Chapter 5
Exploring the Biology and Evolution of *Blastocystis* and Its Role in the Microbiome

Anastasios D. Tsaousis, Emma L. Betts, Abby McCain, Jamie M. Newton, Vasana Jinatham, and Eleni Gentekaki

Abstract *Blastocystis* is a microbial eukaryote, considered to be the most prevalent microbe in the human gut, colonizing approximately one billion individuals worldwide. *Blastocystis* is extremely genetically diverse with 17 distinct genetic subtypes found in birds and mammals. Although *Blastocystis* presence has been linked to intestinal disorders, its pathogenicity still remains controversial due to its high prevalence in asymptomatic carriers. *Blastocystis* can withstand fluctuations of oxygen in the gut and as a result harbors peculiar mitochondrion-related organelles (MROs). These are considered to be an intermediate form between a typical aerobic mitochondrion and an obligate anaerobic hydrogenosome. Genomic analysis has shown that 2.5% of *Blastocystis* genes have been laterally acquired from eukaryotes and prokaryotes. These acquired genes are associated with carbohydrate scavenging and metabolism, anaerobic amino acid and nitrogen metabolism, oxygen-stress resistance, and pH homeostasis. In addition, *Blastocystis* has genes associated with secretion that are potentially involved in infection, escaping host defense and even affect composition of the prokaryotic microbiome and inflammation of the gut. In this chapter, we provide an overview of the state-of-the-art *Blastocystis* knowledge, and present published data that can be used to understand the genomic adaptations of this microbial organism and its role within the microbiome of the hosts.

Keywords *Blastocystis* · Eukaryome · Genetic diversity · Microbiome · Mitochondrion-related organelles (MROs) · Pathogenicity · Prevalencel subtyping

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General Introduction

Blastocystis is a microbial eukaryote colonizing the gastrointestinal tract of a variety of hosts, including humans, artiodactyls, marsupials, perissodactyls, proboscideans and rodents, avian species, reptiles, fish and some insects (Boreham and Stenzel 1993; Stenzel and Boreham 1996). Alexeieff was the first to provide a detailed description of *Blastocystis* from a number of hosts including rats, chickens and reptiles (Alexeieff 1911). At that time, the organism was classified as yeast and named *Blastocystis enterocola*. A number of possible earlier accounts of *Blastocystis* dating as far back as the 1840 London cholera epidemic also exist (Zierdt 1991). Following its discovery in humans, Brumpt (1912) adopted the name *Blastocystis hominis*. The organism is now classified within the stramenopiles, a diverse group of eukaryotes that includes diatoms and oomycetes (Silberman et al. 1996). Specifically, in phylogenetic trees *Blastocystis* groups with opalinids, *Karotomorpha* and *Proteromonas*, all of which reside in the gastrointestinal tracts of metazoans. Though stramenopiles have at some stage of their life cycles a flagellum *Blastocystis* has lost its flagellar apparatus along with any related protein coding genes (Fig. 5.1) (Gentekaki et al. 2017). Four morphologically distinct stages of *Blastocystis* have been identified so far, including the vacuolar, granular, cyst and amoeboid forms (Tan and Suresh 2006; Tan et al. 2010; Tan 2008; Clark and Stensvold 2016). Due to its very small size (being as small as 5 μm and averaging 8–12 μm), immobility and lack of descriptive morphological

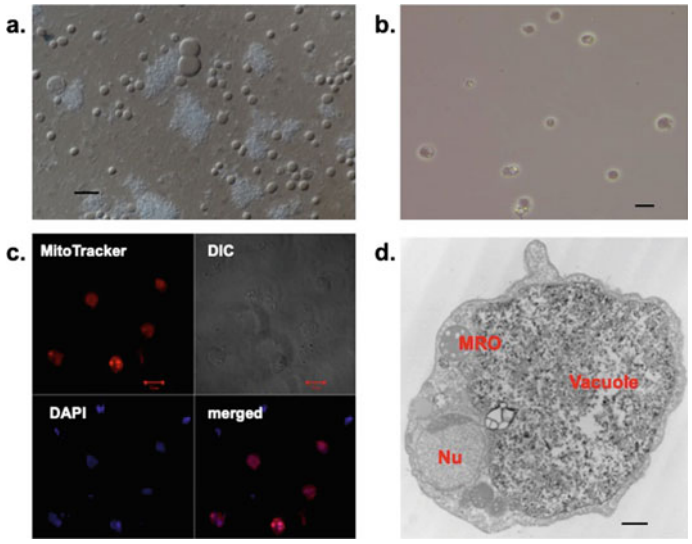

Fig. 5.1 *Blastocystis* cells. **a** Light microscopy of a *Blastocystis* xenic culture in Jones media. Scale bar: 25 μm. **b** Light microscopy of a neutral-red stained *Blastocystis* axenic culture. Scale bar: 20 μm. **c** Staining of *Blastocystis* mitochondrion-related organelles with MitoTracker red, DAPI staining of the nucleus and MROs and a differential interference contrast (DIC) image of *Blastocystis* cells. Scale bar: 10 μm. **d** Transmission electron microscopy picture shown a *Blastocystis* cell with its nucleus (Nu), various MROs and a large vacuole. Scale bar: 500 nm

characters, *Blastocystis* has been often overlooked or mistaken for cell debris when observed using microscopy.

The *Blastocystis* life cycle still requires some elucidation. However, it is widely understood that *Blastocystis* enters the host in the metabolically inactive dormant cyst form via the faecal oral route (Tan 2004). The faecal oral route involves a food or waterborne source being the primary cause of the spread of *Blastocystis*. There is, however, evidence for zoonotic transmission as well. Regardless of the source, once transmission is complete excystation occurs in the large intestine into the vacuolar form and can then further morph into the granular or ameboid form. At this stage *Blastocystis* can replicate by binary fission and can start to proliferate. The organism encysts in the large intestine. The cyst further develops in the faeces losing the fibrillar layer it initially possesses. Once it is released into the faeces, the cyst is free to enter another host.

Blastocystis is one of the most commonly encountered protists in the human gut with an estimated prevalence of one billion (Stensvold and Clark 2016; Clark et al. 2013). Its colonization rate ranges from 20% in Europe (Bart et al. 2013) to over 30% in some developing countries (Alfellani et al. 2013a; Ramirez et al. 2014), with one study showing incidence of 100% in a group of children in Senegal (El Safadi et al. 2014). Numerous animal studies suggest that prevalence of *Blastocystis* is higher in animals (Alfellani et al. 2013c; Betts et al. 2018; Cian et al. 2017). Because asymptomatic carriage is frequent and since presence of other intestinal parasites is not excluded definitively in patients with gastrointestinal symptoms, questions have been raised regarding *Blastocystis* biology, pathogenicity, transmission and possible impacts on the host and its gut microbiota.

Prevalence, Diversity and Biogeography

The advent of molecular methods has revealed unexpected genetic diversity that does not correspond to the morphological stasis of *Blastocystis*; based on SSU rRNA sequences, 17 known subtypes (STs) that colonize mammals and birds (Fig. 5.2) along with various isolates from ectothermic hosts have been identified (Noel et al. 2005; Stensvold and Clark 2016; Yoshikawa et al. 2016). These are all considered separate species. It is highly probable that the genetic diversity of *Blastocystis* is greater, but as yet uncovered due to sampling bias towards specific hosts. Specifically, sampling efforts have centered on humans and animals of importance to us (pets, zoo animals and livestock), while insects, arthropods and other ectotherms remain only sparsely sampled (Alfellani et al. 2013c; Cian et al. 2017; Masuda et al. 2018; Betts et al. 2018; Paulos et al. 2018). Regardless, differences observed in the SSU rRNA gene are also reflected in the genomes of the various *Blastocystis* subtypes. Specifically, not only is the genetic distance between subtypes high, but those also differ in their GC percent content and gene complement (Gentekaki et al. 2017).

At first look, *Blastocystis* does not appear to be host specific (Fig. 5.2). Subtypes 1–9 colonize humans, but these have also been found in several other hosts

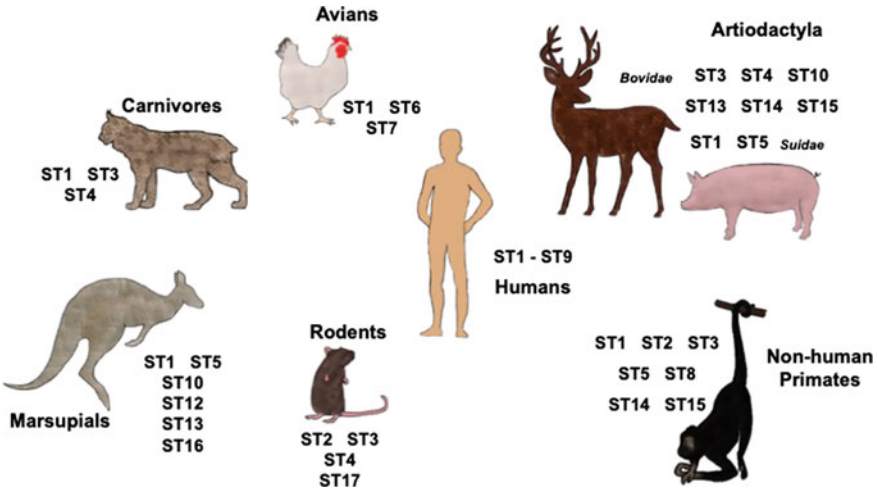

Fig. 5.2 *Blastocystis* subtypes with various host specificities

(Stensvold and Clark 2016). The exception seems to be ST9, which has only been found in humans. ST10 to ST17 have only been found in animals with the exception of ST12, which has been found in humans from South America (Ramirez et al. 2016). Nonetheless, when considering hosts at higher taxonomic levels, then some degree of specificity exists (Alfellani et al. 2013b). For instance, ST10 and ST14 are hallmark subtypes of artiodactyls (Cian et al. 2017; Betts et al. 2018). Notably, sequences from insects and some other ectothermic hosts have yet to be isolated from endotherms. Thus temperature might pose a constraint on host specificity of this organism as it has been speculated for other protists (Jinatham et al. 2019).

The various subtypes colonizing humans are distributed globally, however some patterns have emerged. Subtype 3 is the most abundant and also the most widespread worldwide, while ST4 is mostly restricted in Europe potentially suggesting its recent origin (Stensvold and Clark 2016) (Fig. 5.3). Nonetheless, data suggest that STs present in fowl (ST6, ST7) and porcine hosts (ST5) are more often present in inhabitants from rural regions. Frequent contact between animals and humans in these regions likely leads to transmission of these subtypes between hosts. In contrast, such contact is limited in urban centers.

While many subtyping studies from developed countries exist, those lag behind in low- and middle-income countries (LMICs) (Noradilah et al. 2017; Ramirez et al. 2016; Thathaisong et al. 2013; Yowang et al. 2018). Typically, diagnosis of *Blastocystis* in LMICs is carried out microscopically and is part of large-scale routine parasitology surveys. To our knowledge, currently there are less than 40 reports investigating prevalence and subtyping of *Blastocystis* in LMICs, many of which are focusing on immunocompromised patients or patients with gastrointestinal symptoms (Fig. 5.3). Based on all these studies, the overall prevalence of *Blastocystis* in LMICs of Africa, South America, South East Asia, rest of Asia, and the Middle

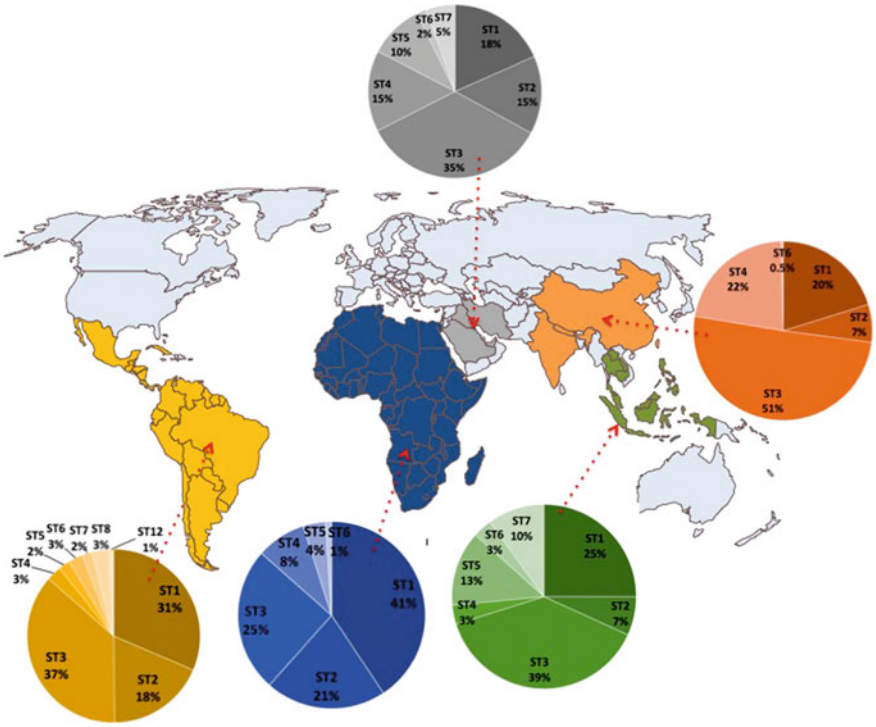

Fig. 5.3 Prevalence and biogeographical distribution of human *Blastocystis* subtypes in low and middle income countries

East averages at around 30% (Fig. 5.3). Matching the global trend, ST3 is the predominant subtype distributed in LMICs, excluding countries in Africa, where ST1 predominates (Di Cristanziano et al. 2019; Abdulsalam et al. 2013). In South America, prevalence rates of ST3 and ST1 are very similar with ST3 being marginally more dominant (Villamizar et al. 2019; Forsell et al. 2017; Oliveira-Arbex et al. 2018). Subtypes ST5 to ST7 are commonly found in all LMICs often in association with fowl and pigs highlighting their zoonotic potential.

Typically, humans seem to be colonized by a single subtype with some exceptions (Meloni et al. 2011; Whipps et al. 2010). Whether this is a methodological issue (direct sequencing versus cloning) or if indeed a single subtype dominates, remains unclear. Though similar information on animals is just emerging, it seems that mixed colonization is more common than in humans (AbuOdeh et al. 2019; Cian et al. 2017; Betts et al. 2018). This information along with ST-specific functional repertoires implies distinct interactions in the human gut ecosystem, an area of study that remains little explored.

Blastocystis *and Microbiome*

Several recent studies have found that presence of *Blastocystis* is strongly correlated to specific microbial profiles. Specifically, *Blastocystis* carriage in individuals without gastrointestinal symptoms has been associated with higher bacterial richness and diversity (Andersen et al. 2015; Audebert et al. 2016; Forsell et al. 2017; Laforest-Lapointe and Arrieta 2018; Nash et al. 2017; Nieves-Ramirez et al. 2018). The lower abundance of *Bacteroides* in *Blastocystis* carriers is a consistent finding across studies from various regions (Andersen et al. 2015; Forsell et al. 2017; Beghini et al. 2017; Tito et al. 2019). In contrast, positive associations exist with *Ruminococcus* and other clostridia, *Prevotella*, and *Methanobrevibacter* (Andersen et al. 2015; Beghini et al. 2017; Nash et al. 2017). The negative association of *Blastocystis* with *Bacteroides* has been attributed to the latter not contributing enough to a "*Blastocystis*-favorable" environment (Stensvold and van der Giezen 2018). When subtype of *Blastocystis* was taken into account in microbiome studies differential associations of STs with specific prokaryotic taxa were noted. Specifically, ST3 was negatively correlated with *Akkermansia*, while ST4 had the opposite relationship (Tito et al. 2019). *Akkermansia* is a mucin utilising bacterium that is considered beneficial. Abundant evidence suggests that this bacterium is indicative of good intestinal health and has protective action against metabolic disorders (Cani and de Vos 2017; Dao et al. 2019; Hanninen et al. 2018). Collectively these results suggest that subtype characterization is essential for accurately determining the relationship between *Blastocystis*, microbiota profiles, and host health.

An important caveat that needs to be taken into account is causality of the observed microbiota changes. Whether it is *Blastocystis* that alters the host prokaryotic microbiota, or another reason (e.g. low-grade inflammation) is as yet unknown (Nieves-Ramirez et al. 2018). Differentiating between the two will greatly advance our understanding of gut ecology and roles of individual component taxa. A good starting point is to undertake studies similar to those of Yason et al. (2019). The authors performed in vitro competitive assays of a laboratory grown strain of ST7 and individual bacteria and extended experiments to cell lines and mice (Yason et al. 2019). The authors consistently showed decrease of *Bifidobacterium longum*, which was attributed to oxidative stress caused by either the host immune system or metabolic activity of *Blastocystis* (Yason et al. 2019). Thus the role of *Blastocystis* in the gut remains unclear hinting at complex interactions that have yet to be defined. A major challenge in accurately defining the role of *Blastocystis* in the gut lies in finding ways to use native microbial flora, which better represents the gut ecosystem rather than lab grown strains.

In that vein, in-depth understanding of *Blastocystis* biology is essential to disentangle its role in the gut and interactions with microbiota.

Blastocystis *Biology*

Currently, the genomes of nine subtypes are available in public databases, but only a few have been investigated in detail. Comparative genomics of as many subtypes as possible would provide considerable insights into their biology and pathogenicity. A recent study showed that up to ~2.5% of *Blastocystis* genes have been laterally acquired from prokaryotic and eukaryotic organisms (Eme et al. 2017). These genes relate to infection and evasion of host defenses, anaerobic amino acid and nitrogen metabolism, oxygen-stress resistance and carbohydrate scavenging, and have likely played key roles in *Blastocystis* adapting to the gut ecosystem (Eme et al. 2017). Of those laterally acquired genes, only one has been cellularly localized and biochemically characterized. The *SufCB* gene encodes for a protein that is involved in the assembly of Fe/S clusters in *Blastocystis.* Similarly to the archaeal taxon Methanomicrobiales, the SUF system of *Blastocystis* has a *SufCB* fusion gene (Tsaousis et al. 2012). In phylogenetic trees, the *Blastocystis* and archaeal homologues cluster together into a strongly supported clade, suggesting a lateral gene transfer event from the Methanomicrobiales. SufCB is also found in the genomes of all *Blastocystis* subtypes, as well as *Proteromonas lacertae*, a close relative of *Blastocystis.* The *Blastocystis* SufCB protein is cytosolic, binds [4Fe-4S] clusters, has ATPase activity, and is overexpressed under conditions of oxygen stress (Tsaousis et al. 2012). This mirrors findings in various bacteria, where the SUF machinery is also overexpressed under oxygen stress and iron depletion (Tsaousis et al. 2014; Mettert et al. 2008; Rangachari et al. 2002).

The alternative oxidase (AOX) is another protein that has played a role to *Blastocystis* dealing with variable oxygen concentrations in the gut. The AOX was shown to localize in the mitochondrion-related organelles (MROs) of *Blastocystis* (Tsaousis et al. 2018). The localization and functional characterization of this protein in *Blastocystis* suggest that the cells themselves do respire oxygen (Tsaousis et al. 2018), questioning previous hypotheses about the "obligate" anaerobic nature of this organism. By having both SufCB and AOX proteins, *Blastocystis* might be able to quickly buffer transient fluctuations of oxygen in the gut. This could also explain the absence of the organism in patients with established inflammatory bowel syndrome (IBS), whereby the gut environment is highly permissive to oxygen (Ramirez et al. 2014; Tito et al. 2019). The peculiarities of *Blastocystis* MROs likely contribute to the organism's ability to survive in the extreme environment of the gut.

Blastocystis *MROs*

In addition to the AOX, *Blastocystis* MROs contain many peculiar and potentially recurrent functions that reflect its unique lifestyle (Fig. 5.4). It is worth mentioning that the MRO is currently the only organelle of *Blastocystis* that has been highly characterized (Makiuchi and Nozaki 2014). These MROs combine metabolic properties

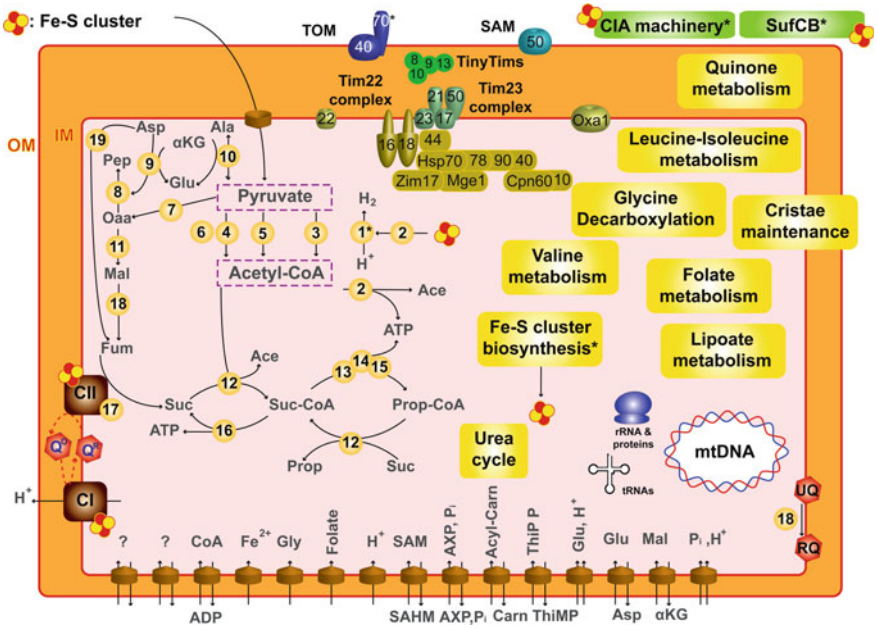

Fig. 5.4 Proposed metabolic map of *Blastocystis* mitochondrion-related organelles (MROs) based on the genome predictions [figure modified from (Gentekaki et al. 2017; Tsaousis et al. 2019)]. Various metabolic features of the *Blastocystis* MRO's roles in energy generation, Fe/S cluster assembly, amino acid and lipid metabolism. Numbers associated with protein descriptions are outlined below: (1) FeFe-hydrogenase, Flavodoxin; (2) iron-only hydrogenase maturation rSAM protein HydE; (3) por, nifJ; pyruvate-ferredoxin/flavodoxin oxidoreductase; (4) dihydrolipoamide succinyltransferase; (5) PDK2_3_4; pyruvate dehydrogenase kinase 2/3/4; (6) 2-oxoglutarate dehydrogenase; (7) Pyruvate carboxylase, alpha subunit; (8) phosphoenolpyruvate carboxykinase (ATP; (9) Aspartate aminotransferase; (10) Alanine aminotransferase; (11) Malate dehydrogenase; (12) Acetate: Succinate CoA transferase; (13) Methylmalonyl-CoA mutase; (14) Methylmalonyl-CoA epimerase; (15) Propionyl-CoA carboxylase alpha subunit; (16) Succinyl-CoA Synthetase; (17) Succinate dehydrogenase subunit 5; (18) Rhodoquinone Biosynthesis enzyme RquA; (19) Aspartate ammonia lyase. Proteins/pathways labelled with an asterix (*) were shown to be localised in *Blastocystis* MROs using immunofluorescence microscopy. Standard amino-acid abbreviations are used: Ace, acetate; ACP, acyl carrier protein; aKG, alpha-ketoglutarate; BCD, branched chain amino acid degradation; CI, Complex I; CII, Complex II; Carn, Carnitine; CDP-DAG, cytidine diphosphate diacylglycerol; CIA machinery: Cytosolic Iron/Sulphur cluster Assembly machinery; CL, cardiolipin; DHAP, dihydroxyacetone phosphate; DHOro, dihydroorotate; Fd, Ferredoxin; Fum, fumarate; Gly3P, glycerol-3-phosphate; Mal, malate; MMC, methyl-malonyl-CoA; Nd(p), NAD(P); mtDNA, mitochondrial DNA; Oaa, oxaloacetate; Oro, orotate; PA, phosphatidic acid; PE phosphatidylethanolamine; Pep, phosphoenol pyruvate; PI, phosphatidylinositol; Prop, propionate; PS, phosphatidylserine; QO/R, quinone/quinol, oxidized or reduced; RQ, rhodoquinone; SAHC, S-adenosylhomocysteine; SAM, S-adenosylmethionine; Suc, succinate; SUF; Sulphur mobilization; THF, tetrahydrafolate; ThiMP, thiamine monophosphate; ThiPP, thiamine pyrophosphate; UQ, ubiquinone

of aerobic mitochondria, anaerobic mitochondria and hydrogenosomes (Stechmann et al. 2008) thus blurring the boundaries between all these organelles, as previously defined (Embley and Martin 2006). In silico predictions have demonstrated that *Blastocystis* organelles do harbor a mitochondrial genome, have elements of both aerobic and anaerobic metabolism having an incomplete tricarboxylic acid (TCA) cycle, pathways of amino acid metabolism, Fe/S cluster biosynthesis machinery, mitochondrial protein import, urea cycle, transporters for exchange of metabolites and quinone metabolism (Fig. 5.4) (Stechmann et al. 2008; Gentekaki et al. 2017). So far, candidates from the following pathways have been localized in *Blastocystis* MROs: the mitochondrial protein import (Tom70) (Tsaousis et al. 2011), components of the Fe/S cluster biosynthesis machinery (Tsaousis et al. 2012; Long et al. 2011; Tsaousis et al. 2014), the anaerobic metabolism (FeFe-Hydrogenase) (Stechmann et al. 2008), the TCA cycle [succinyl-CoA synthesase (SCS)] (Hamblin et al. 2008) and parts of the glycolytic pathway (Río Bártulos et al. 2018) (Fig. 5.4).

Biochemically, a large amount of the current knowledge on *Blastocystis* metabolism has been resolved by functional characterization of its MROs (Lantsman et al. 2008). An unusual feature of its metabolism (Fig. 5.4) is a TCA cycle that runs in reverse to the canonical mitochondrial cycle and with only half of the pathway being present. This pathway terminates with the reduction of fumarate to succinate with fumarate acquiring electrons from fumarate reductase, which is ligated to the membrane bound electron transporter complex II (Tsaousis et al. 2019). The electron transport chain (ETC) also works in reverse to the canonical mitochondrial chain with complex III, complex IV and ATP synthase being absent. Complex I acquires its electrons from NADH (Stechmann et al. 2008), which are then transported to complex II by the reduction of ubiquinone (Q) to ubiquinol (QH_2). QH_2 is oxidised back to Q at complex II and the cycle keeps repeating (Stechmann et al. 2008; Gentekaki et al. 2017). There is no aerobic synthesis of ATP, since ATP synthase is absent, thus the ETC could only be responsible for the production of the proton gradient in the organelle (Denoeud et al. 2011).

Since no chemiosmotic ATP synthesis takes place in *Blastocystis,* ATP is synthesized anaerobically (Denoeud et al. 2011; Stechmann et al. 2008). Though the ATP synthesizing mechanisms of *Blastocystis* are common in anaerobic protists (Muller et al. 2012), its pyruvate metabolism is quite unique (see Fig. 5.4) in that there are three enzymes, which convert pyruvate to acetyl-CoA: pyruvate: ferrodoxin oxidoreductase (PFO) and pyruvate: $NADP^+$ oxidoreductase (PNO), both of which are common in anaerobic protists and pyruvate dehydrogenase (PDH), which is almost universally present in aerobic organisms and canonical mitochondria (Gentekaki et al. 2017; Tsaousis et al. 2019). Although PFO is present in *Blastocystis*, activity of this enzyme has yet to be detected (Eme et al. 2017).

Nonetheless, most biochemical pathways in *Blastocystis* mitochondria remain uncharacterized. An 'omics-based approach, such as metabolomics, could be applied to map the organism's metabolic pathways allowing identification of the metabolites produced by the organism. A metabolomics NMR study has already been conducted to analyze the metabolism of the protozoan parasite *Giardia lamblia* (Vermathen et al. 2018), which would be a practical tool to explore *Blastocystis* metabolism as

well. Previous studies have suggested that *Blastocystis* has a significant impact on the gut microbiome, thus it is not unlikely that its metabolites might underpin specific interactions with the microbiota (Andersen et al. 2015; Hanninen et al. 2018; Yason et al. 2019). Therefore, metabolomic-based studies of *Blastocystis* positive fecal samples, as well as, in vitro metabolomics will contribute significant information.

Conclusion

Blastocystis is a microbial eukaryote that has attracted considerable research interest in the last decade. Despite this, we have only scratched the surface on the role of *Blastocystis* in the complex and extreme habitat of the gut. Significantly, the question of its pathogenicity, which was raised decades ago, remains unanswered. While hypotheses have been brought forth about differential pathogenicity of subtypes, this has not been shown unequivocally. Future studies should include all subtypes of *Blastocystis* and combine in silico analysis, in vitro culturomics and classical cell biology and biochemistry approaches. New tools that will characterize *Blastocystis* and interactions within its native environment (the gut) are urgently needed. Only then can valid conclusions be drawn about the pathogenicity of the different subtypes.

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