (*Amblyomma maculatum*) and the presence of the pathogen *Rickettsia parkeri* (Budachetri et al., 2018). However, what makes the interaction between *M. mitochondrii* and its host unique is the astonishing observation that *M. mitochondrii* is not only present in the cytoplasm, but a portion of its population colonises the intermembrane space of mitochondria (Fig 1) and appears to lead to mitochondrial matrix condensation (Sacchi et al., 2004). Such peculiar localization, never reported in any other animal cell, raises major questions: (1) how and why does *M. mitochondrii* get into mitochondria and how do mitochondria respond? (2) What is the role of *M.mitochondrii* in the cell, and does this benefit the host? (3) why does invasion of mitochondria not lead to cell death? In the following paragraphs, we will address these questions by summarizing the available data and putting forward hypotheses and experimental approaches to test them.

Mitochondria as an unexpected ecological niche

The origin and evolution of the intramitochondrial tropism (IMT) displayed by *M. mitochondrii* are currently unknown. Another *Midichloria* symbiont was reported to be capable of IMT, in *R. bursa* ticks (Epis et al., 2008). Although *Midichloria* are the only bacteria capable of intramitochondrial tropism in a metazoan organism, other bacteria have been reported as intramitochondrial or very closely associated to mitochondria in protists. Examples for IMT/mitochondrial association are found in bacteria that colonize the protists *Halteria geleiana* (Yamataka and Hayashi, 1970), *Urotricha ovata Kahl* (de Puytorac and Grain,

1972) and more recently two *Diplomonas* species, whose bacterial endosymbionts were the founding members of the new genus "Candidatus Cytomitobacter" (Tashyreva et al., 2018). The most parsimonious explanation to multiple phylogenetically unrelated bacteria invading or interacting very closely with mitochondria is the parallel independent evolution of these interactions, while a potential alternative would be an event of horizontal transfer of genes that mediate IMT. Future studies will uncover whether these bacteria employ common strategies to colonize mitochondria. In any case, the localization of bacteria in the mitochondrial IMS raises the question of the physiological reasons and advantages this localization might have. Here, we put forward hypotheses, focusing on the *M. mitochondrii* - *I. ricinus* system and drawn from our knowledge of mitochondrial IMS biology in model systems. In addition, our own unpublished transcriptome data of *M. mitochondrii* and/or *I. ricinus* also support to a certain degree the existence of signaling and metabolic pathways, as well as of homologs of the proteins that are mentioned in the following paragraphs.

The IMS as a protected space: avoiding cytosolic host defence mechanisms and modulating apoptosis

One possible explanation for the localization of *M. mitochondrii* in the IMS is that it potentially confers the ability to actively suppress dangerous pathways originating from mitochondria. In addition, IMT can offer protection by escaping cytosolic defence machineries based on innate immune recognition or xenophagy. It might also give the bacterium the capacity to modulate the host mitochondrion so as to avoid apoptosis induction (Tiwari et al., 2015), e.g by suppressing cytochrome c release and preventing cristae remodeling (Polčic et al., 2017) . This could allow the suppression of mitophagy and Ca^{2+} signaling, which eventually might result in Ca^{2+} overload in mitochondria, followed by permeability transition and cell death (Orrenius et al., 2015). As, in contrast to other tissues, mitochondria are usually quite dormant in oocytes (Tiwari et al., 2015), they could represent a protected space without much reactive oxygen species production taking place. In addition, intramitochondrial *M. mitochondrii* might also modulate the immune response by preventing the release of mitochondrial DNA that is known to induce the innate immune response (Nakahira et al., 2011).

The IMS: a microreactor?

In addition to providing protection, the physical and chemical conditions of the IMS might constitute a favourable environment for *M. mitochondrii*. For example, the proximity to the mitochondrial respiratory chain might under certain conditions provide more suitable conditions for bacterial growth. For example, it has been reported in mammalian cells that inside mitochondria, close to the respiratory chain, the temperature can be significantly above that of the cytosol (Chrétien et al., 2018). Whether this temperature difference is also found in tick oocyte mitochondria remains an open question, and while the overall GC content of the *M.mitochondrii* (37%) does not seem to be specifically tailored for high temperatures, this needs to be confirmed by comparative analysis of its 16S rRNA GC content with that of non-mitochondrial *Midichloriae* (Wang et al., 2006). The IMS might also serve as a hub to exchange metabolites between host and bacterium. Indeed, ATP concentrations in the IMS are comparatively high and might be used directly by the bacterium. The existence of a putative ATP-ADP translocase in the bacterium can support this hypothesis, although *M. mitochondrii* appears to have retained the ability to synthesize ATP (Sassera et al., 2011). In contrast, several other obligate intracellular bacteria, which display highly reduced genomes, have lost

the ability to synthesize nucleotides and therefore rely on ATP import from the host (energy parasitism, shown e.g. by Chlamydiae (Trentmann et al., 2007), *Lawsonia intracellularis* (Schmitz-Esser et al., 2008) and *Rickettsia prowazekii* (Winkler and Daugherty, 1984). In addition, the IMM and the IMS are important sites for cellular lipid metabolism, and the bacterium might benefit from direct lipid supply. Moreover, positioning in the IMS might allow the bacterium to influence AMP signaling (i.e. potentially to increase mitochondrial biogenesis), that is in part driven by the IMS-localized adenylate kinase 2 enzyme (Dzeja and Terzic, 2009).

The IMS as shuttle for transfer to the next generation

Transovarian transmission to the next generation is clearly the reason why many intracellular symbionts reside in female reproductive organs, however in the specific case of *M. mitochondrii* an additional factor contributing to its accumulation in oocytes could be the high number of mitochondria found in oocytes (up to 100 times more than in an epithelial cell, (Monnot et al., 2013). A last hypothesis on the reason for the IM localization is that mitochondria in general might serve as shuttles that allow *M. mitochondrii* to efficiently pass from one generation to the next, as the bacterium would then hijack the obligate maternal inheritance of mitochondria to ensure its own transmission. After fertilization, mitochondria initially do not have to divide but are distributed among cells of the early embryo. This might therefore also form a way for the bacterium to efficiently spread to multiple tissues in the next generation.

Scenarios for mitochondrial colonization by *M. mitochondrii*

Electron micrographs (EM) support inference of various possible scenarios for the life cycle of *M. mitochondrii*, and are complimented by similar work used to understand now well-characterized intracellular bacteria, including pathogens like *Listeria monocytogenes* (Tilney and Portnoy, 1989) or symbionts like *Wolbachia*, a member of the order *Rickettsiales* like *M. mitochondrii* (Avakyan and Popov, 1984). *Wolbachia* accumulate inside a host-derived vacuole (e.g. (Fischer et al., 2014)). It appears that cytosolic *M. mitochondrii* do not (see Fig. 1, (Sacchi et al., 2004)). Although it is also possible that *M. mitochondrii* might temporarily reside in a vacuole, which might allow the bacterium to escape into the cytosol or fuse with the mitochondrial outer membrane, resulting in its observed intermembrane space (IMS) localization (Fig 2). Alternatively, cytosolic bacteria could directly enter mitochondria, possibly hijacking mitochondrial fusion. Unfortunately, EM precludes the analysis of dynamic events, and therefore validation of either model critically depends on the ability to perform functional tests, and therefore on the ability to manipulate *M. mitochondrii* in vitro, which is yet to be developed.

Once inside mitochondria, *M. mitochondrii* appears to manipulate the contacts that normally keep the inner and outer membrane at a constant distance of about 20nm, sometimes leading to substantial separation of the inner and outer membrane (Fig 1C and (Sacchi et al., 2004)). 3D analyses and quantitative light microscopy will be required to address whether *M. mitochondrii* modulates mitochondrial dynamics in addition to affecting mitochondrial ultrastructure. Such approaches will also be instrumental to determine the proportion of cytoplasmic versus mitochondrial *M. mitochondrii*. Strikingly, several examples

of mitochondria colonized by more than one bacterium have been reported, suggesting either multiple rounds of invasion or bacterial division within the IMS. The presence of multiple bacteria appears correlated with altered mitochondrial ultrastructure up to drastic mitochondrial matrix condensation (Fig 1C and (Sacchi et al., 2004)) and it is reasonable to expect these alterations to affect mitochondrial function. Surprisingly, these mitochondrial changes do not correlate with an apparent induction of cell death. We hypothesize that the bacterium actively counters apoptosis, similar to other obligate intracellular bacteria (Rudel et al., 2010) .

While several tick species harbour bacteria belonging to the *Midichloria* genus, currently the only complete genome available for this genus is that of *Midichloria mitochondrii,* a putative symbiont of the hard tick *I. ricinus.* The reduced genome of *M. mitochondrii (*1.2Mb) is indeed typical of rickettsial endosymbionts (Sassera et al., 2011). Comparative genomics between *M. mitochondrii* and closely related species that do not display IMT (e.g. *Midichloria* from *Ixodes holocyclus* (Beninati et al., 2009; Castelli et al., 2016) will therefore be instrumental to uncover whether specific genes are associated with the unique ability of the symbiont to invade mitochondria. However, the *M. mitochondrii* genome already unveiled an intriguing and surprising finding: the presence of flagellar genes, including components of the motor and export apparatus (Sassera et al., 2011). Even though the flagellum of *M. mitochondrii* has never been visualized, hook and filament proteins have been detected experimentally (Mariconti et al., 2012b). A typical PAMP (pathogen associated molecular pattern), the flagellum can be recognized by Toll-like receptor family proteins, which are highly conserved in eukaryotes (reviewed in (Leulier and Lemaitre, 2008). Consequently, flagellar genes have been lost or are not expressed in several intracellular bacteria or endosymbionts, including most members of the *Rickettsiales* (Toft and Fares, 2008). Given the immunogenicity and significant energetic cost associated with flagellar synthesis (Toft and Fares, 2008), it is likely that *M. mitochondrii* flagella play an important role in the bacterial life cycle. Such role may be difficult to imagine, if we only think about the motility function of this structure, but flagella have recently been shown to have also other functions, in particular bacterial adhesion and invasion (Haiko and Westerlund-Wikström, 2013) and symbiotic interactions (Shimoyama et al., 2009). Alternatively, the flagellum of *M. mitochondrii* might actually represent a type three secretion system that could mediate adhesion (e.g. to mitochondria) or contribute to host cell manipulation through the secretion of bacterial proteins, similar to other intracellular bacteria (Ashida et al., 2007; Mueller et al., 2014; Waterman and Holden, 2003; Zhang et al., 2012). The flagellum might thus be the key to the interaction between the symbiont and the organelle.

Possible implications of mitochondrial colonization by *M. mitochondrii*

Genomic information allows to formulate hypotheses concerning the impact of *M. mitochondrii* on its host physiology, which await experimental testing. Ecological studies detected high prevalence and low genetic variability of *M. mitochondrii* (Al-Khafaji et al., 2019; Lo et al., 2006), suggesting it could be beneficial to *I.ricinus*, however this is not proven and its specific impact on the host cell or host physiology are unclear as the mechanisms of interaction with mitochondria. A current hypothesis is that the bacterium improves host

cellular respiration during blood-feeding, during which ixodid ticks are thought to enter a hypoxic state. Indeed, the *M. mitochondrii* genome has been found to encode a complex IV cbb3 cytochrome oxidase that allows oxidative phosphorylation at low oxygen tension (Sassera et al., 2011) . This intriguing finding, together with the presence of a bacterially encoded ATP transporter and an observed increase in the bacterial population in response to low oxygen tension and high metabolic needs in the host has led to propose that *M. mitochondrii* could assist the tick cellular respiration during this specific stage of its life cycle (i.e., feeding; (Sassera et al., 2008)). In addition, *M. mitochondrii* might also provide *I. ricinus* with metabolites or vitamins, as recently proven for another intracellular symbiont of the *Francisella* genus, which provides B vitamins to its host, the African soft tick *Ornithodoros moubata* (Duron et al., 2018). *I. ricinus* apparently lacks all enzymes for porphyrin (haem) biosynthesis except for the last three enzymes (Perner et al., 2016). Curiously, the first enzyme encoded in the *I. ricinus* genome is coproporphyrinogen III oxidase (CPOX). In higher eukaryotes, this enzyme is localized in the IMS and it is tempting to speculate that *M. mitochondrii* provides precursors for this enzyme. *M. mitochondrii* might also play a role in the regulation of mitochondrial reactive oxygen species (ROS) production. Functionally, ROS detoxification by antioxidants has been shown to play a role in maintaining fertility/fecundity in *Drosophila* (Parkes et al., 1998) and mosquitoes (DeJong et al., 2007), and maternally inherited arthropod symbionts such as *Wolbachia* have been shown to regulate ROS levels (Zug and Hammerstein, 2015). In ticks, an example of ROS manipulation by bacteria is given by the pathogen *A. phagocytophilum,* which has recently been shown to inhibit ROS production and apoptosis to preserve its replicative niche (Alberdi et al., 2019)**.**

Midichloria mitochondrii **in non-ovarian tissues**

Recently *M. mitochondrii* has been detected in other tick organs, including salivary glands (Mariconti et al., 2012a), malpighian tubules, tracheae and guts (Olivieri et al., 2019). This finding led to hypothesize different functions for the bacterial populations residing in different organs. *M. mitochondrii* subpopulations could not just be supplying essential nutrients to the host but also enhance the reproductive fitness, helping in anti-oxidative defence, energy production, water balance and homeostasis. More 'selfish' reasons for the multiple localization include ensuring both vertical transmission (*M. mitochondrii* in oocytes) and horizontal transmission to the vertebrate host during the blood meal (*M. mitochondrii* in salivary glands), as well as optimization of energy parasitism. While giving some answers, these findings also open a novel question: is *M. mitochondrii* localized also in mitochondria in tissues other than oocytes? The low bacterial load makes electron microscopy a frustrating endeavor, and correlative approaches will be required to definitively answer this question.

Outlook

Mitochondria are highly conserved across almost all eukaryotes and are intensively studied due to their role in aging, cancer and in a variety of pathologies (Nunnari and Suomalainen, 2012). Despite this, many aspects of mitochondrial biology are unknown and 30% of mitochondrial proteins have an unknown function. Studying the crosstalk between *M. mitochondrii* and mitochondria might provide novel insight into mitochondrial biology, much

like the study of *Listeria monocytogenes* has yielded invaluable insight into fundamental cell biological processes of its eukaryotic host cells (Cossart, 2011).

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Figures

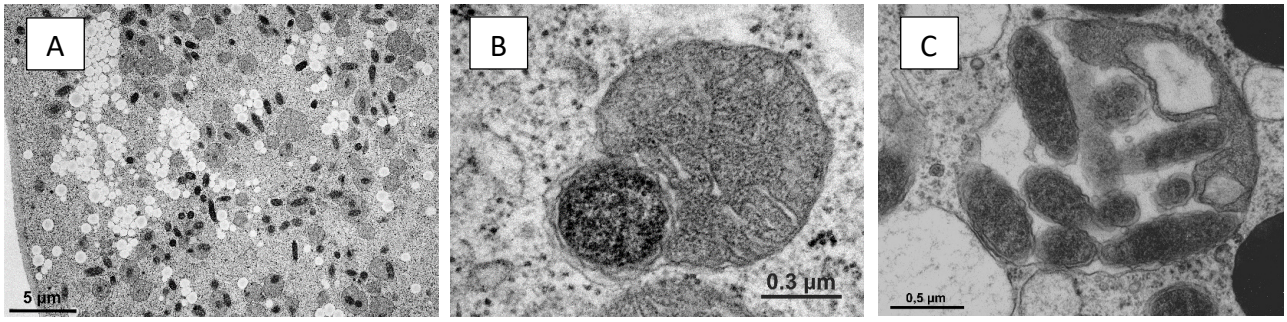

Figure 1

Transmission electron micrograph of *I.ricinus* oocytes colonized by *M. mitochondrii.*

A Low magnification image displaying cytosolic and mitochondrial *M. mitochondrii.* B Mitochondrial intermembrane space localization of a single bacterium. C Multiple bacteria colonize an inflated intermembrane space, while the mitochondrial matrix is highly condensed. Courtesy of Emanuela Clementi and Luciano Sacchi.

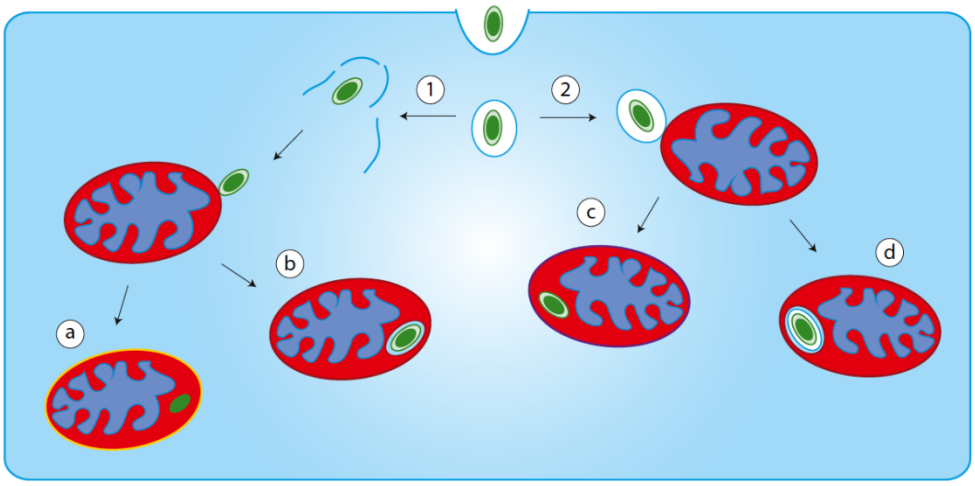

Figure 2

Different scenarios for the life cycle of *M.mitochondrii*. To date no electron micrographs have shown *M. mitochondrii* entering cells, this could occur upon colonization of other tissues or during horizontal transmission. After cell invasion, the bacterium (green) either escapes from the phagosome (1) or remains phagosome-bound (2). After escape, different scenarios could result in colonization of the mitochondrial intermembrane space: (a) of the bacterium and its outer membrane fuses with the mitochondrial outer membrane. (b) The mitochondrion "phagocytoses" the cytosolic bacterium. (c) The phagosomal membrane enclosing the bacterium fuses with the mitochondrial outer membrane. (d) The mitochondrion "phagocytoses" the phagosome containing the bacterium, which would result in two host-derived membranes in addition to the bacterial membranes.

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