Investigating the Biochemical and Organellar Adaptations of *Proteromonas lacertae*

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Abbreviations

ACOX	Acyl-CoA oxidase
ADP	Adenosine diphosphate
AOX	Alternative oxidase
ATP	Adenosine triphosphate
BSA	Bovine serum, adult
°C	Degrees celcius
cDNA	Complementary DNA
CI	Complex 1
CII	Complex 2
CIII	Complex 3
CIV	Complex 4
CO_2	Carbon dioxide
CoA	Coenzyme A
CV	Complex 5
DAO	D-amino acid oxidase
DDO	D-aspartate oxidase
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
ETC	Electron transport chain
FAD	Flavin adenine dinucleotide
FADH	Flavin adenine dinucleotide (semiquinone form)
$FADH_2$	Flavin adenine dinucleotide (hydroquinone form)
FDH	Fumarate reductase
Fe	Iron
FeFe	Iron-only

Fe-S	Iron-Sulphur
GFP	Green fluorescent protein
H_2	Hydrogen
H_2O	Molecular water
HSP	Heat shock protein
IMP	Inner membrane peptidase
IMS	Intermembrane space
ISC	Iron-sulphur (Fe-S) cluster
LB	Luria Broth
MIM	Mitochondrial inner membrane
MOM	Mitochondrial outer membrane
MPP	Mitochondrial processing peptidase
MROs	Mitochondria-related organelles
mtDNA	Mitochondrial DNA
mtHSP	Mitochondrial heat shock protein
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
O_2	Oxygen gas
OD_{600}	Optical density at 600 nm
OXA	Oxidase assembly translocase
P.lacertae	*Proteromonas lacertae*
PAM	Presequence translocase-associated motor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC	Pyruvate dehydrogenase complex

PDH	Pyruvate dehydrogenase
PFO	Pyruvate:ferrodoxin oxidoreductase
Pi	Inorganic phosphate
PNO	Pyruvate:$NADP^+$ oxidoreductase
Q	Quinone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S	Sulphur
SAM	Sorting assembly machinery
SDH	succinate dehydrogenase
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SHAM	Salicylhydroxamic acid
ssRNA	Small subunit RNA
TBS	Tris-buffered saline
TCA	Tricarboxylic acid
TEM	Transmission electron microscopy
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
TPI-GAPDH	triose phosphate isomerase-glyceraldehyde 3-phosphate dehydrogenase fusion
tRNA	Transfer RNA
TTFA	2-thenoyltrifluoroacetone
UQ	Ubiquinone

Abstract

Proteromonas lacertae is an anaerobic, biflagellated microbial eukaryote belonging to Stramenopiles, one of the largest and most diverse groups of eukaryotes, characterised by the presence of tripartite, hair-like structures on the larger of the two flagella. At least one microbial Stramenopile is known to not possess these characteristic features (e.g. *Blastocystis*) which does not resemble other organisms from this group in any way. In-spite them being morphologically very different, *Proteromonas* happens to be the closest-known relative of *Blastocystis*, both being within the opalines. They are also some of the only Stramenopiles known to colonise larger organisms, *Proteromonas* is found in the hindgut of lizards and *Blastocystis* is known to colonise the intestinal tract of a range of animals, yet whether either of them actually cause disease has yet to be confirmed. Not only is their morphology strikingly different, their mitochondria also bear no resemblance to one another. *Blastocystis* possesses multiple anaerobic mitochondria-related organelles (MROs), whereas *Proteromonas* has a single, large lobed mitochondrion closely associated with the nucleus. A striking biochemical observation making *Blastocystis* unique amongst Stramenopiles is the presence of an alternative oxidase, and the absence of complexes III, IV and V of the electron transport chain (ETC). As well as this, it has been predicted to harbour proteins that could establish a reduced/incomplete tricarboxylic acid (TCA) cycle in its MROs. The main focus of this investigation is to explore the mitochondrial protein composition of *Proteromonas* and compare it to *Blastocystis*. In addition, we will attempt to characterise some of the biochemical pathways, including the ETC, proteins involved in the TCA cycle and AOX. Preliminary results on these investigations will be presented. This data shows that, biochemically, *Proteromonas* and *Blastocystis* are very similar, suggesting that these mitochondrial adaptations occurred prior to the diversification of these two organisms.

Keywords: *Proteromonas lacertae*, *Blastocystis*, Stramenopiles, mitochondria/MROs, bioinformatic analysis, biochemical characterisation, metabolic pathways

1.0 Introduction

1.1 Introduction to Proteromonas lacertae and Blastocystis

Proteromonas lacertae is an anaerobic unicellular eukaryote belonging to the Stramenopile (Heterokonta) phylum, the *Opalina* clade, and is closely related to the better known Stramenopile *Blastocystis,* which is commonly known to colonise the human intestinal tract (Silberman *et al.* 1996) as well as the intestines of various other animals. The pathogenicity of both organisms is questionable, and the pathogenicity of *Blastocystis* has been debated for some time, due to the fact that, while it lives within approximately one billion individuals globally, many individuals it colonises are asymptomatic (Gentekaki *et al.* 2017). Despite being very closely related, *Proteromonas lacertae* and *Blastocystis* are morphologically distinct from one another, and *Blastocystis* has seemingly lost characteristics that define Stramenopiles (Gentekaki *et al.* 2017; Denoeud *et al.* 2011).

Stramenopiles are a large group of eukaryotes, containing many different organisms with a great degree of diversity (Denoeud. *et al.* 2011), including diatoms, slimes, algae and water moulds with different characteristics (Gentekaki *et al.* 2017). The organisms within this group can be both unicellular and multicellular, autotrophic and heterotrophic (Leipe *et al*. 1996). All Stramenopiles share two main characteristics, the first characteristic being that they are usually biflagellated during at least one stage of their lifecycle (Denoeud *et al.* 2011) and the second being that these flagella possess tubular, tripartite hair-like structures called mastigonemes (Perez-Brocal, Shahar-Golan and Clark, 2010; Gentekaki *et al.* 2017), which increase the available surface area of the flagella, potentially improving the cells motility. These tripartite hairs were used to demonstrate the closeness between the separate algal species, oomycetes and other heterotrophic lineages. It is theorised that this group, Stramenopiles, formed as a result of the acquisition of these mastigonemes and the group is characterised using the location, biogenesis and ultrastructure of these filaments. Mastigonemes are composed of short basal bodies, with a long tubular shaft and few (2-3) terminal filaments. They are synthesised within the endoplasmic reticulum (ER) and exported to the cell surface, where they are attached to the axonemal (underlying) side of the microtubules (Leipe *et al*. 1996). They are found along the longest (anterior) of the two flagella, and in the cases of *Proteromonas* and *Reticulosphaera*, they are also found on the cell surface (Leipe *et al*. 1996). These structures (now called somatonemes when on the cell surface) appear on the posterior half of *P.lacertae* but not on the anterior surface, which is both bare and corrugated (Brugerolle *et al.* 1988) (*Figure 1*). Each of the corrugated ridges contains a cortical microtubule and myofibril, which likely assist in the migration of the somatonemes and prevent them from attaching to the anterior of the cell. These surface hairs are still produced in the ER and are very similar to

the flagellar mastigonemes in terms of ultrastructure, assembly and microtubule attachment, the main difference between them being their cellular location (Brugerolle *et al.* 1988).

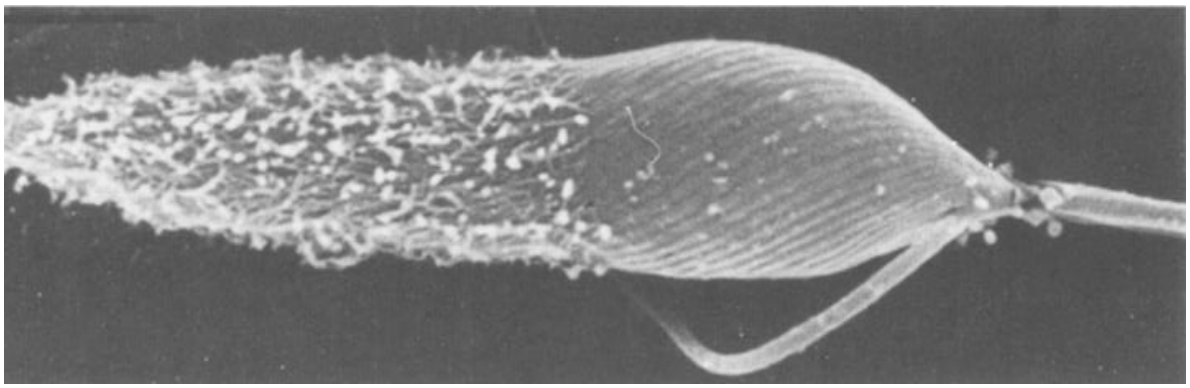

Figure 1. SEM image of *Proteromonas lacertae* (Leipe *et al*. 1996). A SEM image showing the structural features of *P.lacertae*. SEM allows the visualisation of *Proteromonas* in greater detail. The anterior surface of the cell is visibly bare, yet corrugated and the posterior of the cell is covered in the hair like structures called somatonemes. This image also shows the organisation of the flagella, how the anterior flagellum is straight behind the cell, whereas the posterior wraps around the cell.

Interestingly, *Blastocystis* is still characterised within the Stramenopiles, even though it has long since lost the features used to classify Stramenopiles, suggesting that at one time, it may have possessed these flagella, somatonemes, mastigonemes and been morphologically more similar to *Proteromonas*. The reason behind the loss of these characteristics is unknown, perhaps they were lost due to the environment and lifestyle of *Blastocystis*, and perhaps the fact that it is the only Stramenopile known to infect humans may also hint as to why it has changed so drastically in comparison to its close relatives. This is purely speculation, however, and there is no evidence for, all that is known is that *Blastocystis* no longer shares the features used to characterised Stramenopiles.

Blastocystis, as mentioned, is the closest relative of *Proteromonas lacertae* and because of this, comparing these two organisms may help to understand certain adaptations within each of them (Perez-Brocal, Shahar-Golan and Clark, 2010) and potentially gain a sense of evolutionary history of the two organisms. *P.lacertae* colonises the hindgut (posterior intestinal tract) of lizards (Perez-Brocal, Shahar-Golan and Clark, 2010) and is questionable in its pathogenicity. While it has been found within the intestinal tract of lizards it has not yet been documented causing disease. Its morphology better represents a Stramenopile than that of *Blastocystis*. *P.lacertae* is more elongated in shape, being 3 x 13 µm long, and possesses two flagella, one larger than the other, covered in mastigonemes (Perez-Brocal, Shahar-Golan and Clark, 2010). *Proteromonas* has a nucleus, two Golgi, a rhizoplast (a fibrillary structure connecting the nucleus to the kinetosome,

which is the basal body of the flagella) and a single, large lobed mitochondria with tubular cristae and a matrix of varying density (Perez-Brocal, Shahar-Golan and Clark, 2010) (Figure 2).

Blastocystis, however, differs greatly morphologically, in that it is spherical, roughly 5 µm in diameter, non-flagellated and has four forms it takes during different stages of its lifecycle; cyst, vacuolar, granular and amoeboid (Melhorn *et al*. 2012; Tan. 2008). The amoeboid and granular forms are more commonly seen in faecal samples, whereas amoeboid are normally seen cultured *in vitro*. Cyst forms are those that exit the body and are surrounded by a thick wall beneath the outermost membrane (Melhorn *et al*. 2012; Tan. 2008) which protects the cyst from the harsh outside environment, allowing it to survive until it reaches its next host. The inside of the *Blastocystis* cell is also very different, possessing a large, empty vacuole with an unknown function that takes up nearly 80% of the space within the cell, resulting in the cytoplasm and the organelles it contains being pushed up against the cell periphery. In the cyst stage, the vacuole is not observed, in its place instead is a large mass of glycogen (Melhorn *et al*. 2012; Tan. 2008). It has a nucleus, which can vary in number depending on the stage (Melhorn *et al*. 2012; Tan. 2008), Golgi, endoplasmic reticulum. *Blastocystis* has been shown to be lacking the genes required for peroxisomes and therefore are potentially lacking these organelles (Gentekaki *et al.* 2017) however, it is unknown whether *Proteromonas lacertae* peroxisomes are absent. *Blastocystis* has neither the canonical mitochondria, nor even the typical hydrogenosomes (Muller *et al.* 2012; Stechmann *et*

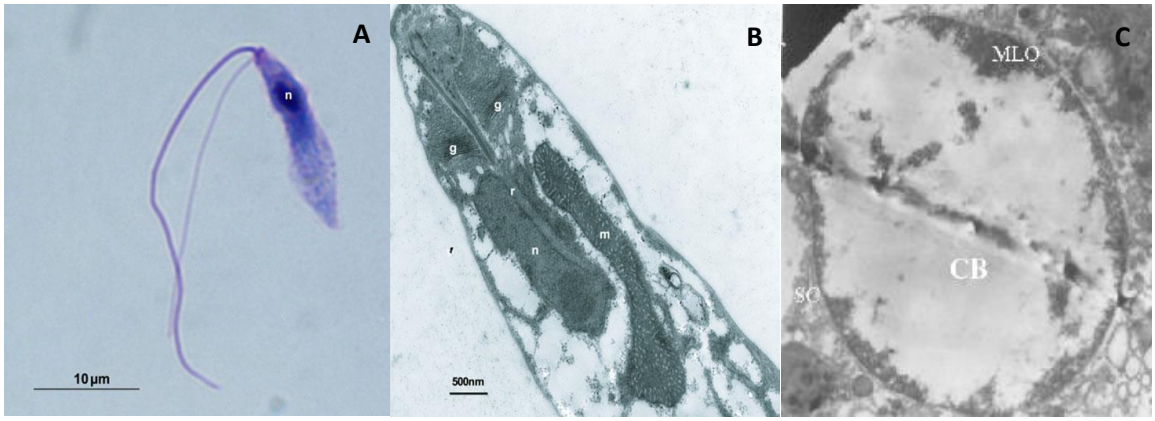

Figure 2. A structural comparison between *Proteromonas lacertae* and *Blastocystis* **(A)** (Perez-Brocal, Shahar-Golan and Clark, 2010) The general morphological features of *Proteromonas* more closely represent Stramenopiles. **(B)** (Perez-Brocal, Shahar-Golan and Clark, 2010) The internal organisation of *Proteromonas*, the most noticeable feature being the single, large mitochondrion very closely associated with the nucleus. **(C)** (Zhang *et al.* 2011) The morphological and internal features of *Blastocystis*, which shares none of the features that *Proteromonas* possesses. As shown, *Blastocystis* has a large vacuole (CB=cell body) taking up the majority of the space within the cell. N,nucleus; G, golgi; R, rhizoplast; M, mitochondrion; CB, cell body; MLO, mitochondria-like organelle.

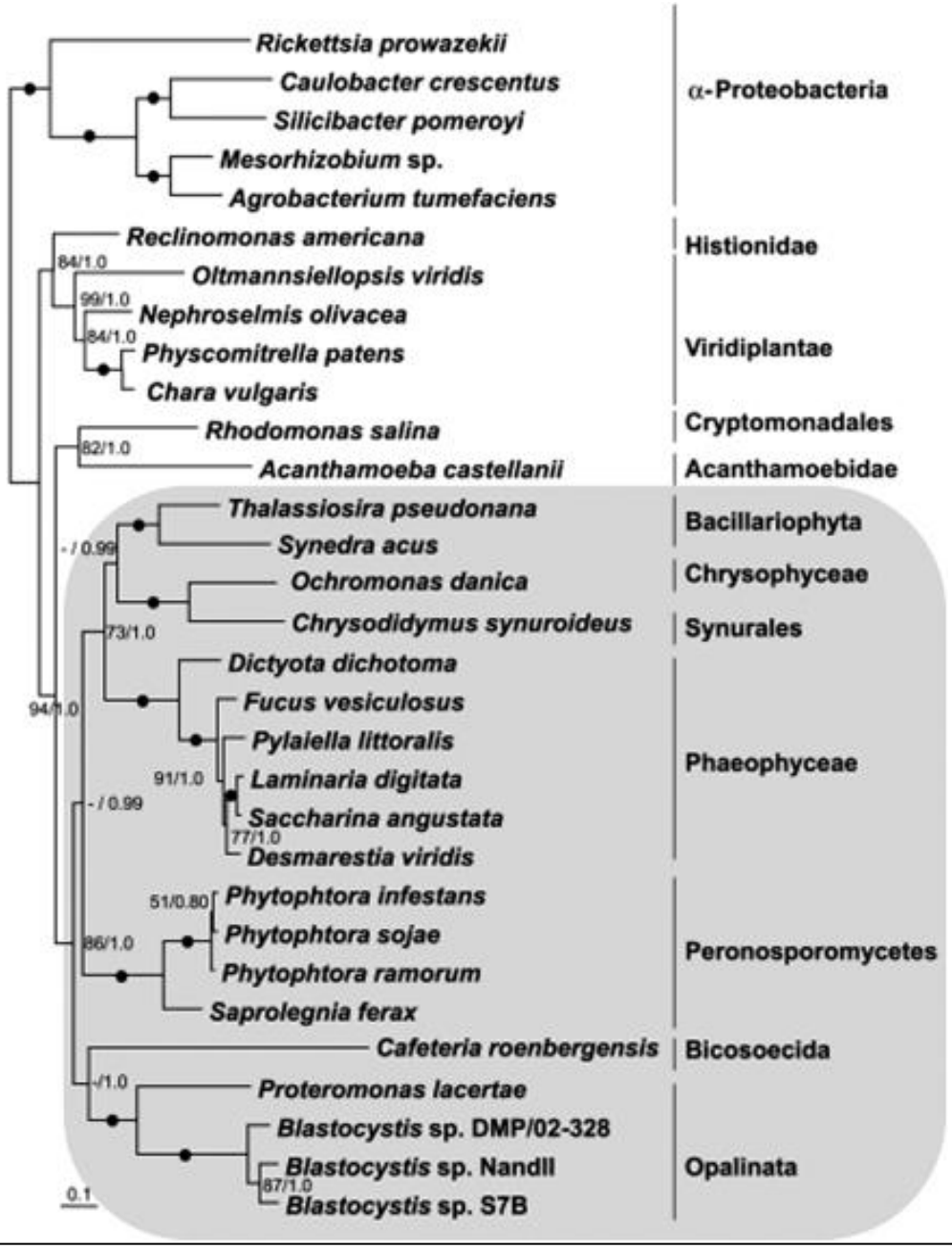

Figure 3. Phylogenetic Tree of Stramenopiles. (Perez-Brocal, Shahar-Golan and Clark, 2008) A maximum-likelihood based phylogenetic tree of the Stramenopiles generated through the use of ETC Complex I *nad* genes. This tree was constructed using the 9 amino acid sequences of NADH dehydrogenase genes found in all Stramenopiles. This phylogenetic tree gives us some idea into the relationship between *Proteromonas* and *Blastocystis* within their clade. It also shows the presence of another closely related organism, *Cafeteria roenbergensis*, which could potentially be compared to *Proteromonas* and *Blastocystis.*

al. 2008), rather it possesses something that is biochemically (Stechmann *et al.* 2008) in-between the two organelles.

As a final note regarding the relationship between *Proteromonas* and *Blastocystis*, it was suggested by Perez-brocal et al. that there may be organisms more closely related to *Proteromonas lacertae*

in the form of *Opalina* and *Protooplaina.* This has yet to be proven, however, and until the genomes of these two organisms are sequenced, *Blastocystis* will remain the closest known relative to *Proteromonas lacertae* (Perez-Brocal, Shahar-Golan and Clark, 2010).

1.2 Mitochondria and Mitochondria-related organelles

All known groups of eukaryotes possess an organelle of mitochondrial origin such as canonical mitochondria and mitochondria-related organelles (MROs), hydrogenosomes or mitosomes (Muller *et al.* 2012), except one, *Monocercomonoides* sp., an organism lacking any form of MRO, and vital pathways typically found in mitochondria, such as iron-sulphur cluster biosynthesis, have been relocated to the cytosol (Karnkowska *et al.* 2016). Typically, the term mitochondria refers to oxygen respiring, double membrane bound organelles with an inner membrane converted into cristae that contain the enzymes required for the tricarboxylic acid cycle and the electron transport chain (ETC or Oxidative Phosphorylation) (Muller *et al.* 2012; Stechmann *et al.* 2008; Modica-Napolitano *et al.* 2002).

Mitochondria are double membrane bound organelles, with the mitochondrial outer membrane enclosing the entirety of the inner mitochondrial content (Modica-Napolitano *et al.* 2002). The mitochondrial inner membrane is organised into a series of folds called cristae, which allow for an increased surface area and the amount of folds varies between cell and tissue types. This increase in surface area allows for more proteins to embed within the mitochondrial inner membrane, such as those involved in process such as oxidative phosphorylation, potentially resulting in a higher yield of ATP or other end products (Modica-Napolitano *et al.* 2002). The main purpose of typical mitochondria is to produce energy for the cell and they play a major biochemical role in the synthesis of ATP using the TCA cycle and the ETC, which is then exported to the cytosol of the cell containing the MRO, with the aid of ATP synthases, which generate the ATP through the use of a proton gradient, and ADP/ATP carriers (Muller *et al.* 2012). However, MROs such as Hydrogenosomes and Mitosomes do not, and use different pathways to generate energy. In anaerobic mitochondria, due to a lack of atmospheric oxygen, a state called anoxia, they do not rely solely upon the use of O_2 as an electron acceptor, rather they use endogenously produced acceptors such as fumarate, and produce succinate as a major by-product (Muller *et al.* 2012).

1.3 Mitochondrial Evolution

For decades now it has been theorised that the mitochondria, plastids and hydrogenosomes have not always been present within eukaryotic cells, and that over time they and their biochemical pathways have been adapted to suit the needs of the cell it resides within. Mitochondria are thought to have once been free-living prokaryotes that were taken up by another cell in an endosymbiotic event that happened roughly two billion years ago yet how this event occurred is

mainly speculated and not exactly known. Eukaryotes, unlike bacteria and archaea, are defined by their membrane enclosed organelles which compartmentalises their internal proteins and reactions, whereas bacteria and archaea, termed prokaryotes, have all of their internal components freely floating in the cytosol, such as the nuclear material which is cytosolic in bacteria yet enclosed within the nucleus in eukaryotes (Gabaldon and Pittis, 2015).

The mitochondria is undoubtedly one of the more integral organelles, being responsible for multiple different functions within the eukaryote, the most notable of which is the generation of cellular energy in the form of ATP. The origin of the mitochondria is suspected to be α-proteobacterial and to have been acquired by eukaryotes in a form of endosymbiotic event, in which the host cell either engulfed the mitochondrial ancestor or the mitochondrial ancestor invaded the host, avoided degradation by the host and over time developed into the mitochondria (Gabaldon and Hyunen, 2004). Despite being of α-proteobacterial origin, only 10-20% of mitochondrial proteins appear to be α-proteobacterial, and as a result of the retailoring process by eukaryotes over-time, the mitochondrial proteome does not have particularly obvious bacterial protein homologues (Gray, 2014). While a large portion of the mitochondrial proteome is nucleus encoded, with preproteins being transported and imported into the mitochondria, the proteins encoded by the mitochondrial genome, while a relatively small amount (organisms such as *Plasmodium* can be as low as 3 proteins, whereas it can be as high as 66 in the excavate *Andalucia godoyi*), are essential in proper mitochondrial function (Gray, 2014). *Proteromonas* is such an example, as its genome has 27 protein encoding genes, but these proteins belong to both ribosomes, necessary for protein translation and NADH dehydrogenase, a component of the electron transport chain (Perez-Brocal, Shahar-Golan and Clark, 2010). Due to the miniscule portion of mitochondrial proteins encoded by the mitochondrial genome, it is theorised that the mitochondrial genome of the last eukaryotic common ancestor encoded no less than 70 proteins, and as exemplified by the mitochondrial genome of *Proteromonas*, the typical eukaryote mitochondrial genome is essential in that it supports the translation of proteins that are part of important mitochondrial functions (Gray, 2014).

Similar to this project, in which the mitochondrial proteome of *Proteromonas* will be generated through bioinformatics, using a predicted proteome consisting of proteins predicated from both nuclear and mitochondrial genomes, the mitochondrial proteome of most eukaryotes can be predicted using this method and another, being tandem mass spectrometry and both techniques can support one another as they both have their strengths and limitations (Warnock, Fahy and Taylor, 2004; Richley, Chinnery and Leister, 2003), in spite of these techniques however, the knowledge regarding many mitochondrial proteomes is incomplete as eukaryotes are extremely diverse. Although the information regarding mitochondrial proteomes is lacking, one this is clear,

that there is a remarkable amount of heterogeneity among mitochondria and mitochondria related organelles, regarding the size of the mitochondrial genome and proteome and the overall functionality of the mitochondria (Gray, 2014).

In order to be able to identify what the last mitochondrial common ancestor was, the proteome and genome was investigated, and compared to modern prokaryotes. At first, the metabolic activity of mitochondria was compared to prokaryotes, and those found to be most similar were Bdellovibrio, a γ-proteobacterium, and paracoccus, an α-proteobacterium, and as a result of their metabolic similarity, they became the first proposed models of proto-mitochondria (Gabaldon and Huynen. 2004). Later, phylogenetic analysis of the ssrRNA and respiratory complex proteins was performed, the result confirmed α-proteobacteria as the mitochondrial ancestor. Next step was to find out which modern α-proteobacteria most closely resembles mitochondria through sequencing the genome of Rickettsia prowazekii, the result of which showed that members of the Rickettsia genus as the mitochondria's closest relatives (Gabaldon and Huynen. 2004; Roger, Muñoz-Gómez and Kamikawa, 2017). Rickettsiales are obligate intracellular parasites, and it has been suggested that mitochondrial ancestor underwent convergent evolution, adapting to become what the host needed (Gabaldon and Huynen. 2004). Other groups such as Wang and Wu also undertook the task of finding the LMCA, using a more expanded database, although the conclusion was the same, the last ancestor of the mitochondria belongs to the Rickettsiales order (Wang and Wu, 2014). Following endosymbiosis, the bacteria underwent metabolic streamlining, the main focus of which was energy generation/conversion, amino acid and protein synthesis (Gray, 2014)

One of the major steps in the formation of the mitochondria is the reduction of the endosymbionts genome. Since the endosymbiotic event, the genome of the mitochondria has reduced significantly, and one of the questions that arose following the discovery of the MCA was just how much of the mitochondrial proteome was of α-proteobacterial origin. As mentioned previously, only 10-20% of the mitochondrial proteome, leading the the theory of lateral gene transfer, a method by which the mitochondria acquired new proteins (Gray, 2014). Eukaryotic genomes, have been affected along with mitochondrial and have expanded thanks to donations of DNA made by the shrinking mitochondrial genome in a process called endosymbiotic gene transfer (Timmis *et al.* 2004). This process of endosymbiotic gene transfer has essentially remodelled and rebuilt the eukaryotic genome, yet it is not restricted to the ancient event of the endosymbiotic event, as it still occurs today, observed through the use of marker genes that show the transfer mitochondrial genes to the nuclear genome in transformed yeast cells (Dyall, Brown and Johnson. 2004; Timmis *et al.* 2004) and occurs with high frequency.

There are multiple theories as to how the mitochondria came to exist inside the eukaryote. One of them, the hydrogen hypothesis proposes that the endosymbiont managed to invade the host

without being eliminated, perhaps due to the endosymbiont offering something that the host needed such as aiding the host in obtaining essential compounds such as hydrogen (Dyall, Brown and Johnson. 2004) and that anaerobic pathways resulted in organelles such as hydrogenosomes or hydrogen producing mitochondria (Embley *et al.* 2003), and the mitochondrial ancestor invaded under anoxic or hypoxic conditions. The second is the aerobic hypothesis, that suggests the aerobic mitochondrial ancestor rescued the host from oxygen tension (Dyall, Brown and Johnson. 2004), resulting in an aerobic organism. While these theories differ, a couple of things remain the same, that mitochondria are of α-bacterial origin and that they came to be within eukaryotes through an endosymbiotic event.

1.4 Major Functions of the mitochondrion

The mitochondrial protein import system

While mitochondria themselves do possess a genome, this genome does not encode everything necessary for them to function and as such will require proteins and other molecules to be transported into them from elsewhere in the cell. In order to be able to do this, mitochondria, in both their outer and inner membranes, possess specialised import machineries, collections of proteins whose function is to allow passage and import of what the mitochondria requires.

Mammalian mitochondria have multiple different pathways they use in order to import precursor proteins synthesised in the cytosol of the cell. These pathways include the presequence, Cys rich, β-barrel, carrier and α-helical pathways (Wiedemann and Pfanner. 2017), however, the presequence pathway will be the main focus here as the Cys, β-barrel and α-helical pathways involve the insertion of these structures into their respective membranes.

The presequence pathway, also known as the classical pathway involves the use of TOM and Tim23 to import protein sequences transcribed in the cytosol that still possess their N-terminal presequences (which can vary in length from <10 amino acids to 100 amino acids in length), which act as targeting signals. Upon entering the TOM complex, the presequence translocase-associated motor (PAM) drives translocation from the intermembrane space into the mitochondrial matrix. A major component of the PAM complex is mtHsp70 which has both an ATPase domain and a binding domain, which allow for recognition of the preprotein (Kulawiak *et al*. 2013). Once in the matrix, a heterodimeric mitochondrial processing peptidase (MPP) will cleave the presequence from the protein, resulting in the formation of the matrix protein. Once they enter the membrane, the heterodimeric MPP will cleave the presequence. The presequence pathway is also the only one of these five sequences that carries any form of N-terminal presequence, but all pathways still have targeting signals (Wiedemann and Pfanner. 2017; Kulawiak *et al*. 2013).

The TOM complex is the main import machinery in the mitochondrial outer membrane and is formed of the major pore, Tom40 and three sensory subunits, Tom20 which is responsible for recognising of the N-terminal presequences and acts as the initial receptor, Tom22 assists Tom20 in binding the protein presequences by binding the positively charged surface of the N-terminal and Tom70 binds proteins with internal targeting sequences (Tsaousis *et al.* 2010). Once the proteins have bound to these recognition proteins, they are directed towards to the β-barrel Tom40, where they are translocated into the intermembrane space. All three of the mentioned receptors have overlapping specificities and, if necessary, can function as the others would. Upon passing through Tom40, the preprotein will interact with the intermembrane domain of Tom22. As well as these four major subunits, there are three smaller, less essential proteins, Tom5, 6 and 7. While these subunits do not contribute to the function of the main complex, they do stabilise the structure (Wiedemann and Pfanner. 2017).

Once they have been translocated through TOM into the intermembrane space, the preproteins interact with TIM23, which is a multisubunit complex responsible for both recognising the N-terminal sequences and transporting the preproteins into the matrix and the insertion into the inner membrane. Another TIM protein, Tim50, acts as receptor, recognising and binding the presequences of preproteins entering the intermembrane space from Tom40. Tom50 also aids Tim21 and Tim23, which is the inner membrane pore of the TIM complex (Wiedemann and Pfanner. 2017). Tim23 is formed of the main pore which is a C-terminal domain domain embedded in the membrane and has an N-terminal domain that extends into the intermembrane space and interacts with Tim50, and Tim50 assists with keeping the pore channel closed. Tim17 is structurally similar to Tim23 in that they both have similar membrane domains and consist of four α-helical transmembrane segments. As well as this they contain motifs necessary for maintaining structural integrity of the complex (Wiedemann and Pfanner. 2017). Tim17 interacts with Tim23 and initiates the release of proteins (Bolender *et al.* 2008). All of the presequences are cleaved proteolytically by the previously mentioned MPP, which can remove these sequences when the preprotein has entered the matrix, or during transport through Tim23 and following cleavage, these presequences are degraded by a presequence protease (Prep). Following initial cleavage by MPP, some proteins can be cleaved again by an inner membrane peptidase (IMP) which removes the hydrophobic sorting sequence, after which the protein is released into the IMS or can anchor to the membrane itself (Wiedemann and Pfanner. 2017).

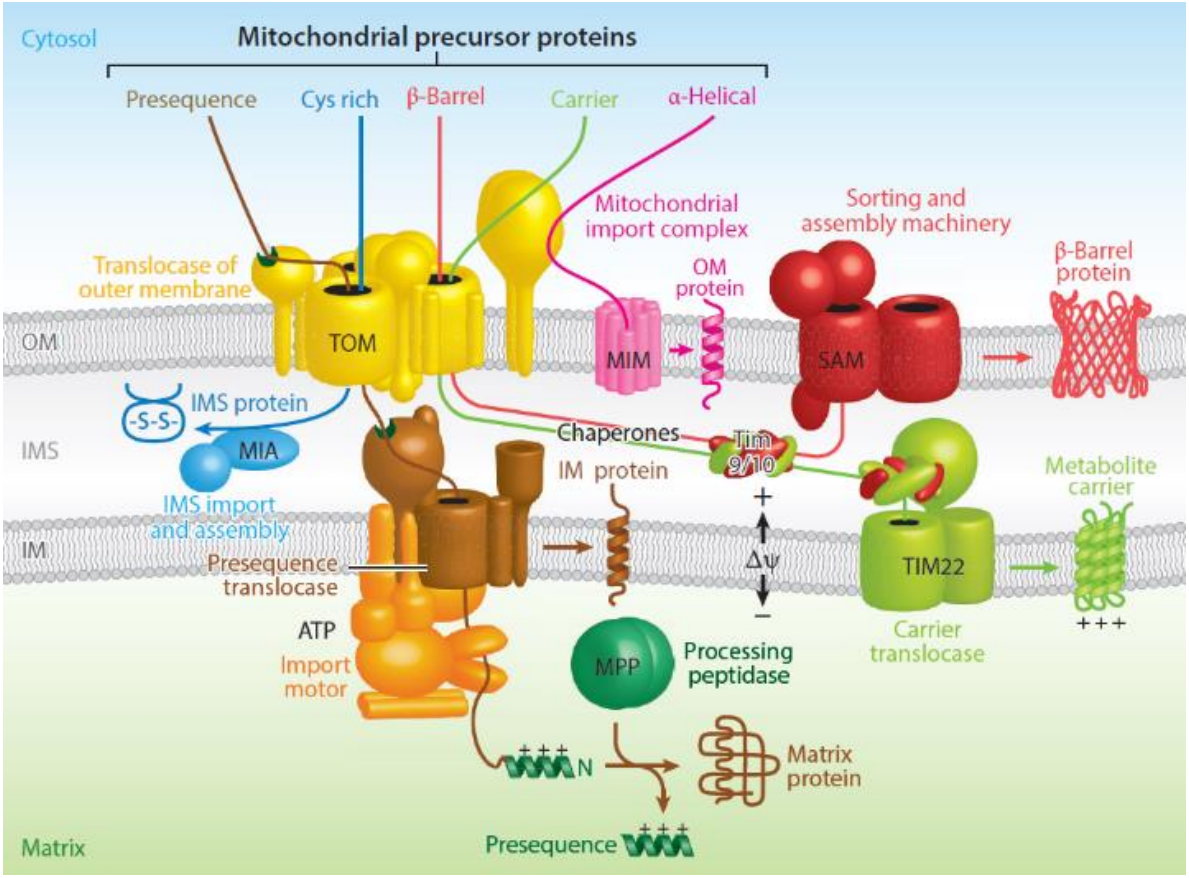

Figure 4. The Mitochondrial Import System (Wiedemann and Pfanner. 2017). The mitochondrial import system consists of protein complexes in the inner and outer membrane, which are responsible for recognising preproteins and importing them into the outer membrane, intermembrane space, the inner membrane or the matrix.

Another essential protein within the mitochondrial import system is the oxidase assembly translocase (OXA), the main component being Oxa1, which is responsible for the transport of proteins from the mitochondrial matrix and insertion into the inner membrane. Oxa1 along with Mba1 will bind to ribosomes and aid in the insertion of proteins into the inner membrane. It also mediates the sorting of inner membrane proteins encoded by the nucleus and imported by TIM23. Its final function is to aid in the assembly of Tim22, and defects in Oxa1 impairs biogenesis of noncleavable proteins (Hildenbeutel *et al.* 2012).

The import system of *Blastocystis* has been illustrated by Gentekaki *et al.* (Figure 5) when they were investigating its genome. In comparison to the standard mammalian model, there are many similarities, but also many differences. When comparing their TOM complexes, *Blastocystis* lacks Tom 20, 22, 5, 6 and 7. It lacks SAM 35 and 37 but has the major components of both complexes, possessing what appears to be reduced forms. As far as the TIM complex is concerned, there appear to be no differences, *Blastocystis* NandII even possesses similar Tiny Tims and Oxa (Gentekaki *et al*. 2017). The composition of *Proteromonas* import however, is unknown. Given how close they are within their clade, it could be theorised that their import systems could be similar, but until the

protein and biochemical contents of the mitochondria have been characterised, it is not possible to say.

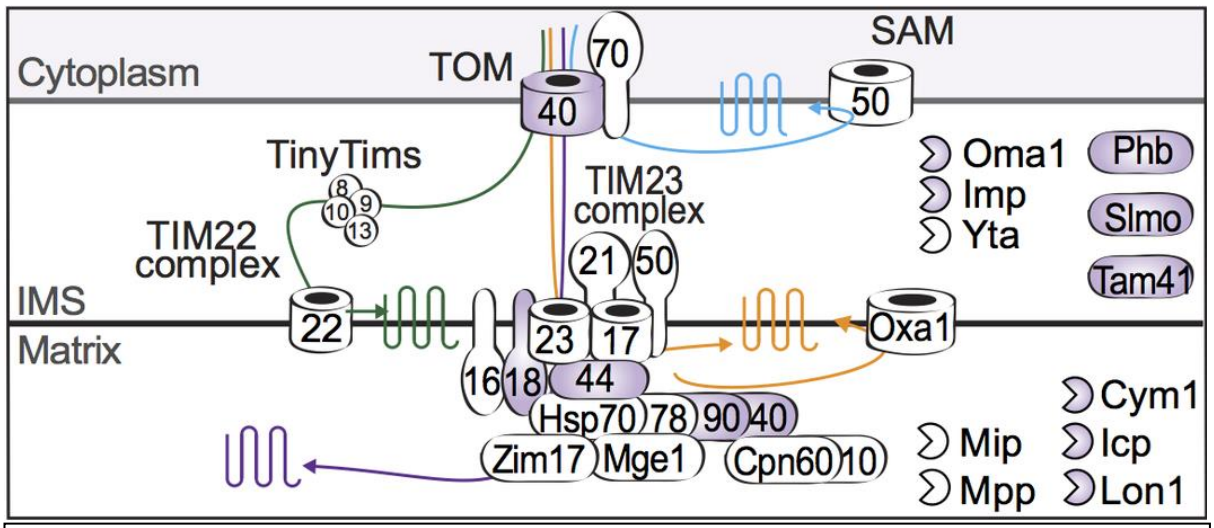

Figure 5. The predicted mitochondrial import system of *Blastocystis* NandII (Gentekaki *et al.*2017). The import system present within *Blastocystis* MROs appears to be similar to the import system present in mammalian mitochondria, only it is reduced by comparison.

Electron Transport Chain

Energy metabolism by mitochondria and MROs is an essential process that allows the survival of organisms both multicellular and unicellular. Described below are the two main metabolic process that lead to the synthesis of ATP, a vital molecule that fuels many cellular biochemical pathways. Most free energy produced in through glucose oxidation is in the form of NADH and FADH (Dolezal *et al.* 2005), which act as coenzymes. These products, NADH and $FADH_2$, are produced I the cytosolic glycolysis pathway (Dolezal *et al.* 2005) which will then go on to participate in other metabolic cycles, namely the electron transport chain.

Mitochondria have two membranes, an outer membrane, which is permeable to small molecules and ions through the use of pores and channels, and an inner membrane which is impermeable to most molecules including H^+, which must enter through specific transporters (Lodish *et al.* 2000). Embedded within this inner membrane are the proteins required for certain metabolic pathways carried out within the mitochondria, such as those of the electron transport chain (Complexes I-V) (Lodish *et al.* 2000).

Within the matrix of the mitochondria resides other important proteins, such as the pyruvate dehydrogenase complex. This complex is responsible for the conversion of pyruvate to acetyl-CoA, the starting point of the citric acid cycle, a main producer of NADH (Lodish *et al.* 2000). It contains ribosomes required for protein synthesis, especially of hydrophobic proteins that cannot be imported through the mitochondrial membranes, such as NADH dehydrogenase complexes found in *Proteromonas lacertae*, as they are encoded within the mitochondrial genome. The enzymes of

the TCA cycle, the enzymes used in fatty acid β-oxidation and enzymes required for amino-acid oxidation also reside within the mitochondrial matrix (Lodish *et al.* 2000).

The ETC is responsible for the transfer of electrons from electron donors through the use of different electron acceptors, which will ultimately lead to the generation of new molecules of ATP. The generation of this ATP is coupled to the oxidation of $FADH_2$ and NADH and in the later stages, the reduction of O_2 to H_2O (Lodish *et al.* 2000; Nelson and Cox, 2013). The electron transfer process begins with the electrons form the NADH generated through the TCA cycle entering the first complex, NADH dehydrogenase (or also called NADH-Q oxidoreductase), which is the largest of the membrane-bound complexes (Rutter *et al.* 2010; Berg *et al.* 2002). NADH dehydrogenase is a multi-subunit protein with some of the NAD genes being encoded by the mitochondrial genome, and the others being encoded by the nuclear genome (Berg *et al.* 2002). It is a large 'L' shaped structure, with part of it being embedded in the mitochondrial inner membrane, and the rest projecting into the mitochondrial matrix (Rutter *et al.* 2010; Berg *et al.* 2002). In the first stage of the reaction, the electrons are transferred from NADH to the FMN prosthetic group, forming the reduced version, $FMNH_2$. Following electron transfer to FMN, the electrons are transferred from $FMNH_2$ to Fe-S clusters where they are then shuttled to coenzyme Q (During the transfer of these two electrons, four H^+ ions are pumped from the matrix of the mitochondria, into the intermembrane space) forming QH_2. These electrons are then released to a mobile quinone in the membrane (resulting in the uptake of two H^+ from the matrix) (Rutter *et al.* 2010).

The next enzyme in the ETC is succinate dehydrogenase. The eukaryotic SDH complex consists of four different subunits, SDHA, B, C and D. SDHA and SDHB form the hydrophilic head of the complex, which protrudes into the matrix of the mitochondria, it is also the main catalytic site for the conversion of succinate to fumarate during the TCA cycle as they contain the necessary redox cofactors the enable electron transfer from the donors to ubiquinone (Nelson and Cox, 2013).. SDHA is the main binding site for succinate and contains a FAD, SDHB contains 3 iron/sulphur clusters that transfer the electrons to ubiquinone (Nelson and Cox, 2013).. These subunits can also work in the reverse, as opposed to converting succinate to fumarate, they can convert fumarate to succinate and therefore possess fumarate reductase activity. Fumarate reductase can replace succinate dehydrogenase in the respiration of anaerobic organisms (Nelson and Cox, 2013). Subunits SDHC and SDHD are a part of the membrane anchor complex and are embedded within the mitochondrial inner membrane, it is also the site of transfer of electrons to Q (Nelson and Cox, 2013). No protons are pumped from the matrix to the IMS during this stage of the ETC.

Complex III, or ubiquinol cytochrome c oxidoreductase, is formed of eleven subunits and contains three different haem groups, b_L (low affinity), b_H (high affinity) and c_1. The enzyme also contains an iron sulphur protein with a 2Fe-S centre for electron transfer (Rutter *et al.* 2010). Electron transfer

in this part of the chain is a cycle in itself, the Q cycle, in which the electrons are transferred to the cytochrome *c* from Q. The quinone binds one of the two quinone binding sites, Q_o and Q_i, at this stage it binds Q_o, it then transfers its electrons. The first of the two electrons transfers to the Fe-S centre and then to cytochrome c_1 and finally cytochrome *c* from which it diffuses (Lodish *et al.* 2000; Rutter *et al.* 2010). The second electron first transfers to cytochrome b_L then to b_H and finally to a quinone currently bound to the Q_i site. This repeats until a second molecule of QH_2 is formed and a total of four protons are pumped from the matrix to the IMS (Rutter *et al.* 2010).

The final stage of the ETC involve s complex IV, cytochrome C oxidase, which catalyses the oxidation of the reduced cytochrome *c*, a reaction which is coupled to the reduction of O_2 to H_2O. The four electrons proceed through the complex at different stages, and two protons are pumped from the matrix into the IMS. Oxygen acts as the terminal electron acceptor in aerobic organisms as it has a high affinity for electrons (Rutter *et al.* 2010), whereas other molecules such as fumarate are used

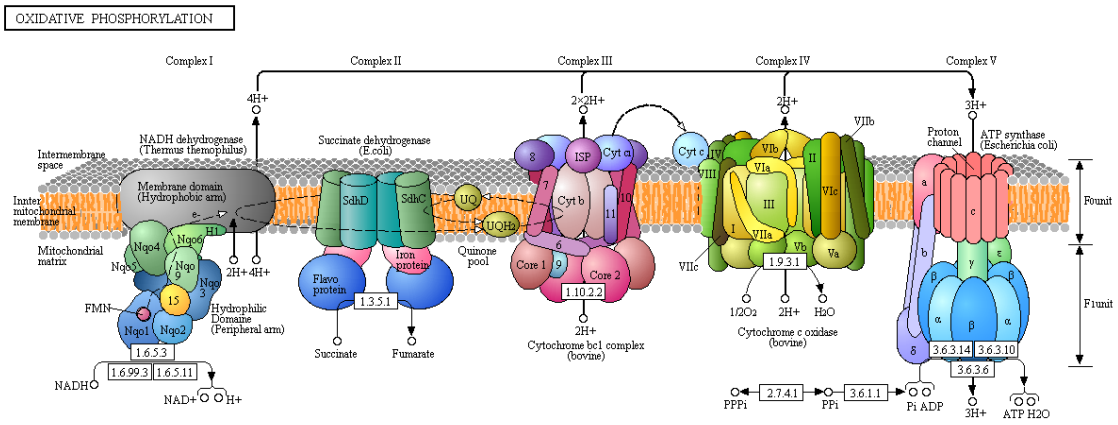

Figure 6. The mammalian electron transport chain. (KEGG database- https://www.genome.jp/kegg-bin/show_pathway?map=map00190&show_description=show) The mammalian electron transport chain is more complex and has more complexes than that found in *Blastocystis*, having a total of five main protein complexes as opposed to the two complexes and AOX found in *Blastocystis.* This ETC results in a greater yield of ATP through increased proton pumping.

as electron acceptors in anaerobic organisms. Following this stage of the electron transfer chain, the protons that have been pumped from the matrix into the IMS now pass into complex V, ATP synthase, which drives the production of ATP using molecules of ADP + Pi. Therefore, the combined effort of glycolysis, TCA cycle and ETC result in the generation of ATP.

Blastocystis has an abnormal electron transport chain, in that is reduced in comparison to the standard five complex mammalian ETC. While *Blastocystis* has complexes I and II, it lacks III, IV and ATP synthase, instead these complexes are replaced by an alternative oxidase, a protein present in the mitochondria of Trypanosomes, Microsporidia, fungi and plants (Mcdonald and Vanlerberghe, 2004; Muller *et al.* 2012). AOX is an inner membrane protein present within the mitochondria and

acts as a member of the ETC, functioning similarly to complex IV, in that is catalyses the reduction of oxygen to water through accepting electrons from ubiquinone (Mcdonald and Vanlerberghe, 2004). Unlike *Blastocystis*, however, many of the other organisms in possession of an AOX also still have electron transport chain complexes III, IV and V and in *Blastocystis*, seems to replace complex IV, reducing oxygen instead. AOX have multiple functions and are not limited to the reduction of O_2. Complex IV is sensitive to cyanide, which inhibits its activity, but AOX is cyanide resistant (Pennisi *et al.* 2016), many organisms can use the alternate AOX pathway to avoid killing by certain fungicides or any other complex III or IV inhibitors as AOX is unaffected by such compounds. While the AOX compound acts as a detour, it is also less efficient as using it instead of the final set of complexes results in fewer protons being pumped into the intermembrane space, and in organisms with a ATP synthase would result in a much lower yield of ATP (Mcdonald, Vanlerberghe and Staples, 2009). As well as O_2 reduction and protection against certain compounds, in some organisms, AOX is vital in reducing cellular stress, such as in anoxic or hypoxic conditions, by regulating reactive oxygen species production. AOX reduces ROS by preventing the over-reduction of the ETC by dissipating excess energy as heat, preventing over reduction of the quinone pool (Mcdonald and Vanlerberghe, 2004; Vishwakarma *et al.* 2015).

Glycolysis

Along with the electron transport chain, glycolysis is a major energy generation pathway within the cell, unlike the ETC however, glycolysis is cytosolic and energy production occurs through the metabolism of glucose. In glycolysis, a single molecule of glucose is degraded in a series of sequential reactions, and at certain points during this sequence, energy is released in either the form of ATP or NADH. In glycolysis, there are 10 steps and two main phases, the preparatory phase and the payoff phase with the preparatory phase requiring ATP and the payoff phase yielding both ATP and NADH (Nelson and Cox, 2013). The preparatory phase, the first five steps, involve the phosphorylation of glucose by hexokinase to glucose 6-phosphate, the conversion of G6P by phosphohexose isomerase to fructose 6-phosphate which is then phosphorylated by phosphofructokinase-1 into fructose 1,6-bisphosphate. Both phosphorylation steps require the consumption of a single molecule of ATP, which is converted to ADP (Nelson and Cox, 2013). During the fourth step, fructose 1,6-bisphosphate is split by aldolase into two separate molecules, glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, the latter of which is isomerised by triose phosphate isomerase into a second molecule of G3P, following which glycolysis has left the preparatory phase and entered the payoff phase (Nelson and Cox, 2013).

The payoff phase is the energy production phase of glycolysis (Nelson and Cox, 2013). The two molecules of G3P are both oxidised and phosphorylated by Pi and G3P-dehydrogenase, forming 1,3-bisphosphoglycerate, following which both molecules are converted by phosphoglycerate

kinase to 3-phosphoglycerate. 3-phosphoglycertae is the converted to 2-phospholgycerate by phosphoglycerate mutase, which is converted to phosphoenolpyruvate by enolase and then pyruvate by pyruvate kinase. The steps involving the conversion of 1,3-bisphosphoglycerate to 3-phosphoglycerate and phosphoenolpyruvate to pyruvate are the energy generation steps, each one producing 2 molecules of ATP, which yields 6 in total, since 2 are consumed in the preparatory phase and 8 are generated in the payoff phase. Pyruvate is the main end product of glycolysis and has multiple fates upon being formed, the first being its oxidation into acetyl-CoA which is a major component within the tricarboxylic acid cycle (Nelson and Cox, 2013). The second is via lactic acid fermentation, which occurs in states of hypoxia as NADH cannot be oxidised to form NAD^+ which is required for the electron transport chain, so instead pyruvate will be reduced to form lactate, accepting these electrons from NADH, allowing the formation of NAD^+. The third and final fate is common in plants, protists and other organisms in which pyruvate is fermented to ethanol (Nelson and Cox, 2013).

The information regarding glycolysis in *Blastocystis* is somewhat lacking, however, Hamblin *et al.* in their supplemental data have shown that the proteins expressed within the glycolytic pathway are the same as those in mammalian glycolysis, with the exception being that no pyruvate kinase has been expressed (Hamblin *et al.*, 2008). Curiously though, while the majority of the pathway appears to be identical to that found in mammals, there is one major difference, *Blastocystis* is in possession of neither triose phosphate isomerase or glyceraldehyde-3-phosphate dehydrogenase as individual proteins, rather is has a fusion of the two, referred to as TPI-GAPDH fusion protein which, due the presence of mitochondrial targeting signals, was assumed to be located in the MROs of *Blastocystis*, and was later confirmed through the use of GFP and confocal microscopy, showing localisation within the MROs (Bartulos *et al.*2018). Following the discovery of TPI-GAPDH localisation within the MROs mead to the analysis of the localisation of the remaining glycolytic proteins, the result of which shows that, only the proteins in the payoff phase (second half) of the pathway were found in the mitochondria (Bartulos *et al.*2018).

The Tricarboxylic acid cycle

Pyruvate, as previously mentioned, has three main fates: lactic acid fermentation, alcohol fermentation and oxidation to acetyl-CoA. Pyruvate is oxidised to acetyl-CoA by a multi-enzyme complex called pyruvate dehydrogenase, consisting of three enzymes: E_1, E_2 and E_3, which acts to dehydrogenate and decarboxylate pyruvate. The oxidative decarboxylation of pyruvate is an irreversible step, and in the process the carboxyl group is removed and a molecule of acetate and CO_2 is formed, and a molecule of NAD^+ is reduced to NADH, which will then be oxidised by complex I of the electron transport chain. Each of the enzymes in the PDH complex have a separate role, E_1 decarboxylates pyruvate, forming hydroxyethyl-TPP and then oxidising the hydroxyethyl group to

an acetyl group (Nelson and Cox, 2013). E_2 then catalyses the formation of acetyl-CoA through the transfer of the newly formed acetyl group to coenzyme-A and E_3 regenerates lipoate. The citric acid cycle has 8 steps following the formation of acetyl-CoA: 1) condensation of acetyl-CoA by citrate synthetase, 2a) citrate is dehydrated by aconitase to *cis*-aconitate and then 2b) rehydrated by aconitase again to form isocitrate. Steps 3) and 4) both involve oxidative decarboxylation of isocitrate to α-ketoglutarate by isocitrate dehydrogenase and α-ketoglutarate to succinyl-CoA by an α-ketoglutarate dehydrogenase complex respectively. 5) Involves the substrate level phosphorylation of succinyl-CoA by succinyl-CoA synthetase to form succinate. Step 6) is where the TCA cycle and ETC crossover as succinate undergoes a dehydrogenation by succinate dehydrogenase (complex II) which results in electrons being transferred to quinone, and forming fumarate in the TCA cycle. Step 7) fumarate is hydrated into malate which is the 8) dehydrogenated into oxaloacetate, which is the final step (Nelson and Cox, 2013). The oxaloacetate will then go onto accept the acetyl group from acetyl-CoA catalysed by citrate synthase in the first step of the cycle, restarting the cycle once again. The main products of the citric acid cycle are NADH produced during steps 3, 4 and 8, ATP (or GTP) produced at step 5 and $FADH_2$ by complex II at step 6. (Nelson and Cox, 2013)

It has been shown that the TCA cycle in *Blastocystis* is not quite the same as the one present within mammals, which is to be expected as most of the other metabolic functions and pathways are reduced as well. *Blastocystis* is in possession of the multi subunit pyruvate dehydrogenase, which is significant as hydrogenosomes and mitosomes do not possess this complex. *Blastocystis* has two ways to form acetyl-CoA through pyruvate decarboxylation; the first uses PDH which connects with the electron transport chain, and the second uses PFO and having both of these enzymes allows the organism to convert pyruvate to acetyl-CoA in a broader range of oxygen levels (Stechmann *et al.* 2008). The entire standard TCA cycle is not present within the MROs of *Blastocystis*, however, and through analysis of expressed sequence tags, the following were not found: citrate synthase, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase (Stechmann *et al.* 2008), meaning that *Blastocystis* is essentially missing the first half of the TCA cycle. The absence of the proteins has led to a slight reconstruction of the pathway, and where the first few steps should be, the initial step in the *Blastocystis* TCA cycle is the conversion of acetyl-CoA to acetate by acetate:succinate CoA transferase which transfers the coenzyme A to succinate, forming succinyl-CoA, which can then be converted back to succinate by succinyl-CoA synthetase (which is coupled with Pi to form ATP). The succinate then goes on the participate in the TCA cycle by interacting with succinate dehydrogenase, being converted to fumarate, generating $FADH_2$, fumarate is converted to malate and malate to oxaloacetate (Stechmann *et al.* 2008; Tsaousis *et al.* 2010). The oxaloacetate can then be converted to phosphoenolpyruvate by phosphoenolpyruvate carboxykinase (Gentekaki *et al.* 2017).It is also possible that the pathway proceeds in reverse.

Pyruvate carboxylase carboxylates pyruvate to from oxaloacetate, which can then be converted to malate, fumarate, and then succinate by their respective enzymes (Tsaousis *et al.* 2010; Stechmann *et al.* 2008). If it was to proceed this way, this would mean that succinate dehydrogenase acts as a fumarate reductase instead. Both glycolysis and the citric acid cycle are more complex than this simple overview, however, a basic understanding is all that is needed to be able to see just how different mammalian cycles are in comparison to protists such as *Blastocystis*.

Fe-S cluster biosynthesis

Iron-sulphur clusters are essential cofactors found in nearly all cell and are involved in major metabolic processes such as protein translation, DNA maintenance, regulation of gene expression, energy production etc. (Braymar and Lill, 2017). In eukaryotes, Fe-S cluster biosynthesis machinery is found in the cytosol, mitochondria and other mitochondria-related organelles. In some organisms such as *Cryptosporidium* (Dellibovi-Ragheb, Gisselberg and Prigge, 2013), *Microsporidia* (Tsaousis *et al*, 2008) and *Giardia* (Pyrih *et al,* 2016), the mitochondria have been heavily reduced into mitosomes and contains only Fe-S machinery. Due to the damaging redox reactions that occur with excess iron and sulphide, a strict balance must be maintained in order to prevent organellar and cellular damage (Braymar and Lill, 2017).

There are multiple steps involved in the synthesis of iron-sulphur clusters. The first requires the synthesis of [2Fe-2S] clusters with the help of Isu1, a scaffold protein, cysteine desulphurase to donate sulphur and frataxin to donate the iron (Dellibovi-Ragheb, Gisselberg and Prigge, 2013; Braymar and Lill, 2017). The second involves the release of the cluster from the scaffold through the use of a chaperone, which then assists in binding to a downstream Grx5 iron-sulphur cluster transfer protein. Following this, the cluster is inserted into machinery that facilitates the synthesis of [4Fe-4S] clusters. The final two steps, [2Fe-2S] clusters are converted into [4Fe-4S] clusters, which can then be inserted/used as required by the cell (Braymar and Lill, 2017).

Like other eukaryotes, *Blastocystis* has been found to be in possession of Fe-S cluster machinery (Tsaousis *et al.* 2012). SufCB, a protein fusion, was identified in *Blastocystis*, which was acquired through lateral gene transfer from a methanoarchaea. It localises within the cytosol of *Blastocystis* and is involved in the maturation of iron-sulphur cluster proteins, displaying normal cysteine desulphurase activity and exhibiting ATPase activity in the presence of magnesium chloride (Tsaousis *et al.* 2012). As well as investigating the localisation and functions of *Blastocystis* SufCB, Tsaousis *et al.* investigated the localisation of the iron-sulphur cluster system. It was found to be localised within the MROs of *Blastocystis*, although many different anaerobic parasitic protists such as *Trachipleistophora* and *Entamoeba* have relocated their Fe-S cluster machinery to their cytosol (Tsaousis *et al,* 2012). It has been shown that *Blastocystis* possesses homologues of yeast

mitochondrial ISC machinery and, using mitotracker, the machinery was found to localise within its MROs (Tsaousis *et al.* 2012).

1.5 *Blastocystis* MROs

In order to be able to properly investigate the mitochondrial adaptations of *P.lacertae*, the properties of *Blastocystis* mitochondrion-like organelles must first be looked at, given how *Blastocystis*, as mentioned, is the closest relative of *P.lacertae*.

Even though *Blastocystis* is strictly anaerobic, it still possesses a cristae bearing MROs, which under the electron microscope appear to be fairly typical (Tsaousis *et al.* 2010). These cristae were visualised through using varies dyes which were accumulated within the mitochondria, suggesting that the MRO actively maintains an electrochemical proton gradient across the mitochondrial membranes (Muller *et al.* 2012). Stramenopile mitochondria resembling standard mitochondria is apparently quite remarkable, given how, to survive in environments that are either hypoxic or anoxic, anaerobic organisms have replaced their mitochondria with either mitosomes or hydrogenosomes.

Blastocystis MROs are also in possession of a genome, something that hydrogenosomes do not normally have, which is roughly 27,000-29,000 bp in size, which is small for stramenopiles as their genomes typically range from 31,000-59,000 bp (Tsaousis *et al.* 2010). The genome of *Proteromonas* is known, and is roughly 48,000 bp (Perez-Brocal, V., Shahar-Golan, R. and Clark, G. 2010) which more resembles other stramenopiles as opposed to *Blastocystis*, suggesting that *Blastocystis* has, along with loss of characteristic Stramenopile features, streamlined its genome, losing anything considered unnecessary.

Blastocystis MROs appear to have incomplete versions of energy metabolism cycles present in canonical mitochondria (Figure 7). The first note is that the acetyl-CoA metabolism pathway appears to be an incomplete Krebs cycle (while this may not be the case, this is what it appears to be), as it has the stages from Succinyl-CoA to malate yet does not have the earlier stages, it also seems to be reversed, going from malate to succinyl-CoA instead. As well as this, the MRO has an incomplete ETC. Complex I (NADH dehydrogenase ubiquinone oxidoreductase) is present, as well as complex II (four complex II subunits were identified), however, it is unknown whether this complex functions as a succinate dehydrogenase or a fumarate reductase. In contrast to other stramenopiles, *Blastocytsis* lacks the genes *cox* 1-3 and *cob* which encode parts of the cytochromes that make up complexes III and IV of the electron transport chain, and genes encoding complex V, ATP synthase, are also not present showing an incomplete electron transport chain (Tsaousis *et al.* 2010). In the place of these final three complexes sits an AOX, a protein typically found in organisms

that may need to cope with oxygen stress, the activity of which is similar to complex IV, acting as the terminal electron acceptor in the ETC (Tsaousis *et al.* 2010; Pennisi *et al.* 2016).

Due to *Blastocystis* possessing MROs resembling traditional mitochondria, to confirm they are anaerobic, two main enzymes were investigated, being [FeFe] hydrogenase and PFO, as these enzymes are typically found in anaerobic organisms and would aid in the classification of its MROs (Tsaousis *et al.* 2010). Within the *Blastocystis* genome, both PFO and [FeFe] hydrogenase homologues were identified, the latter of which is responsible for the production of H_2 gas, a major electron acceptor within certain anaerobic mitochondria and hydrogenosomes, and in *Blastocystis* it is suspected to accept electrons from PFO (Stechmann *et al.* 2008). The [FeFe] hydrogenase possesses an additional C-terminal domain, which is homologous to a class of protein called flavodoxins, electron transfer protein contain a flavin mononucleotide, which is typically a unique domain arrangement found in [FeFe] hydrogenases. These flavodoxins are also electron transfer proteins and may be used as an alternative redox partner for ferredoxins, acting as a substitute in the transfer of electrons from PFO to [FeFe] hydrogenases in *Blastocystis* (Stechmann *et al.* 2008). Biochemically localised within the organelle is a PNO that facilitates the conversion of pyruvate (which is produced during glycolysis and transported into the MRO) to acetyl-CoA. The synthesis of ATP is driven by the conversion of acetyl-CoA, via substrate level phosphorylation, to acetate through the use of an acetate:succinate-CoA transferase and a succinyl-CoA synthetase cycle (Muller *et al.* 2012). It was predicted that, due to the presence of a lactate dehydrogenase within the *Blastocystis* cytosol, both acetate and lactate are major end products of ATP synthesis, although this has yet to be confirmed (Lantsman *et al.* 2008).

Whether *P.lacertae* shares these features or more closely represents an anaerobic mitochondrion currently unknown, but the aim of this investigation is to identify the mitochondrial protein composition of *P.lacertae* in order to confirm or rule out whether the two organism share similarities in this organelle.

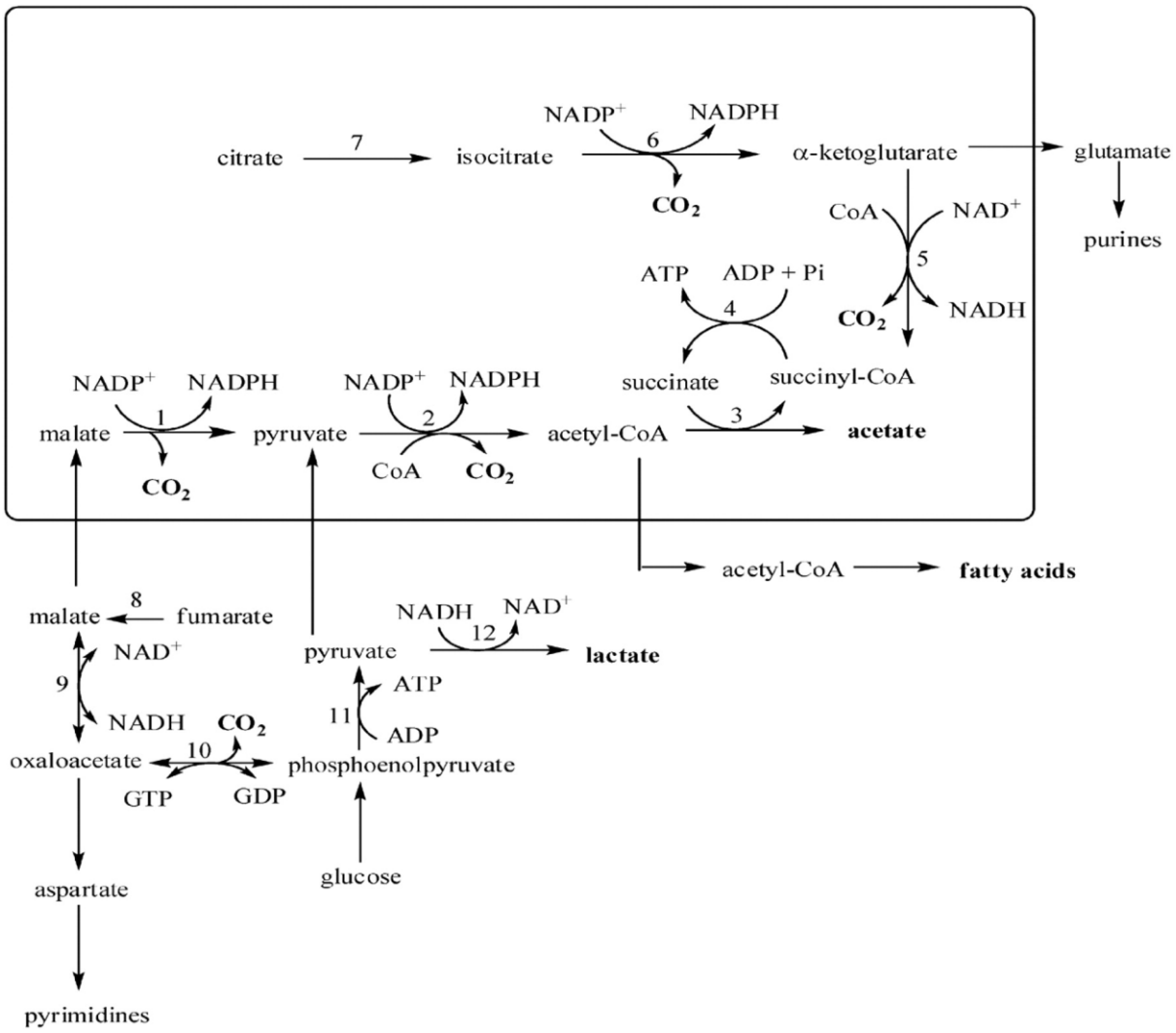

Figure 7. Metabolic processes within the *Blastocystis* MRO, simplified. (Lantsman et al. 2008) The proposed biochemical pathways within the MRO of *Blastocystis*. While simplified, it shows the major metabolic pathways within the MROs with the only exception being the proteins involved in the electron transport chain. (The enzymes involved in these pathways are numbered and the matching enzymes have been taken from the original Figure legend). (1) Malic enzyme, (2) PNO, (3) ASCT, (4) STK, (5) α-ketoglutarate dehydrogenase, (6) isocitrate dehydrogenase, (7) aconitase, (8) fumarate hydratase, (9) malate dehydrogenase, (10) PEPCK, (11) pyruvate kinase, (12) lactate dehydrogenase.

1.6 Peroxisomes

Peroxisomes are membrane bound organelles that are present in nearly all eukaryotes and play a major role in lipid and ROS metabolism (Fransen *et al.* 2011). They are dynamic and plastic organelles, in that they can rapidly alter their enzyme content and number to suit the needs of the cell and as soon as they are no longer needed, they can be rapidly degraded in a process called pexophagy (Dunn *et al.* 2005), but a balance between biogenesis, degradation and multiplication is necessary and imbalance could be indicative of disease within organisms such as humans (Fransen. 2012).

Peroxisome biogenesis is mediated by several different peroxisomal proteins called peroxins. One such protein is Pex11p, which is involved in peroxisome fission, and defects in this peroxin can result in fewer and larger peroxisomes due to the lack of division (Goodman. 2005). Other peroxins such as Pex3p are involved in the docking of the peroxisome to cytosolic Pex19p, which helps to direct and organise the peroxisomes, Pex16p also interacts with Pex19p, but aids in peroxisomal membrane biogenesis. There are over 30 different peroxin proteins, numbered in the order they were discovered, which are either located on the peroxisomal membrane like Pex3p, or localised in the cytosol such as Pex19p. Peroxins are variable in their functions and can be involved in regulating the assembly, size, biogenesis, docking, and protein import of the peroxisome (Goodman. 2005). The biogenesis of peroxisomes takes place in the ER and the three mentioned peroxins, Pex3p, Pex16p and Pex19p are suggested to play a vital role in the synthesis of peroxisomal membranes. Pex19p is suggested to act as a guide, directing peroxisomal membrane proteins to the newly synthesised membrane, and absence of any three of these proteins will result in the complete absence of peroxisomes (Goodman. 2005; Fransen. 2012).

As well as the synthesis of membranes, peroxisomes require a large number of matrix proteins that enable them to execute their metabolic functions, which are synthesised in the cytoplasm. These proteins possess targeting motifs allowing them to be directed to the peroxisomes, by either PTS1 or PTS2. PTS2 is less conserved than PTS1 and more variable in its sequence. Unlike standard import pathways in the mitochondria, peroxisomal proteins will fold and assemble into oligomers prior to translocation through the import apparatus (Goodman. 2005). Pex5p is an import receptor that interacts with PTS1 (Pex7p recognises PTS2 proteins) and, once bound, will transport the cytosolic peroxisome protein into the matrix, the process is complex, and relies on a series of protein-protein interactions, cargo recognition/docking, translocation and the release of the protein. Pex19p also acts as a chaperone for proteins bound to the peroxisome matrix (Fransen. 2012).

Peroxisomes are versatile in their functions and are involved in many different key metabolic pathways. They play a key role in fatty acid oxidation, where it is involved in the shortening of the

acyl-CoAs in order to produce acetyl-CoA, and the process is mediated by two proteins, both of which are bifunctional and both of which are acyl-CoA oxidases (Acyl-CoA oxidase 1 and 2). ACOX1 is the main enzyme involved in the oxidation of very long chain fatty acids and dicarboxylic acids whereas ACOX2 is involved in oxidation of pristanic acid, di- and trihydrocholestanoic acid. (Wanders, Waterham and Ferdinandusse. 2016). As mentioned, the end product of this fatty acid oxidation is acetyl-CoA, which is then used in multiple different pathways, especially within the mitochondria (Figure 8). Peroxisomes are linked to mitochondria by way of their metabolic pathways, namely the use of NAD^+, NADH and NADPH. Peroxisomes are involved in the reoxidation of NADH, which is a necessary compound for peroxisomes as it is crucial in the oxidation of fatty-acids. The reoxidation of NADH into NAD^+, however, must be carried out in the mitochondria, and must be shuttled there. As well as the reoxidation of NADH, peroxisomal NADP is reduced into NADPH through the use of redox shuttles that require NADP-linked isocitrate dehydrogenases present in the mitochondria, cytosol and peroxisomes, which transport it using malate, to the mitochondria. Mitochondria are also the oxidation site of many other peroxisomal acyl-CoA esters such as acetyl-CoA which needs the TCA cycle and other -CoAs which need the ETC to be appropriately oxidised (Wanders, Waterham and Ferdinandusse. 2016).

Peroxisomes are also responsible for the degradation of D-amino acids and utilise two main enzymes; D-amino acid oxidase (DAO) which has a broad specificity and reacts with amino acids such as D-serine and D-alanine. The other enzyme, D-aspartate oxidase (DDO), is highly specific, interacting with D-aspartate and D-glutamate. Both enzymes are suggested to regulate levels of amino acids and the typical products of their reactions with amino acids are hydrogen peroxide and ammonia (Wanders, Waterham and Ferdinandusse. 2016). Finally, Peroxisomes are also involved in ROS metabolism, which is essential given how they also produce ROS as a by-product is some of their reactions, such as those carried out by the acetyl-CoA oxidases. To combat ROS, peroxisomes are in possession of a range of antioxidants such as thioredoxin 2, and thioredoxin reductase which neutralise the harmful ROS before they can damage the organelle (Wanders, Waterham and Ferdinandusse. 2016).

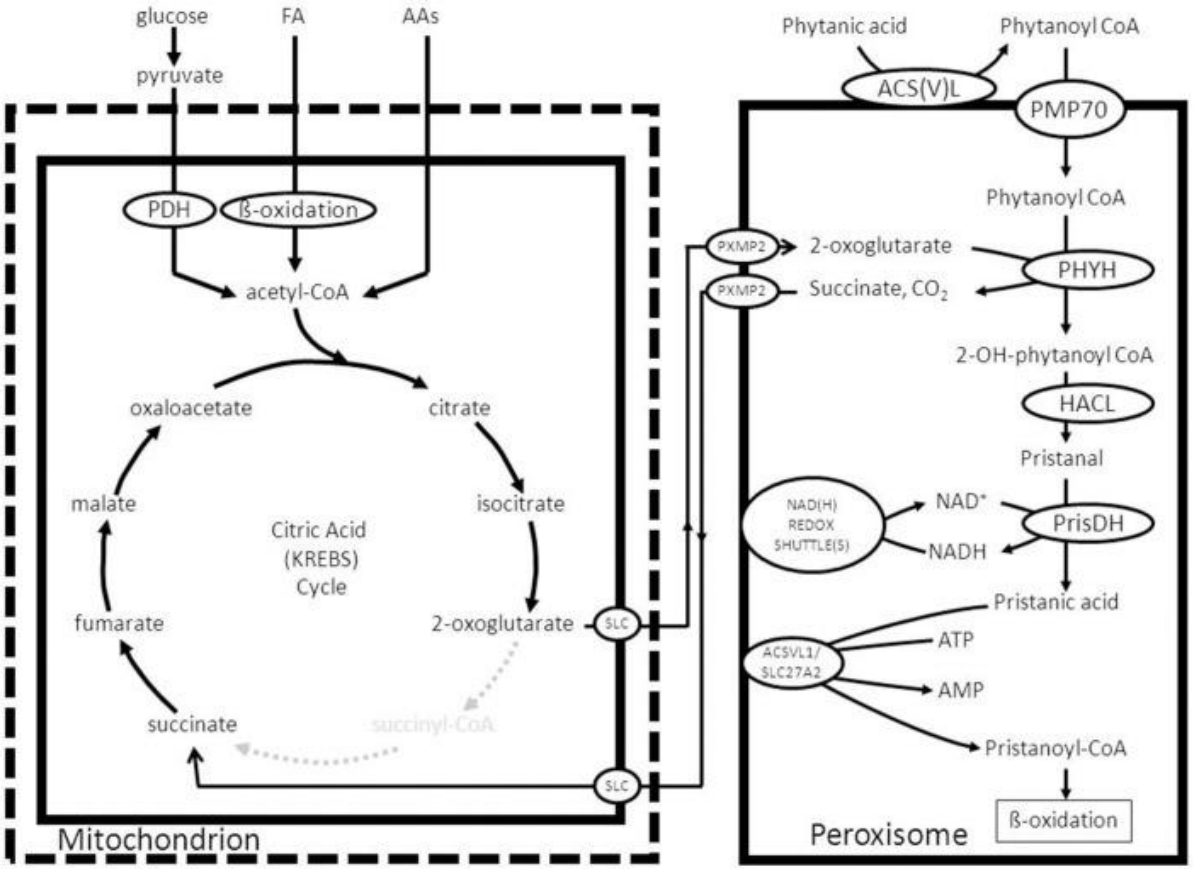

Figure 8. Metabolic connection between peroxisomes and mitochondria (Wanders, Waterham and Ferdinandusse. 2016) The biochemical connection between peroxisomes and the mitochondria in humans. Peroxisomes play a role in the KREBS cycle through the production of Acetyl-CoA, a major component.

In *Blastocystis*, there appears to only be a single subunit, Dsl1, of a multisubunit tethering system, involved in transport between the peroxisome and the ER, (Perry *et al.* 2009) and absence or reduction of this complex is thought to occur in organisms that lack peroxisomes (Gentekaki *et al.* 2017). *Blastocystis*, like other parasitic organisms, does not appear to possess peroxisomes, and they have not been characterised in detail for other organisms within the SAR (Stramenopiles, Alveolates and Rhizaria) clade. Genome data on *Blastocystis* was not able to identify any proteins associated with the presence of peroxisomes. In contrast, preliminary data from *P.lacertae* have shown that at least four proteins might be encoded, raising questions about the presence of this organelle in *Proteromonas*.

1.7 Project Aims

Proteromonas lacertae is poorly characterised in comparison to *Blastocystis* and in terms of biochemistry and morphology, nearly nothing is known about it. Throughout this project, my main aim is to characterise the biochemical pathways within the *Proteromonas* mitochondrion through bioinformatic analysis to identify the proteins present and construct the biochemical pathways within the mitochondrion. Considering the closeness of their relationship, I will be comparing this data to the predicted mitochondrial proteome of *Blastocystis* in order to identify how similar or different they are and whether certain adaptations occurred prior to these organisms diversifying or after. As well as investigating the biochemical and structural features of the *Proteromonas* mitochondrion, the presence of peroxisomes will also be investigated, due to them not having been found in *Blastocystis*.

2.0 Methods

Bioinformatic Analysis

The predicted FASTA sequences for *Proteromonas* proteome was supplied by Dr. Andrew Jackson (University of Liverpool) and processed using BLASTP, processing roughly 50 sequences at a time. Results were either confirmed as being mitochondrial or not mitochondrial by using the TargetP 1.1 server Mitoprot to predict mitochondrial targeting sequences and likelihood of protein being mitochondrial and MitoMiner 4.0 (links below).

Software	**URL link**
BLASTP (NCBI)	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins
TargetP 1.1	http://www.cbs.dtu.dk/services/TargetP/
Mitoprot	https://ihg.gsf.de/ihg/mitoprot.html
Mitominer 4.0	http://mitominer.mrc-mbu.cam.ac.uk/release-4.0/begin.do

Proteromonas culturing

Proteromonas lacertae cells were cultured in 15 ml LY media with 15 % adult bovine serum and 2 % vitamin mix. Each culture was left for two weeks for the cells to grow enough and then were transferred from the current media into new autoclaved tubes with fresh media. During the transfer, it was imperative that no contamination occur, so this was carried out under a hood that was treated with ultraviolet light for one hour. Following treatment, the inside of the hood was sprayed with 70 % ethanol, and items being placed in the hood were also sprayed. New media (previously stored at -20 ^{o}C) was melted in a water bath, and 15 ml bovine serum adult and 2 ml vitamin mix was added. Roughly 12 ml of this mixture was added to the autoclaved glass tubes. Cells were transferred from old to new media using a strippette, where the cells were removed without resuspension and gently added to the new tubes. Media was then added to nearly fill the tube, leaving a small amount of O_2 at the top. The lid was placed on (it was ensured that the inside of the lid or the neck of the tube was not touched, autoclaved tweezers were used if necessary) and tightly secured. The cells were then stored at room temperature for the next two weeks until they needed to be transferred again.

To check the state of the cells, they were often observed under a light microscope.

DH5α cell culturing

BL21 (DE5) cells were originally stored in glycerol stocks, and were streaked across a Luria broth agar plate (10 mg/ml of H_2O Tryptone (Oxoid LP0042); 5mg/ml Yeast extract (Oxoid LP0021); 10 mg/ml NaCl and 15mg/ml Difco^{TM} granulated agar-autoclaved and left to cool slightly and poured

onto agar plates near a flame until set) and left overnight at 37 °C to incubate. The following day, colonies were inoculated in 5 ml LB liquid media (10 mg/ml of H_2O Tryptone (Oxoid LP0042); 5mg/ml Yeast extract (Oxoid LP0021) and 10 mg/ml NaCl-autoclaved and left to cool), and left incubating, and shaking, overnight at 37 °C. 2 ml overnight culture was inoculated in 50 ml LB and grown, while both shaking and incubating, until they reached OD_{600}-0.6. They were then spun in a megafuge for 10 minutes at 4,500 xg at 4 °C. Pellets were resuspended in 17.5 ml ice cold 0.1 M $CaCl_2$ and incubated for 20 minutes on ice. They were then pelleted at 45 xg, for 10 minutes at 4 °C and resuspended in 3.5 ml $CaCl_2$ which was then aliquoted into 1.5 ml eppendorfs (80-100 µl) and stored at -80 °C.

LYI-S media preparation

LY media is used to grow *Proteromonas* axenically, and contains the nutrients necessary for its growth and survival. The following ingredients were all added to a 1 L glass Duran bottle: 1 g potassium phosphate dibasic, 0.6g potassium phosphate monobasic, 1 g cysteine, 0.2 g ascorbic acid, 2 g NaCl, 10 g glucose, 5 g liver digest (Oxoid:LP0027) and 25 g yeast extract (Sigma YI625). 2 ml ferric ammonium citrate (22.8g/ml (45.6 mg in 2 ml distilled water)) was added. 700 ml water was added to the bottle along with a large magnetic flea and mixed using a VWR magnetic stirrer until ingredients are fully dissolved. Once mixed, and while still stirring, the pH of the solution should be adjusted to pH 6.8 using 1 M NaOH. Once adjusted, make up to 880 ml with water and pour into 10x 100 ml glass Duran bottles (each with 88 ml) and autoclave. Once autoclaved, seal the bottle tight and store at -20 °C.

The second component of the media is Bovine Serum, Adult (BSA-Sigma: B9433). If frozen, it should be defrosted at 37 °C. BSA needs to be heat inactivated at 56 °C for 30 minutes, and then aliquoted into either 15 ml into 15 ml falcon tubes or 45 ml into 50 ml falcon tubes.

Vitamix is the final component to the complete LYI media. Vitamin mix is made from 4 main solutions, each made separately and combined later on. Solution 1 is made using 45 mg nicotinamide, 4mg pyridoxal HCL, 23 mg calcium pantothenate, 5 mg thiamine HCL and 1.2 mg vitamin B12, which should be dissolved in water to 25 ml. Solution 2 is made by dissolving 7 mg riboflavin and 5.5 mg folic acid in roughly 500 µl 0.1 M NaOH (if a precipitate forms then too much was used) and made up to 45 ml with water. Solution 3 is made by dissolving 2 mg D-biotin in 45 ml water. Solutions 1-3 were mixed together. Solution 4 was made by dissolving 1 mg DL-6,8-Thioctic acid in 5 ml 95 % ethanol, and adding roughly 500 mg Tween-80, then making up to 30 ml with water. All solutions were combined and sterile filtered, being aliquoted 12 ml into 15 ml falcon tubes.

Fixation of *Proteromonas*

Proteromonas lacertae had yet to be fixed, therefore protocols used to fix other eukaryotes must be tested in order to identify a viable method of fixation for Proteromonas. The below protocol was adapted from the immunolocalisation protocol used by Tsaousis et al. 2010

The Proteromonas culture was spun at 800 xg for 10 mins in a megafuge and re-suspended it in 14 ml of fresh LY media without bovine serum adult. The cells were then incubated in 1:5000 dilution of Mitotracker for 20 mins at 37 °C. The cells were spun again at 800 xg for 10 mins and then re-suspended in 5 ml of 1x PBS (1x PBS tablet (OXOID BR0014G per 100ml water)) (pH 7.4.). The cells were spun a third time at 800 xg for 10 mins and re-suspended cells in 3 ml of 3.7 % formaldehyde/0.5 % acetic acid and then fixed for 15 min at 37 °C inside the tube. The re-suspended cells were transferred to pre-treated poly-L-lysine slides, which were then incubated slides at 37 °C in the anaerobic chamber for 1h. Slides were then washed for 5 min in 1x PBS and then washed for 5 min in PBS/0.5 % Tween-20 three times. The slides were then incubated with 0.1 % Triton X-100 for 5 min three times. Triton X was removed, and the slides were incubated overnight at 4 °C with primary antibody. The following day, they were washed three times with PBS-Tween 80 and incubated for 1 hour with secondary antibody at room temperature (1/1,000 dilution). They were washed twice with PBS-Tween 80 and then twice with 1X PBS. Cover slips were mounted with antifade mounting medium containing DAPI and observed under a laser scanning confocal microscope (Zeiss LSM 510 Meta) using a 100× oil immersion lens.

Extraction of *Proteromonas* RNA

Total RNA was extracted from *Proteromonas* using RNeasy mini kit (QIAGEN) according to the manufacturers Yeast protocol

Complementary DNA synthesis

The cDNA synthesis was performed using the protocol found on Thermo Fisher Scientific, titled: SuperScript™ II Reverse Transcriptase, according to the manufacturer's instructions.

PCR of *Proteromonas* DNA using specific primers

Multiple PCRs were carried out in order to isolate the DNA sequence for the desired protein, which would differ slightly based on how many base pairs there are in the DNA sequence. The initial protocol was provided, along with the Taq Ultra Mix Red, by PCRBIO. Each PCR mix was made using 25 µl PCRBIO Ultra Mix Red (diluted from 2X to 1X overall), 2 µl (0.4M) forward primer, 2 µl (0.4M) reverse primer, 20 µl autoclaved water and 1 µl DNA, to make up a total volume of 50 µl per reaction. A mastermix was usually made, which involved the addition of the taq, forward and reverse primers and the water, multiplied by how many samples were needed + 1 for spare. 49 µl

was added to each PCR tube with 1 µl DNA being added after, with a positive control of DNA and a negative control of 1 µl water.

- AOX: The primers used for PCR of AOX were EcoR1 Forward 5'-GCGCGAATTCATGAATAGATTACTAGCCGTC -3' and Xho1 Reverse 5'-ATAAAATTATATAAATCTATTTGTCTCGAGGCCG -3'. As with all of the PCR protocols, the lid had an initial step where it needed to heat up to 105 °C. The protocol involves an initial denaturation step at 95 °C for 3 minutes, then a denaturation step of 95 °C for 15 seconds, Anneal step of 58 °C for 15 seconds and an Extension step of 72 °C for 50 seconds. The denaturation, anneal and extension steps were repeated 40 times. Following the main cycle, there was a final step at 72 °C for 10 minutes before it holds at 8 °C.
- Pex19: The primers used for PCR of Pex19 were Nde1 Forward 5'-GCGCCATATGATGACATCTGTTATTGAAACAG -3' and BamH1 Reverse 5'-GCTATGCAAGGGTGTATAATAATGGGATCCGGCC -3'. For Pex19 there was an initial denaturation step at 95 °C for 3 minutes, then a denaturation step of 95 °C for 15 seconds, Anneal step of 56 °C for 15 seconds and an Extension step of 72 °C for 40 seconds. The denaturation, anneal and extension steps were repeated 40 times. Following the main cycle, there was a final step at 72 °C for 10 minutes before it holds at 8 °C.

Gel electrophoresis of PCR product

Gel electrophoresis was carried out using an agarose gel of 1-1.5 %, made using agarose powder (MELFORD) and 1X TAE (Tris base, acetic acid and Ethylenediaminetetraacetic acid) buffer (50X=0.5M EDTA, 242g TRIS-base and water to 1L, diluted 1:20 for 1X). The gel was made using 10 mg Agarose per 1 ml TAE buffer. The gel was heated in a microwave until the agarose had fully dissolved, and left to cook. When cool enough to hold, 2-6 µl (depending on the gel volume) of ethidium bromide (10mg/ml SIGMA E1510) was added, and the gel was poured into a mould to set. Once set, it was placed into the gel tank and covered in 1X TAE buffer. The PCR reaction (roughly half) was pipetted into the wells, with 1KB ladder running alongside it. The gel was run at 80V until the dye front reached then end. Upon completion, it was then visualised over UV and any bands were cut out, ready for gel extraction. Gel extraction was performed using QIAprep gel extraction kit (QIAGEN) according to manufacturer protocol.

Ligation of replicated target DNA sequence

The ligation was performed using 1.5 µl gel extraction. 1.5 µl gel extraction, 0.5 µl PGEMT vector, 0.5 µl T4 DNA ligase and 2.5 µl x2 buffer (Promega) were added to a PCR tube and left at either room temperature for 1.5 hours or at 4 °C overnight. Following ligation, the sample can be stored at -20 °C.

Cloning of Ligated samples using DH5α *E.coli* cell transformation

Competent DH5α *E.coli* cells were defrosted, on ice, for 5-10 minutes and 40-50 µl was pipetted, near a flame, into autoclaved 1.5 ml Eppendorf tubes and kept on ice. 2.5 µl of the required ligation was pipetted into the cells, the tube was flicked twice and placed on ice for 10 minutes. A heat block was heated to 42 °C and the cells were heat shocked at this temperature for 45-60 seconds, and were immediately put on ice for 2 minutes following heat shock. 250 µl of LB media was pipetted into the eppendorf, near a flame, and was left incubating at 37 °C while shaking, for 1 hour. After 1 hour, and near a flame, 60 µl of the culture was pipetted onto an agar plate with 0.1 % antibiotic, and the rest was spread on another plate. They were spread using as glass spreader that had been washed with 70 % ethanol and heated over a flame. Prior to the plating of the cells, the spreader was cooled on the plate. After the cells had been spread, they were incubated at 37 °C overnight.

The following day, the cultures were removed from incubation and stored at 4 °C until needed. 5 ml LB was added to a 15 ml falcon tube, along with 0.1 % (5 µl) the desired antibiotic (100mg/ml ampicillin was used here). A wire loop was heated over a flame, and cooled. Once cool, a single white colony was taken from the plate using the loop and transferred into the LB. An alternative method is to briefly heat a pipette tip, scrape a colony and eject the tip into the media. They were left overnight, shaking, at 37 °C. The following day, they were miniprepped using a Thermofisher miniprep kit.

The 15 ml falcon tube was centrifuged at 5,000 xg for 10 minutes, and the supernatant was removed. The pellet was resuspended in 250 µl resuspension solution and transferred to a 1.5 ml Eppendorf. 250 µl lysis solution was added, and the tube was inverted 4-6 times, and shortly after (no longer than a few minutes) 350 µl neutralisation solution was added, and the tube was inverted again. The tube was centrifuged in a microfuge at 12,500 xg for 5 minutes. The supernatant was then transferred into a GeneJET spin column, taking care to not disturb the pellet, and the tube was microfuged at 12,500 xg for 1 minute. Three wash steps were performed following this, which involved adding 500 µl wash solution to the tube and spinning for 60 seconds, discarding the flow through after each wash step. The tube was spun once more, while empty, to remove any residual wash solution. The column was transferred into a fresh Eppendorf, and 50 µl elution buffer was pipetted onto the membrane. The tube was left to stand for a few minutes and then centrifuged for 1 minute. The plasmid can then be stored at -20 °C.

Digest of DNA fragment for cloning

Unlike a standard digest, this one takes place over a few days. Day 1 involves an initial digest with the enzyme matching the reverse primer. In an autoclaved PCR tube, 4 µl 10X buffer, 2 µl restriction

enzyme (i.e. Xho1 for AOX), 20 µl plasmid and 14 µl autoclaved water were mixed and divided into 2 tubes, both containing 20 µl. The same must be carried out for the required pET vector, such as for AOX, the 20 µl plasmid must be replaced with 20 µl pET29b plasmid. These were incubated at 37 °C overnight. On the second day, the digests were recombined, mixed with 12X green loading dye and pipetted into a 1-1.5 % agarose gel, and run slowly at 60-70V for roughly 3 hours. The bands were cut out and extracted using QIAprep gel extraction kit (QIAGEN) and used in the second digest. The ingredients were the same, only the gel extract was used in place of plasmid and the second restriction enzyme (such as EcoR1 for AOX) was used, and incubated at 37 °C for 3 hours. They were recombined, mixed with loading dye and run in another gel for 3 hours. The following day, the bands for the plasmid and pET vector were extracted, where they were then used in a ligation.

Ligation using pET vectors

The second ligation was performed using the pre-selected vector, either pET16b or pET29b, depending on whether the primer sequences matched the vector. Two types of ligation were carried out, fast and overnight. The fast ligation involved adding 2 µl of the DNA insert to 2 µl of the matching vector (for SDHA and Pex19 the vector was pET16b and for AOX the vector needed was pET29b) 1 µl of T4 DNA ligase was added and along with 5 µl of 2x buffer. For a control, the insert was replaced with H_2O. They were left at room temperature for 2 hours and then used in a transformation.

The overnight sample was similar but required there to be 3x as much insert to vector. 2 µl of pET was added to 6 µl of insert (or H_2O for the control), 1 µl of 10x buffer and 1 µl of T4 ligase were both added. This sample was left at 4 °C overnight, and transformed the following day.

Following ligation, a transformation was carried out and then a miniprep. This miniprep would then be used in an expression transformation.

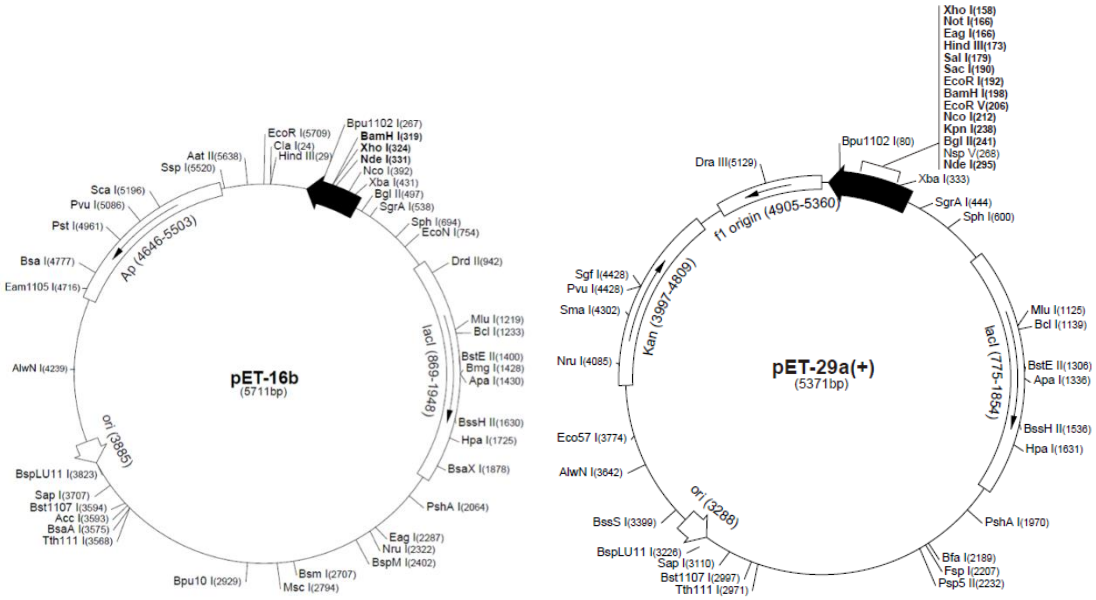

Figure 9. NOVAGEN pET vectors with their respective enzymes and cutting points. pET vectors were used in order to express certain proteins in expression cell lines and had to be cut using the same enzymes used to cut the protein sequences.

Expression transformation

The protocol for an expression transformation is similar to a standard transformation, only the plasmid obtained following cloning is added to expression cells, in this case BL21 (DE3) P-lysosyme cells were used. 1 µl plasmid was added to 40-50 µl cells. Following this step, the protocol is the same as standard transformation. The plates required two antibiotics when using expression cells, the initial antibiotic (such as ampicillin (100 mg/ml) for Pex19 or kanamycin (50 mg/ml) for AOX) and chloramphenicol (50 mg/ml), 1% of each is needed.

Expression of target protein in BL21 (DE3) P-lysosyme cells

50 ml LB media with the appropriate antibiotic (1/100) was added to an autoclaved 250 ml flask sealed with a foam stopper and covered in tin foil. To the media, 2 ml of the overnight culture was added, 1 ml was removed and the sample was read at OD_{600} to get the initial reading. The culture was incubated, shaking, at 37 ^{o}C and every hour, 1 ml was removed and the OD recorded. Once the absorbance was between 0.5 and 0.7 (Prior to induction, 1 ml culture was removed, pelleted at 12,500 xg for 3 minutes and stored at -20) 1 M IPTG was added (1/1000) to induce the culture. It was incubated for a further 4 hours, and the absorbance checked every hour. After 4 hours, the final absorbance was recorded, another 1 ml was pelleted and stored and the remaining culture was pelleted at 1,000 xg for 10 minutes and stored at -20 ^{o}C as well.

Before running the samples through a gel, dye was added and the sample was boiled. 100µl NuPAGE buffer was added to the pre-induction sample and 200µl to the post induction. The samples were passed through a fine syringe and were then boiled at 96 °C for 10 minutes, but resuspended on a vortex after 5 minutes. Once boiled, the sample was pelleted at 12,000 xg for 3 minutes and 10 µl was loaded in a 12 % SDS gel.

Target protein Extraction

Two 15 ml tubes of *Proteromonas* culture were pelleted in 15 ml falcon tubes at 1000 xg for 10 minutes. The supernatants were removed and one sample was resuspended in 1 ml 1x PBS ad transferred to the other tube, both were resuspended in another 8 ml of 1x PBS, and 1 ml DNAase/RNAase mix (50 mM $MgCl_2$, 0.5 M Tris-HCl pH7.0) and 10 µl of EDTA free protease inhibitor was added, the whole solution was kept on ice throughout. This was pelleted again at 1000 xg for another 10 minutes. Supernatant was removed, and the pellet was resuspended in 500 µl PBS/10 % DNAase/RNAase mix and 10 µl protease inhibitor was added. The sample was transferred to a 1.5 ml Eppendorf and was centrifuged in a microfuge at 4500 xg for 2 minutes. The supernatant was removed and the pellet was resuspended in 400 µl 1X NuPAGE LDS sample buffer and passed through a fine syringe. The sample was boiled on a 96 °C for 10 minutes, but was resuspended on a vortex after 5 minutes. Following boiling, the sample was pelleted at 12,000 xg for 3 minutes. It was stored at -20 °C. 10 µl of the protein extraction supernatant was added to 90 µl 1X NuPAGE buffer, 10 µl of which was run in chosen wells. The sample was run in a 0.75 mm, 12 % acrylamide gel at 25 mA for 1-2 hours, or until the dye front had run off of the gel.

Target protein identification using western blotting

Following protein extraction and subsequent sodium dodecyl sulphate (SDS) gel electrophoresis, the proteins from the gel needed to be transferred to a *BIO-RAD* Immun-Blot PVDF (polyvinylidene difluoride) membrane. The transfer process requires the gel to be "sandwiched" with the membrane. A gel holder cassette, which has both a clear side and a black side was used. Starting in order from the black side, a fibre pad, then filter paper was placed. Both of which were soaked in transfer buffer for 5 minutes. On the filter paper, the gel was placed and, after soaking in methanol for 5 minutes then transfer buffer for a further 5 minutes, the membrane was gently placed on the gel. Another piece of transfer buffer soaked filter paper was placed on the membrane, and the final fibre pad on that. The holder was closed and clamped, and placed into the electrode module, with the black side of the holder facing the black side of the module. The module was placed inside of a *BIO-RAD* buffer tank, which was nearly filled with transfer buffer, with a frozen (kept in the -20) cooling unit was placed next to the electrode module in the buffer, to prevent overheating. The blot was run at 200mA for 1 hour. Following transfer, the membrane was removed from the holder and placed in a clear container, and stained with ponceau G stain for 1 minute, to visualise the

bands on the membrane, then washed with water. The membrane was cut, depending on how many lanes and ladders were used. The first step was to block. The membrane was blocked with 5 % Tris buffered saline (with Tween)-milk for 1 hour on a Denley orbital mixer. The membrane was washed with water, then washed in 0.5 % TBST-milk for 30 minutes. Antibody was then diluted (i.e. 1/400 depending on the strength of the antibody) in 1 % TBST-milk and the membrane was washed at 4 ^{o}C overnight. The membranes were washed, on the following day, three times in 1 % TBST-milk. The secondary antibody depends on the primary antibody, such as if the primary antibody was from a rabbit, then the secondary antibody would need to be specific to that animal. The membranes were washed in secondary antibody in 1 % TBST-milk for 1 hour. The blot was then washed 5 times in TBST.

The blots were then covered in equal volumes of Amersham^{TM} ECL^{TM} Prime Western Blotting Detection Reagent (GE Healthcare-RPN2232), 2 ml of both solutions A and B shortly (2-5 minutes) before visualisation, and then visualised.

Fixation for TEM-Transmission Electron Microscopy

Three tubes of growing *Proteromonas lacertae* culture (4-10 days post passage) were pelleted at 800 xg for 10 minutes at 20 ^{o}C. The supernatant was removed and each pellet was resuspended in 2 ml 2.5 % glutaraldehyde in 100 mM sodium cacodylate buffer pH 7.2 and left to fix for 2 hours at room temperature. Following fixation, the sample was pelleted in a microfuge at 1900 xg for 2 minutes and washed in cacodylate buffer for 10 minutes to remove the fixative, this step was repeated once more. Once pelleted, the buffer was removed and the pellet was resuspended in 500 µl buffer, after this, 50 µl was transferred into another tube and warmed in a 55 C water bath for a few minutes. 50 µl of 3 % low melting point agarose was added to the cells, and using a glass pipette, the mixture was quickly transferred into previously made gaskets (plastic cut and sandwiched between two glass slides to allow the gel to form a thin layer which were clamped together) and stored at 4 ^{o}C for 5-10 minutes until the gel had set. Once removed from the fridge, the gel was cut into very thin pieces and transferred into a drop of Alcain blue-0.1 % acetic acid dye, after which it was gently removed from the dye using a bent toothpick and placed in 3 ml of buffer to remove excess dye. Using a glass pipette, the buffer was removed, and care was taken not to remove gel fragments. 1-1.5 ml Osmium tetroxide (made up from 1 ml 4 % osmium tetroxide, 1 ml milli-Q water and 2 ml 200 mM cacodylate buffer) was added and the sample was left at room temperature for 1 hour. Following this step, the osmium tetroxide was removed and the sample was washed for 10 minutes once in 50 % ethanol, and was left overnight in 70 % ethanol at 4 ^{o}C.

The following day, the sample was washed once in 90 % ethanol and then three times in 100 % ethanol. Following this wash step, the ethanol was removed and the fragments were washed twice in 3 ml propylene oxide. This was removed and 50 % propylene oxide/50 % LV resin was added and

left for 30 minutes at room temperature. (LV resin= 12 g LV resin, 4 g VH1 hardener, 9 g VH2 hardener and 0.63 g LV accelerator). 50/50 mx was removed and 100 % LV resin mixture was added and left for 90 minutes, following this 10-12 fragments were transferred into fresh LV and left for another 90 minutes. Using a Pasteur pipette, 6 ml LV resin was put in a small mould and fragments were placed a small distance from the edge of the mould and gently pushed to the bottom using a bent toothpick. They were then placed in a 60 ^{o}C oven for 20-24 hours in preparation for sectioning. To section, the mould was cut where the cells were most concentrated (following light microscopy) and were superglued onto blank resin capsules where it was filed down and the edges of the capsule were cut away using a glass knife to leave the mould raised. The knife has a boat at the back which was filled with milli-Q water. The automated knife was used to cut very thin (few microns thick) slices from the block, which would stack in the water. To expand them, they were exposed to chloroform vapours. Once enough had been collected, roughly seven slices were attached to a slot grid coated in plastic.

To stain, a rectangle of dental wax with labelled columns was covered in milli-Q water and then sealed in parafilm. In each of the columns, a drop of 4.5 % uranyl acetate was placed at the top of each, then below that a drop of milli-Q water, then another below that. The slot grid was placed on the uranyl acetate to stain for 45 minutes, then washed gently under milli-Q then placed on the drop of milli-Q, and repeated. It was dried using filter paper. On another grid of wax, which was also wrapped in parafilm, two drops of milli-Q were placed below a drop lead acetate (in this container, the space was filled with potassium hydroxide). The slot grid was placed in the lead for 7 minutes and transferred to the first, then second milli-Q drop, dried on filter paper and left for a short while underneath a light (while being kept in the air by forceps). After this, they were stored or visualised under the transmission-electron microscope.

Seahorse Agilent XFp-AOX/SDHA

These tests were carried out using the Seahorse XFp Mitofuel Flex Test Kit protocol (User manual Kit 103270-100). The antibiotics used were varying concentrations (1.0-5 mM) Salicylhydroxamic acid (SHAM) and 5.4 mM 2-thenoyltrifluoroacetone (TTFA) to test the activity of alternative oxidase and succinate dehydrogenase subunit A respectively.

Antibodies used:

Anti-AOX antibody: The AOX antibody used was the same as the one found in: Monoclonal Antibodies to the Alternative Oxidase of Higher Plant Mitochondria. Elthon, T., Nickels, R. and McIntosh, L. (1989)

Anti-Pex19 antibody: Abcam Rabbit Anti-PEX19 antibody [EPR9266(B)] (ab137072)

Anti-SufCB antibody: The SufCB antibody used was the same as the one found in: “Evolution of Fe/S cluster biogenesis in the anaerobic parasite Blastocystis.” Tsaousis, A. *et al.* (2012).

3.0 Results

Due to the lack or complete absence of information regarding the biochemical contents of the *Proteromonas* mitochondrion, the main purpose of this investigation was to generate a comprehensible series of data using roughly 40,000 predicted protein sequences that can illustrate the proteins and potential biochemical pathways within the mitochondrion. We would then attempt to characterise specific proteins through investigation of their activity, characterisation through polymerase chain reaction of cDNA and western blotting of extracted proteins, as well as localisation following fixation and the use of specific antibodies. The mitochondrial structure of *Proteromonas* was also found to be unusual and would be investigated, through the use of TEM and serial sectioning. After comparing this data with the predicted proteome of *Blastocystis*, it was found that these two organisms are very similar biochemically and have a similar number of possible mitochondrial proteins, with *Proteromonas* having 303 and *Blastocystis* having 283 with a total of 229 proteins being shared between them: 75 % of *Proteromonas'* proteins are shared with *Blastocystis* and 81 % of the *Blastocystis'* mitochondrial proteins are shared with *Proteromonas.* Investigation of the mitochondrial structure of *Proteromonas* revealed some curious features, and an unexpectedly close association with the nucleus.

3.1 Bioinformatic Analysis of *Proteromonas* and *Blastocystis* mitochondrial proteome

In order to be able to generate the best picture of the protein contents of *Proteromonas*, the predicted proteome/metabolome was analysed bioinformatically. Predicted sequences were analysed using the online protein database BLASTP, and the resultant protein was checked against Mitoprot, Mitominer 4.0 and TargetP 1.1 to confirm whether they could be localised in the mitochondrion. Mitochondrial proteins were organised into their respective biochemical pathways and functions, such as whether they were part of the electron transport chain complexes or were involved in pyruvate metabolism. The proteins were also compared to *Blastocystis* homologues, and a comparative analysis between the two organisms (whether the protein was present in *Proteromonas* only, *Blastocystis* only or both) was carried out.

The tables below (1-18) were all organised based on the organisation of data used by Gentekaki *et al.* which was initially organised as found on the KEGG database.

The graph in Figure 10 compares the total proteins in each biochemical group within the mitochondria of both *Proteromonas* and *Blastocystis*. The number of proteins is mostly the same, aside from the import machinery and proteins involved in ribosome transcription and translation.

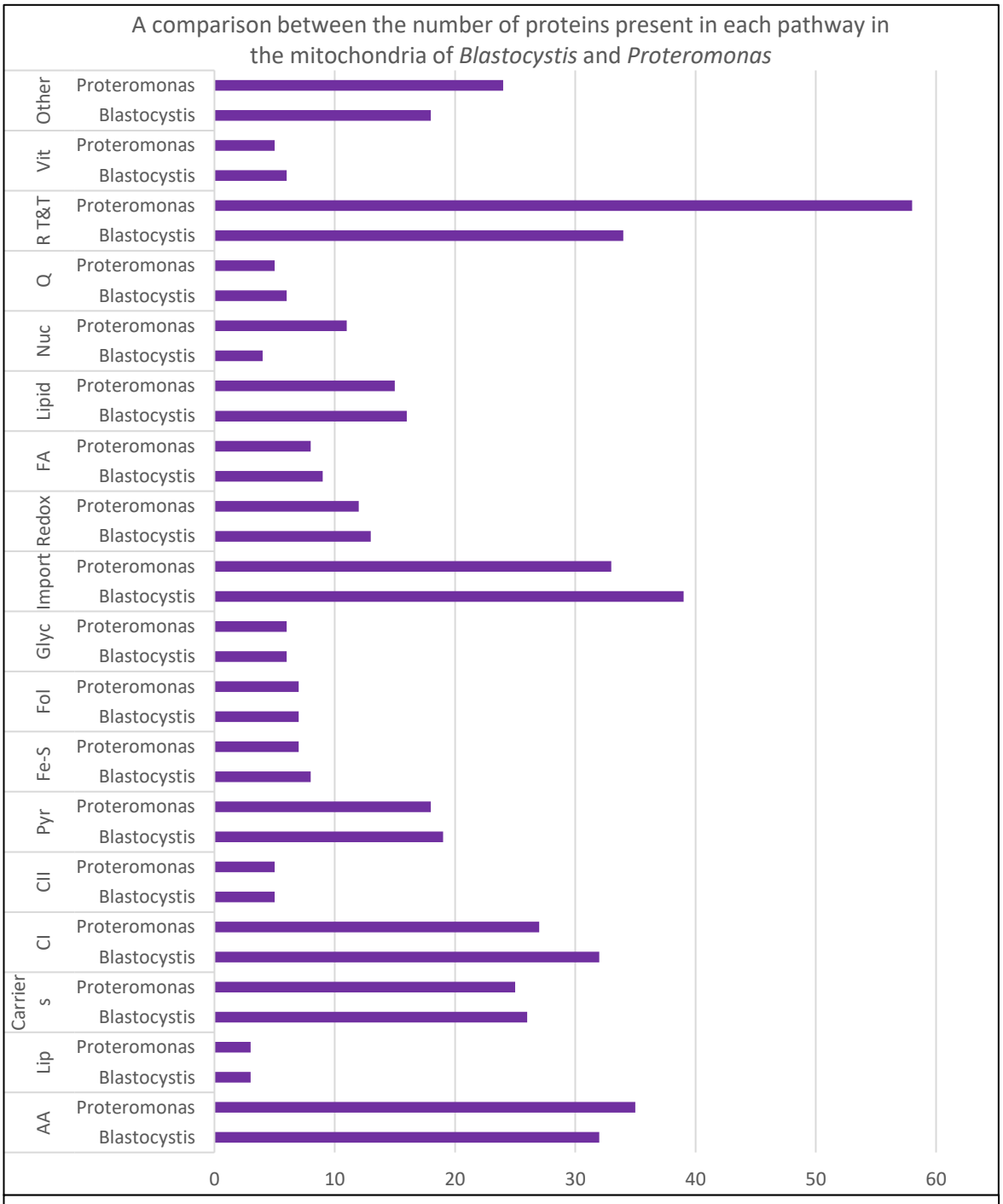

Figure 10. A graph comparing the amount of proteins in each biochemical group within the mitochondria of *Proteromonas and Blastocystis.* A graph to compare the total amount of proteins within each biochemical group within the mitochondria of these two organisms. Abbreviations: AA, Amino acids; Lip, lipoate; CI, complex I; CII, complex II; Pyr, Pyruvate metabolism; Fe-S, iron-sulphur cluster biosynthesis; Fol, Folate; Glyc, glycolysis; FA, fatty acid biosynthesis; Nuc, Nucleotide; Q, Quinone metabolism; R T&T, ribosome transcription and translation and Vit, Vitamins.

As shown in Table 1, a total of 28 proteins associated with amino acid synthesis and metabolism are shared between the two organisms.

Table 1. Predicted proteins involved in amino acid metabolism. A comparison between the proteins involved in amino acid metabolism and biosynthesis in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Amino Acid	L-asparaginase	Present	Absent
	Aspartate aminotransferase	Present	Present
	Alanine aminotransferase	Present	Present
	Aspartate ammonia lyase	Present	Present
	Branched amino acid amino transferase	Present	Present
	Glutamine synthetase	Present	Present
	Threonine dehydrogenase	Present	Present
	2-amino-3-ketobutyrate CoA ligase	Present	Present
	Glycine cleavage system, P protein	Present	Present
	Glycine cleavage system, T protein	Present	Present
	Glycine cleavage system, L protein	Present	Present
	Glycine cleavage system, H protein	Present	Present
	Glycine hydroxymethyltransferase	Present	Present
	dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase	Present	Present
	PDH E1 alpha subunit	Present	Present
	PDH E1 beta subunit	Present	Present
	PDH E3	Present	Absent
	PDH E2	Present	Present
	3-methylcrotonyl-CoA carboxylase alpha subunit	Present	Present
	Isovaleryl-CoA dehydrogenase	Present	Present
	Enoyl-CoA hydratase/isomerase	Present	Present
	3-hydroxyacyl-CoA dehydrogenase	Present	Absent
	malonyl-CoA/methylmalonyl-CoA synthetase	Present	Absent
	3-hydroxyisobutyrate dehydrogenase	Present	Present
	Enoyl-CoA hydratase	Present	Present
	Glycerate Kinase	Present	Present
	Thiosulfate/3-mercaptopyruvate sulfurtransferase 1, mitochondrial	Present	Present
	glyoxylate/hydroxypyruvate reductase	Present	Present
	methylcrotonoyl-CoA carboxylase	Present	Present
	Isovaleryl-CoA dehydrogenase	Present	Present
	3-hydroxyisobutyrate dehydrogenase	Present	Present
	carbamoyl-phosphate synthase	Present	Present
	Guanine amidotransferase	Absent	Present

	Delta-1-pyrroline-5-carboxylate dehydrogenase	Absent	Present
	UDP-N-acetylglucosamine-2-epimerase	Absent	Present
	Homoserine dehydrogenase	Absent	Present
	Class I HMG-CoA	Absent	Present
	nitrilase/cyanide hydratase	Absent	Present
	aspartyl/glutamyl-tRNA amidotransferase, A subunit	Absent	Present

Table 2 shows that all of the proteins that interact with lipoate are identical in both organisms

Table 2. Predicted proteins involved in lipoate metabolism. A comparison between the proteins associated with lipoate in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Lipoate	Lipoyl synthase, mitochondrial (LipA)	Present	Present
	Lipoyltransferase (LipB)	Present	Present
	lipoate-protein ligase	Present	Present

Table 3 below shows all of the predicted carriers. All but one of the carrier proteins in the *Blastocystis* MROs are present in *Proteromonas* with each organism having a total of 26 and 25 respectively.

Table 3. Predicted carrier proteins. A comparison between the predicted carrier proteins in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Carriers	Mrs2/Mrs3; SLC25A37, mitoferrin1	Present	Present
	mitochondrial glutamate carrier 1-like (mitochondrial glutamate transporter),	Present	Present
	SLC25A3, PHC, PIC; solute carrier family (mitochondrial phosphate transporter)	Present	Present
	SLC25A38; solute carrier family 25	Present	Present
	S-adenosylmethionine transporter	Present	Present
	SLC25A27, UCP4; (mitochondrial uncoupling protein)	Present	Present
	mitochondrial coenzyme A transporter SLC25A42-like	Present	Present
	SLC25A24; (mitochondrial carrier; phosphate carrier)	Present	Present
	SLC25A20_29, solute carrier family 25 (mitochondrial carnitine/ acylcarnitine transporter)	Present	Present
	mitochondrial carnitine/ acylcarnitine carrier protein	Present	Present

	SLC25A26; (mitochondrial S-adenosylmethionine transporter)	Present	Present
	Calcium-binding mitochondrial carrier protein (mitochondrial aspartate/ glutamate transporter)	Present	Present
	SLC25A32	Present	Present
	SLC25A12, mitochondrial aspartate/glutamate	Present	Present
	SLC25A19; Thiamine pyrophosphate carrier	Present	Present
	2-oxoglutarate/malate carrier protein	Present	Present
	SLC25A43; solute carrier family 25	Present	Present
	Mitochondrial carrier protein	Present	Present
	solute carrier family 25 member 51-like	Present	Present
	Mitochondrial Tricarboxylate Carrier	Present	Present
	mcfH; mitochondrial substrate carrier family protein	Present	Present
	ATP-binding cassette sub-family B member 10, mitochondrial	Present	Present
	Sodium/potassium/calcium exchanger 6	Present	Present
	Mdm38/Letm1	Present	Present
	MMT1-Mitochondrial iron transport protein	Present	Present
	sideroflexin-5-like	Present	Absent

Table 4 compares the proteins that associate and comprise complex I, NADH dehydrogenase, of the electron transport chain. While they are mostly the same, it is still not yet known if the subunits NuoC, NuoD, NuoG and NuoK are present in *Proteromonas* and as a result, the predicted structure of the complex appears to have a few less proteins.

Table 4. Predicted Complex I proteins. A comparison between the predicted proteins that form the ETC complex I in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
ETC Complex I	subunit NDUFA2	Present	Unknown
	subunit NDUFA5	Present	Present
	subunit NuoE (NDUFV2)	Present	Present
	subunit NDUFA9	Present	Present
	subunit NDUFA9	Present	Present
	subunit NDUFA9	Present	Present
	subunit NuoI (NDUFS8)	Present	Present
	subunit NDUFB9	Present	Present
	subunit NuoF (NDUFV1)	Present	Present
	subunit NuoF (NDUFV1)	Present	Present
	subunit NuoB (NDUFS7)	Present	Present

	subunit NDUFA6	Present	Absent
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 12	Present	Present
	subunit NuoA (ND3)	Present	Present
	subunit NuoC (NDUFS3)	Present	Unknown
	subunit NuoD (NDUFS2)	Present	Unknown
	subunit NuoG (NDUFS1)	Present	Unknown
	subunit NuoH (ND1)	Present	Present
	subunit NuoJ (ND6)	Present	Present
	subunit NuoK (ND4L)	Present	Unknown
	subunit NuoL (ND5)	Present	Present
	subunit NuoM (ND4)	Present	Present
	subunit NuoN (ND2)	Present	Present
	NADH dehydrogenase iron-sulfur protein 6, mitochondria	Present	Absent
	NDUFAF6; NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 6	Present	Present
	NADH dehydrogenase (ubiquinone) complex I, assembly factor 5	Present	Present
	NADH dehydrogenase (ubiquinone) complex I, assembly factor 5	Present	Present
	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex assembly factor 7	Present	Present
	NADH dehydrogenase [ubiquinone] Fe-S protein 4	Present	Present
	NADH dehydrogenase Fe-S protein 8	Present	Present
	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2	Present	Absent
	Ind1	Present	Present

Table 5 shows that all of the proteins that make up complex II are shared by both *Blastocystis* and *Proteromonas,* meaning the complex structure in both organisms is identical.

Table 5. Predicted Complex II proteins. A comparison between the predicted proteins that form the ETC complex II in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
ETC Complex II	SDHA	Present	Present
	SDHB	Present	Present
	SDHC	Present	Present
	SDHD	Present	Present
	SDH5	Present	Present
	acetate non-utilizing protein 9, mitochondrial precursor	Present	Present

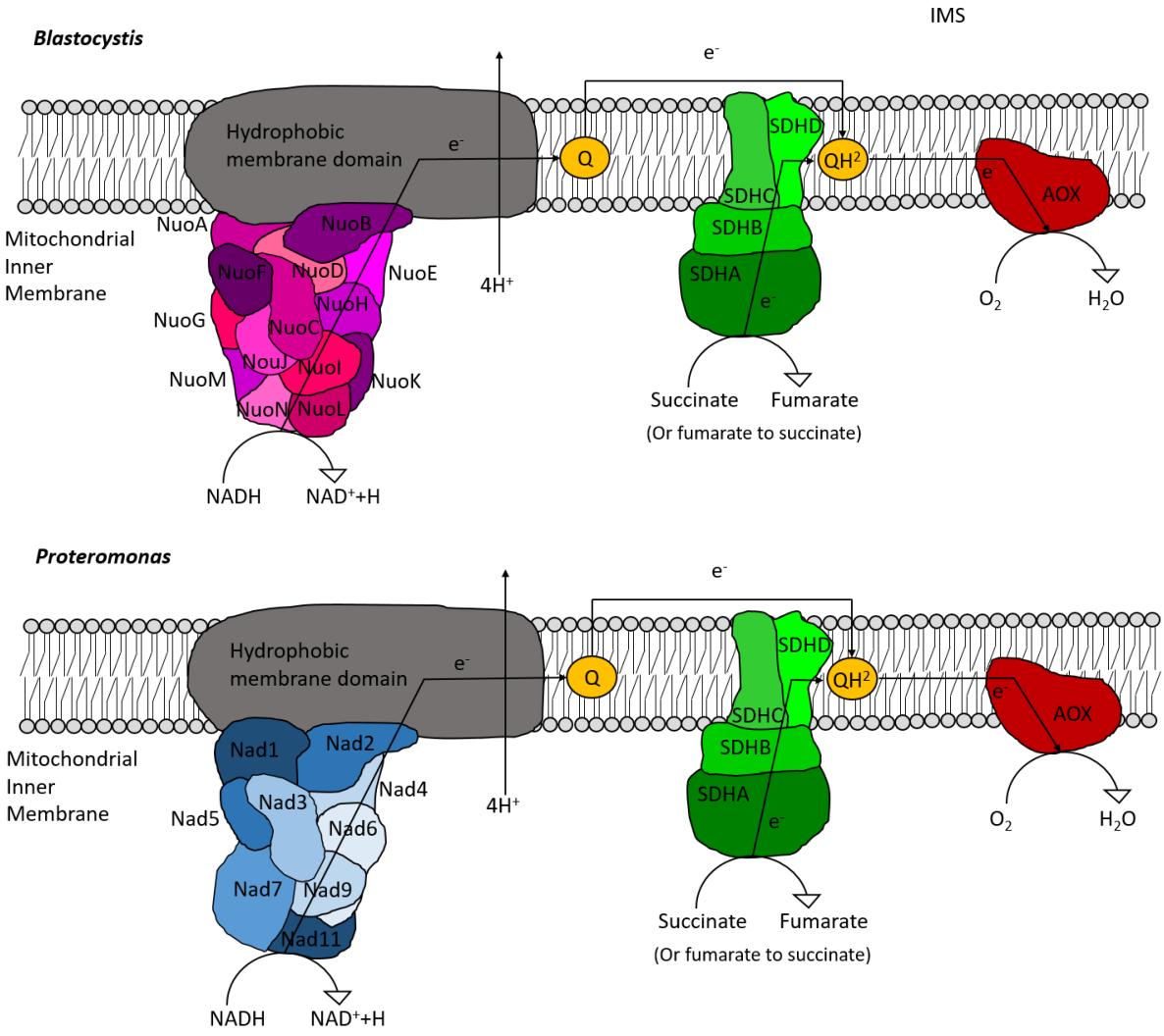

Figure 11. A comparison between the electron transport chains of Blastocystis and Proteromonas. The electron transport chains of both *Blastocystis* and *Proteromonas* are nearly identical, with the only main difference being that *Blastocystis* has 11 complex I subunits compared to the 9 found in *Proteromonas*. Their complex II make-up is the same and they are both in possession of AOX.

As shown in Table 6, most of the proteins involved in the TCA cycle and pyruvate metabolism are shared, also showing how the reduced TCA cycle of *Blastocystis* is also present in *Proteromonas.*

Table 6. Predicted proteins involved in pyruvate metabolism. A comparison between the predicted proteins involved in pyruvate metabolism and the TCA cycle in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Energy + Pyruvate	Fe-only hydrogenase, Flavodoxin	Present	Present
	Hydrogenase, flavodoxin	Present	Present
	Fe-only hydrogenase maturation rSAM protein HydE	Present	Present
	Pyruvate:ferredoxin/flavodoxin oxidoreductase	Present	Present
	Pyruvate dehydrogenase kinase 2/3/4	Present	Absent
	Pyruvate carboxylase, alpha subunit	Present	Present
	Phosphoenolpyruvate carboxykinase	Present	Present
	Fumarate hydratase Class I, anaerobic	Present	Present
	Malate dehydrogenase	Present	Present
	Acetate:succinate CoA transferase 1B	Present	Absent
	Acetate:succinate CoA transferase 1C	Present	Present
	Methylmalonyl-CoA mutase	Present	Present
	Propionyl-CoA carboxylase beta, partial	Present	Present
	Propionyl-CoA carboxylase alpha subunit	Present	Present
	Propionyl-CoA carboxylase beta	Present	Present
	Succinyl-CoA Synthetase beta subunit	Present	Present
	Succinyl-CoA Synthetase alpha subunit	Present	Present
	2-oxoisovalerate dehydrogenase subunit beta	Absent	Present
	acetyl-CoA synthetase (ADP-forming)	Present	Present
	methylmalonyl-CoA decarboxylase	Present	Present

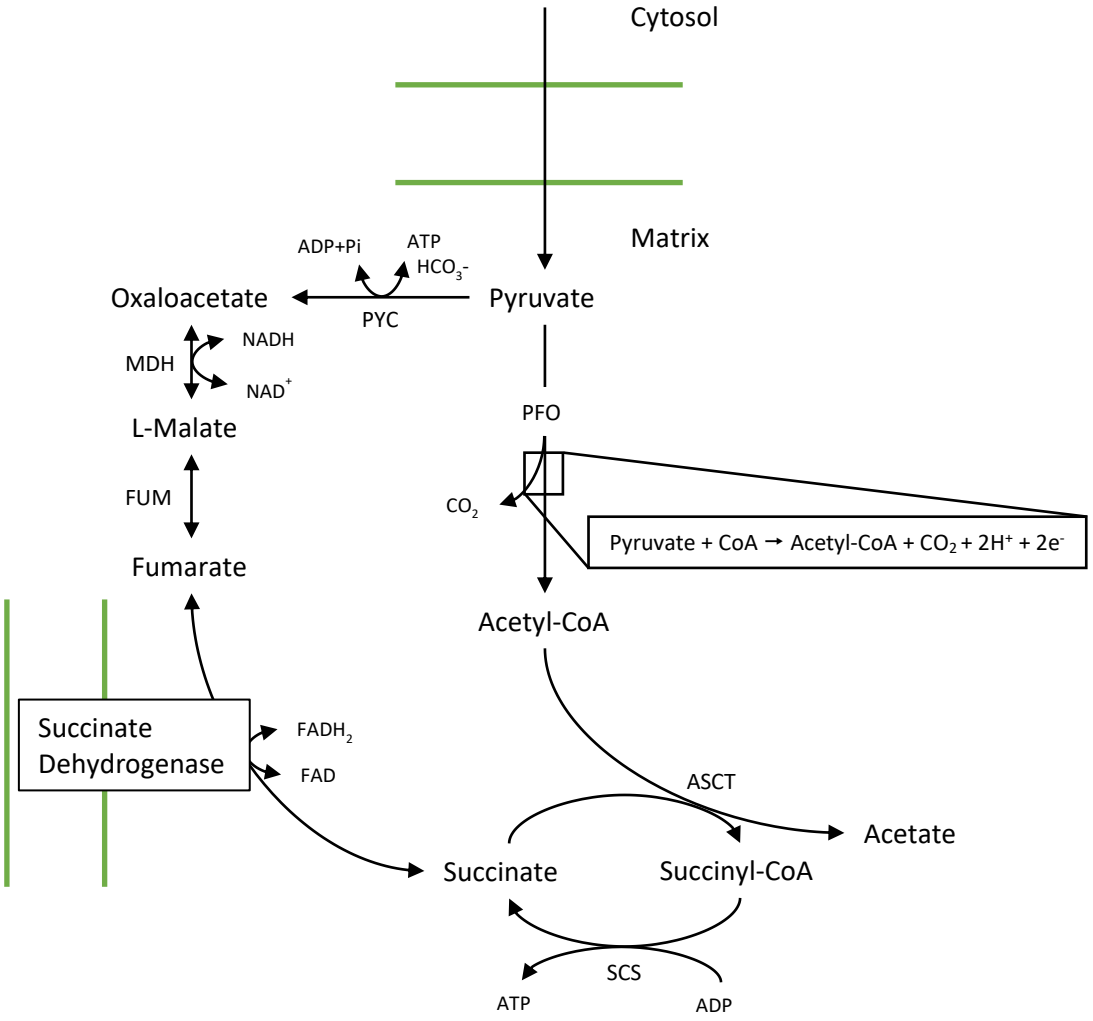

Figure 12. The TCA cycle of *Blastocystis* and *Proteromonas*. The citric acid cycle of *Blastocystis* and *Proteromonas* is reduced in comparison to the mammalian one, possessing roughly half of the cycle. In comparison to one another, however, there are no differences. Abbreviations: PYC, pyruvate carboxylase; MDH, malate dehydrogenase; FUM, fumarate hydratase; SCS, succinate-CoA synthetase; ASCT, acetate:succinate CoA transferase; PFO, pyruvate:ferredoxin oxidoreductase.

As shown in Table 8, the proteins involved in Fe-S cluster biosynthesis are nearly identical, with the one exception being the absence of frataxin in *Proteromonas.*

Table 7. Predicted Fe-S cluster biosynthesis proteins. A comparison between the proteins predicted to be involved in Fe-S cluster biosynthesis in the mitochondria of *Blastocystis* and *Proteromonas.*

Table 7		**Organism**	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Fe-S cluster biosynthesis	NifU-like protein (NFU1)	Present	Present
	Fe-S cluster biosynthesis protein,	Present	Present
	Cysteine Desulfurase; IscS	Present	Present
	Scaffold protein IscA1	Present	Present
	Frataxin	Present	Absent
	Fe-S cluster biosynthesis protein, ISCU	Present	Present
	Erv1	Present	Present
	Scaffold protein IscA2	Present	Present

Table 8 shows the proteins involved in folate metabolism, all of which are identical in both *Blastocystis* and *Proteromonas.*

Table 8. Predicted proteins involved in Folate metabolism. A comparison between the proteins associated with folate in the mitochondria of *Blastocystis* and *Proteromonas.*

Table 8		**Organism**	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Folate	bifunctional dihydrofolate reductase-thymidylate synthase	Present	Present
	Bifunctional methylenetetrahydrofolate dehydrogenase/cyclohydrolase	Present	Present
	5-formyltetrahydrofolate cyclo-ligase	Present	Present
	dihydrofolate reductase	Present	Present
	c-1-tetrahydrofolate synthase	Present	Present
	Dihydropteroate synthase	Present	Present
	Folylpolyglutamate synthase	Present	Present

Table 9 compares the proteins involved in the payoff phase of glycolysis, located in the mitochondria of these two organisms. While largely the same, *Blastocystis* has a TPI-GAPDH fusion that *Proteromonas* does not, instead *Proteromonas* possesses the separate proteins, being triose phosphate isomerase and glyceraldehyde 3-phosphate dehydrogenase. The reason for TPI not being in the above table is due to it being a member of the preparatory phase and therefore cytosolic.

Table 9. Predicted proteins involved in Glycolysis. A comparison between the proteins predicted to be involved in the payoff phase of glycolysis in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Glycolysis	Enolase	Present	Present
	6-phosphofructokinase	Absent	Present
	bifunctional 6-phosphofructo-2-kinase/ fructose-2,6-bisphosphate 2-phosphatase	Absent	Present
	glyceraldehyde 3-phosphate dehydrogenase	Present	Present
	phosphoglycerate kinase	Present	Present
	TPI-GAPDH fusion	Present	Unknown

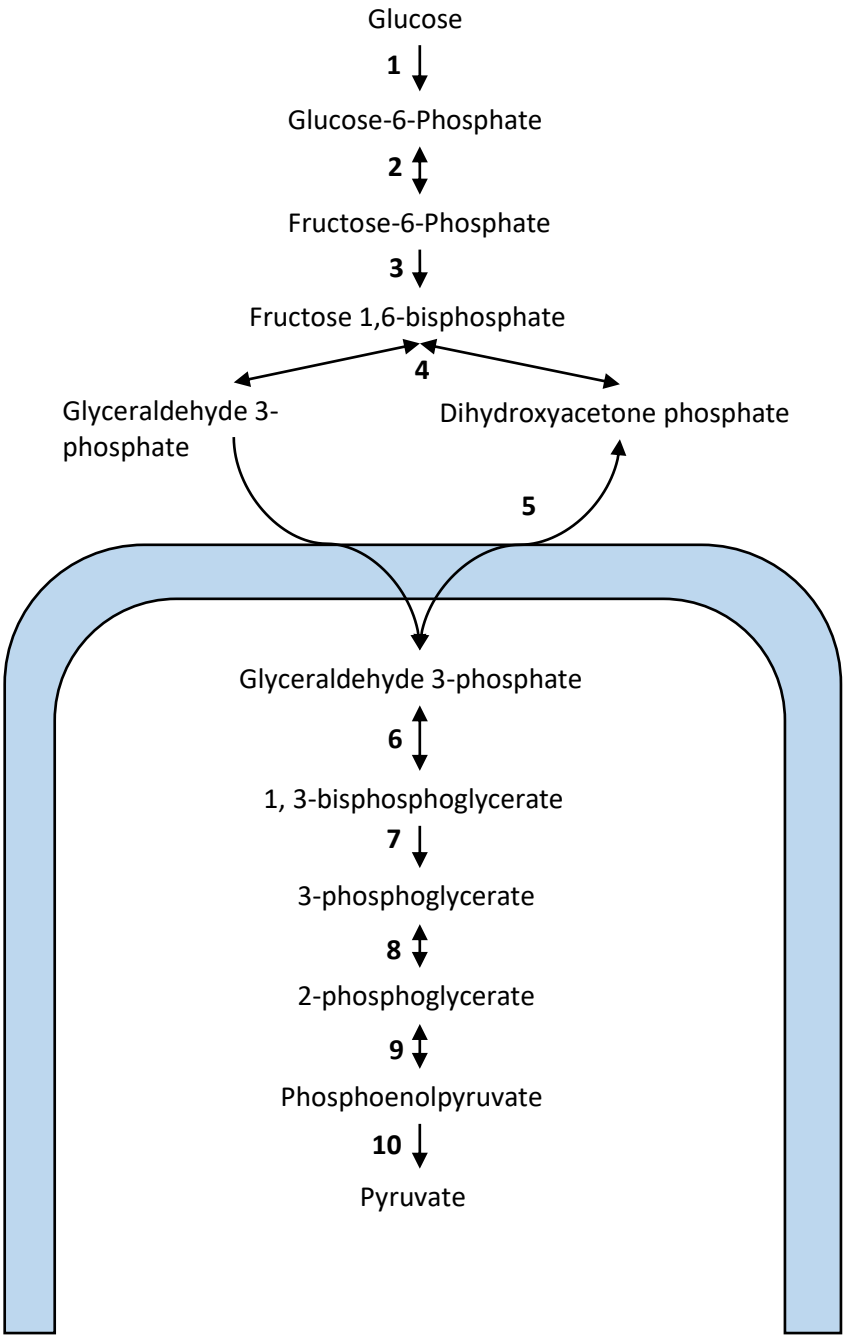

Figure 13. The proposed glycolysis pathway in *Proteromonas.* Glycolysis is split into two main parts, the preparatory and payoff phases. The enzymes in the payoff phase have been found, with mitochondrial targeting signals, to be located in the mitochondrion of *Proteromonas*. The above figure is theoretically what the pathway could look like in *Proteromonas*. In *Blastocystis*, steps 5 and 6 would be mitochondrial, due to the location of the TPI-GAPDH fusion. **1)** Hexokinase; **2)** phosphohexose isomerase; **3)** phosphofuctokinase-1; **4)** aldolase; **5)** triose phosphate isomerase; **6)** glyceraldehyde 3-phopshate dehydrogenase; **7)** phosphoglycerate kinase; **8)** phosphoglycerate mutase; **9)** enolase and **10)** pyruvate kinase.

Table 10 shows another significant difference in a mitochondrial machinery of *Proteromonas* and *Blastocystis*. *Blastocystis* has 39 total import proteins and *Proteromonas* has 33, with only 25 bein shared. The most significant reason for this is that *Proteromonas* completely lacks an outer membrane import system and is missing some, although few, inner membrane import proteins.

Table 10. Proteins predicted to comprise the mitochondrial import machinery. A comparison between the protein import systems in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Import	PHB2; prohibitin 2	Present	Present
	Cpn10; groES, HSPE1; chaperonin GroES	Present	Absent
	Cpn60; groEL, HSPD1; chaperonin GroEL	Present	Present
	pitrilysin metallopeptidase 1	Present	Present
	Hsp40	Present	Present
	dnaJ; molecular chaperone DnaJ	Present	Present
	Hsp70	Present	Present
	Hsp78	Present	Present
	Hsp90/Hsp75	Present	Present
	Hsp68	Absent	Present
	xaa-pro dipeptidase app	Present	Present
	Innermembrane space protease (IMP)	Present	Present
	lon peptidase 1	Present	Present
	GrpE/Mge	Present	Present
	mitochondrial intermediate peptidase	Present	Present
	MPPb	Present	Present
	MPPa	Present	Present
	mitochondrial metalloendopeptidase OMA1	Present	Absent
	Oxa1	Present	Present
	PHB1; prohibitin 1	Present	Present
	Sam50	Present	Absent
	Slowmo homologue	Present	Present
	Slowmo protein, PRELI domain-containing protein 1	Present	Present
	TIM23	Present	Present
	TIM22	Present	Absent
	TIM14-like	Present	Absent
	Tim16/Pam16	Present	Absent
	Tim17	Present	Absent
	Tim21	Present	Present
	Tim44	Present	Present
	Tim50	Present	Present
	Tim10	Present	Absent
	Tim8-13	Present	Absent
	Tim9-10	Present	Absent

	Tom40	Present	Absent
	Tom70	Present	Absent
	Tom70	Present	Absent
	mitochondrial inner membrane AAA protease Yta12	Present	Present
	Zim17; DNLZ, Tim15	Present	Present
	Phospholipid transporting ATPase	Absent	Present
	ABC transporter G fa	Absent	Present
	potassium transporter	Absent	Present
	monocarboxylate transporter 9-like	Absent	Present
	pleitropic drug resistance protein 2	Absent	Present
	AFG1-like ATPase	Absent	Present
	oxalate:formate antiporter-like	Absent	Present

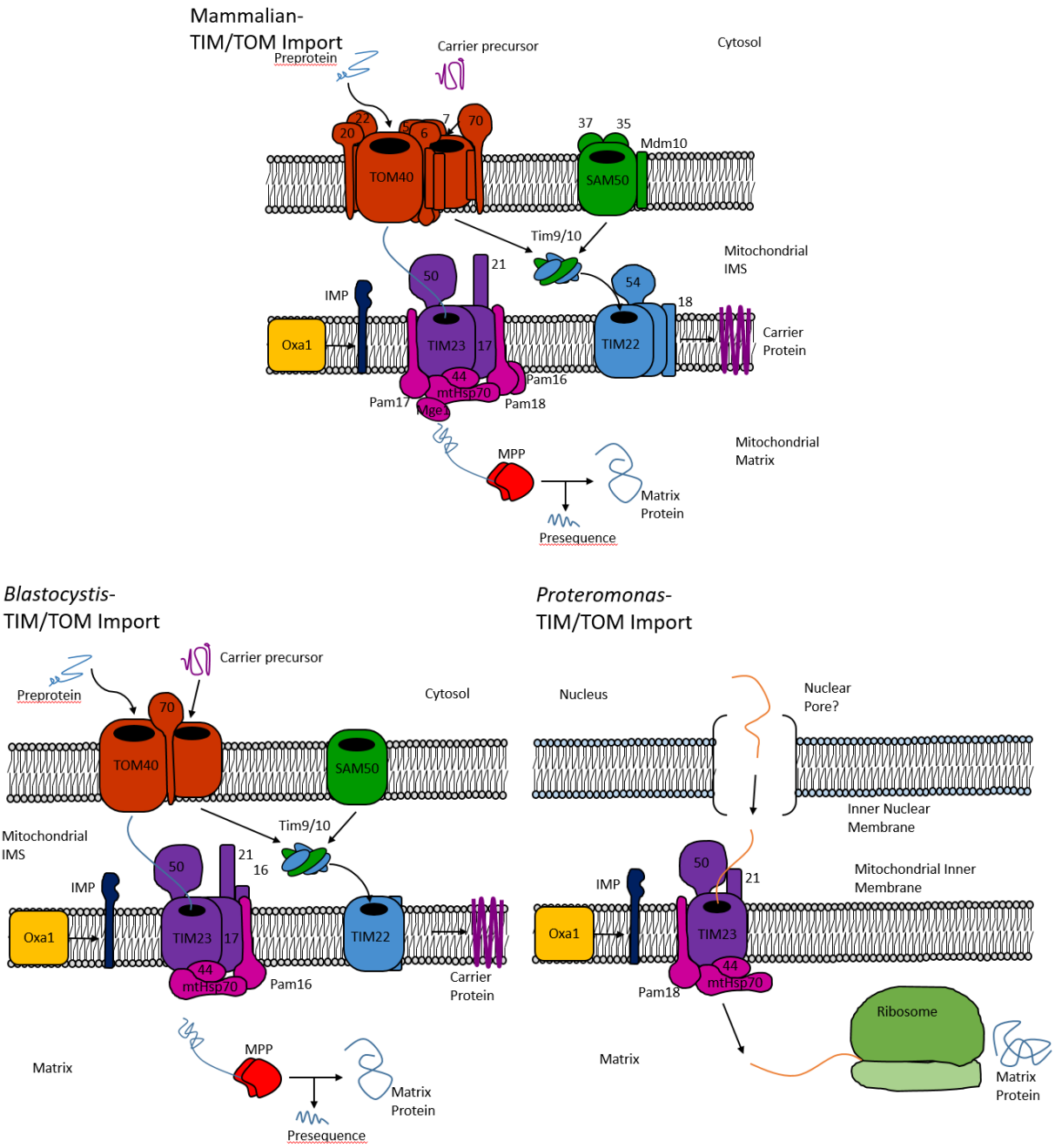

Figure 14. A comparison between the mammalian, *Blastocystis* and *Proteromonas* import systems. When comparing mammalian import to *Blastocystis*, it can be seen that the *Blastocystis* is reduced, but it still has the main components of the import system. *Proteromonas* is missing the outer membrane import system, and due to its close proximity to the nucleus, the method of RNA import has been proposed since the mitochondrion outer membrane has no known method of protein import.

As shown in Table 11, proteins involved in redox pathways are mostly shared by these two organisms, with the exceptions being oxidoreductase and leucyl aminopeptidase.

Table 11. Predicted proteins involved in Redox pathways. A comparison between the redox proteins in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Redox	Ferredoxin	Present	Present
	Thioredoxin	Present	Present
	Glutaredoxin	Present	Present
	Monothiol glutaredoxin (Grx5)	Present	Present
	Superoxide dismutase	Present	Present
	putative iron-dependent peroxidase	Present	Present
	FAD-dependent pyridine nucleotide-disulphide oxidoreductase	Present	Present
	Thioredoxin-dependent peroxide reductase	Present	Present
	Thioredoxin reductase 2	Present	Present
	oxidoreductase	Present	Absent
	hydroxyacylglutathione hydrolase	Present	Present
	leucyl aminopeptidase	Present	Absent
	Glutathione reductase	Present	Present

Table 12 shows proteins involved in fatty acid synthesis, which are mostly shared between the two organisms with *Proteromonas* having 8 proteins, *Blastocystis* having 9 and sharing 7 of them.

Table 12. Predicted proteins involved in fatty acid biosynthesis and metabolism. A comparison between the proteins involved in fatty acid synthesis/metabolism in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Fatty Acid	3-ketoacyl-ACP reductase	Present	Present
	Acyl carrier protein	Present	Absent
	Long-chain fatty acid CoA ligase	Present	Absent
	2-enoyl-[acyl-carrier-protein] reductase (FabI)	Present	Present
	3-oxoacyl-[acyl-carrier-protein] synthase (FabF)	Present	Present
	fatty acid elongase 3	Present	Present
	trans-2-enoyl-CoA reductase (MECR)	Present	Present
	[acyl-carrier-protein] S-malonyltransferase FabD	Present	Present
	long-chain acyl-CoA synthetase	Present	Present
	2,4 dienoyl-CoA reductase	Absent	Present

As shown in Table 13, the majority of proteins involved in lipid synthesis and metabolism are shared by *Proteromonas* and *Blastocystis* with 14 of the 17 proteins being shared.

Table 13. Predicted proteins involved in lipid synthesis and metabolism. A comparison between the proteins involved in the synthesis and metabolism of lipids in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Lipid	3-ketosteroid reductase	Present	Present
	glycerol kinase	Present	Present
	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase	Present	Present
	Phosphatidylglycerophosphatase GEP4, mitochondrial	Present	Present
	cardiolipin synthetase	Present	Present
	Phosphtaidylserine decarboxylase	Present	Present
	TAM41, mitochondrial translocator assembly and maintenance protein 41	Present	Present
	phosphatidylinositol synthase	Present	Present
	choline-phosphate cytidylyltransferase B	Present	Present
	Lysophosphatidic acid phosphatase	Present	Present
	tafazzin-like protein; K13511 monolysocardiolipin acyltransferase	Present	Present
	Sphingosine kinase	Present	Present
	lysocardiolipin and lysophospholipid acyltransferase	Present	Present
	Acyl-protein thioesterase 1	Present	Absent
	2-aminoethylphosphonate-pyruvate transaminase	Present	Absent
	hydroxysteroid (17-beta) dehydrogenase 14	Present	Present
	phospholipid-translocating P-type ATPase	Absent	Present

Table 14 shows the proteins involved in the synthesis and metabolism of nucleotides. A comparison of the two organisms shows that *Proteromonas* has more proteins than *Blastocystis* with 11 proteins compared to 4 respectively.

Table 14. Predicted nucleotide synthesis/metabolism proteins. A comparison between the proteins associated with either the synthesis or metabolism of nucleotides in the mitochondria of *Blastocystis* and *Proteromonas.*

Table 14		**Organism**	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Nucleotide	adenylate kinase	Present	Present
	Ribose-phosphate pyrophosphokinase	Present	Present
	Dihydroorotate oxidase (ubiquinone)	Present	Present
	Ribose-phosphate pyrophosphokinase	Present	Present
	cGMP-dependatn protein kinase 2	Absent	Present
	nucleoside monophosphate kinase	Absent	Present
	mannose-1-phosphate guanylyltransferase	Absent	Present
	glucose inhibited divison protein	Absent	Present
	NAD-dependant epimerase	Absent	Present
	N-glycosylase/DNA lyase	Absent	Present
	DNA-(apurinic or apyrimidinic site) lyase, chloroplastic-like	Absent	Present

Table 15 shows the proteins involved in quinone metabolism, which are mostly present in both organism, with the only exception being the absence of glycerol-3-phosphate dehydrogenase in *Proteromonas*. Like *Blastocystis, Proteromonas* also has an alternative oxidase, the protein that replaces complexes III and IV in the electron transport chain of *Blastocystis.*

Table 15. Predicted proteins associated with quinone metabolism. A comparison between the proteins associated with quinone metabolism in the mitochondria of *Blastocystis* and *Proteromonas.*

Table 15		**Organism**	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Quinone metabolism	electron transfer flavoprotein-ubiquinone oxidoreductase	Present	Present
	electron transfer flavoprotein, alpha	Present	Present
	Rhodoquinone Biosynthesis enzyme RquA	Present	Present
	Glycerol-3-phosphate dehydrogenase	Present	Absent
	Alternative oxidase	Present	Present
	Ubiquionone biosynthesis protein	Present	Present

Table 16 above compares all of the predicted proteins involved in transcription and translation within the mitochondrion of both *Proteromonas* and *Blastocystis.* The difference between the numbers of proteins in either of these organisms is significant, with *Blastocystis* having 34 proteins, *Proteromonas* having many more at 58, and only 28 being shared. The majority of these proteins are ribosomal proteins and tRNAs, of which *Proteromonas* has considerably more than *Blastocystis*.

Table 16. Proteins predicted to be involved in ribosomal transcription and translation. A comparison between the transcriptional and translational machineries in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Ribosome Transcription and Translation	tRNA modification GTPase	Present	Present
	aminomethyltransferase; K06980 tRNA-modifying protein YgfZ	Present	Absent
	DUS2; tRNA-dihydrouridine synthase 2	Present	Present
	mitochondrial translation factor Tu	Present	Present
	mRpL12, GB14723; mitochondrial ribosomal protein L12	Present	Present
	FIS1, TTC11, MDV2; mitochondrial fission 1 protein	Present	Absent
	mitochondrial helicase twinkle, putative	Present	Present
	elongation factor G-2, mitochondrial	Present	Present
	SIRT3; sirtuin 3; K11413 NAD-dependent deacetylase sirtuin 3	Present	Present
	mitochondrial 28S ribosomal protein	Present	Present
	MRPL43; large subunit ribosomal protein L43	Present	Absent
	MRPL49, NOF1; large subunit ribosomal protein L49	Present	Absent
	RP-L21, MRPL21, rplU; large subunit ribosomal protein L21	Present	Present
	MRPL46; large subunit ribosomal protein L46	Present	Absent
	ribosomal protein L27a	Present	Present
	Mitochondrial ribosomal protein L37 isoform 1	Present	Absent
	Ribosomal protein	Present	Present
	RP-L11, MRPL11, rplK; large subunit ribosomal protein L11	Present	Present
	tRNA dimethylallyltransferase, mitochondrial-like	Present	Present
	23S rRNA (uridine2552-2'-O)-methyltransferase	Present	Present
	PTH2; peptidyl-tRNA hydrolase	Present	Present
	tRNA pseudouridine38-40 synthase	Present	Present
	leucyl-tRNA synthetase	Present	Present

	valyl-tRNA synthetase	Present	Present
	isoleucyl-tRNA synthetase	Present	Present
	Methionine--tRNA ligase, mitochondrial	Present	Present
	Methionyl-tRNA formyltransferase	Present	Present
	Glutamate--tRNA ligase	Present	Present
	Alanine-tRNA ligase	Present	Present
	Tryptophan--tRNA ligase, mitochondrial	Present	Present
	elongation factor Tu, mitochondrial	Present	Present
	ATP-dependent DNA helicase PIF1	Present	Present
	NAD-dependent protein deacetylase sirtuin-3	Present	Present
	single subunit mitochondrial RNA polymerase	Present	Present
	40S ribosomal protein S13	Absent	Present
	60S ribosomal protein L5	Absent	Present
	60S ribosomal protein L3	Absent	Present
	phenylalanine-tRNA ligase	Absent	Present
	Nop14-like protein	Absent	Present
	CCR4-NOT transcription complex subunit 1-like	Absent	Present
	Prolyl-tRNA synthetase	Absent	Present
	Ribosomal Protein S4	Absent	Present
	Ribosomal protein S9	Absent	Present
	Ribosomal protein S10	Absent	Present
	Ribosomal protein S12	Absent	Present
	Ribosomal protein S13	Absent	Present
	Ribosomal Protein L2	Absent	Present
	Ribosomal Protein L5	Absent	Present
	Ribosomal Protein L6	Absent	Present
	Ribosomal Protein L14	Absent	Present
	Ribosomal Protein L16	Absent	Present
	Brix-domain-containing protein	Absent	Present
	tetratricopeptide repeat domain containing protein	Absent	Present
	ATP dependant RNA helicase	Absent	Present
	elongation factor-like GTPase 1	Absent	Present
	DNA polymerase delta subunit 1	Absent	Present
	cysteine-tRNA ligase, variant 3	Absent	Present
	probable RNA-binding protein 19	Absent	Present
	Splicing factor 3B subunit 2	Absent	Present
	elongation factor-like GTPase 1	Absent	Present
	tyrosine-tRNA ligase	Absent	Present
	TFIIH basal transcription factor complex helicase subunit, partial	Absent	Present
	pre-mRNA-processing factor	Absent	Present
	Prolyl-tRNA synthetase	Absent	Present

As shown in Table 17 above, the proteins involved in vitamin synthesis and metabolism are nearly identical in both *Proteromonas* and *Blastocystis*, with only one exception, being holo-[acyl-carrier protein] synthase.

Table 17. Proteins predicted to be involved in vitamin synthesis/metabolism. A comparison between the proteins associated with either the synthesis or metabolism of vitamins in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Vitamins	HLCS; biotin--protein ligase	Present	Present
	type II pantothenate kinase	Present	Present
	3-methyl-2-oxobutanoate hydroxymethyltransferase	Present	Present
	phosphopantothenoylcysteine decarboxylase	Present	Present
	holo-[acyl-carrier protein] synthase	Present	Absent
	Cob(I)yrinic acid a,c-diamide adenosyltransferase, mitochondrial	Present	Present

Table 18 below shows the proteins that do not fit into any of the biochemical groups above, and have thus been separated. As a result, there are many proteins here that *Blastocystis* has that *Proteromonas* does not and vice versa, with only 11 of these 31 proteins being shared between the two organism.

Table 18. Predicted proteins not associated with any major pathway. A comparison between the proteins not associated with any of the major biochemical pathways or machineries in the mitochondria of *Blastocystis* and *Proteromonas.*

		Organism	
Protein Pathway	**Protein**	***Blastocystis***	***Proteromonas***
Other	Mitofilin	Present	Present
	ES1 protein homolog, mitochondrial-like	Present	Absent
	Hydroxymethylglutaryl-CoA reductase	Present	Absent
	mitochondrial Rho GTPase; K07870 Ras homolog gene family, member T1	Present	Absent
	Iron-containing alcohol dehydrogenase; yqhD; NADP-dependent alcohol dehydrogenase	Present	Present
	Inorganic pyrophosphatase	Present	Present
	Fumarylacetoacetate hydrolase	Present	Present
	mitochondrial inner membrane protein, MDM33	Present	Absent

	NADP-dependent succinate-semialdehyde dehydrogenase	Present	Absent
	CARKD; carbohydrate kinase domain containing; K17757 ATP-dependent NAD(P)H-hydrate dehydratase	Present	Present
	Presenilins-associated rhomboid-like protein	Present	Absent
	Galactokinase; galK; galactokinase	Present	Present
	gamma carbonic anhydrase 1, mitochondrial	Present	Present
	methyltransferase-like protein 17, mitochondrial	Present	Present
	Succinate-semialdehyde dehydrogenase, mitochondrial	Present	Absent
	methylmalonic aciduria	Present	Present
	CysJ-like protein	Present	Present
	Citrate lyase beta	Present	Present
	Chromosome segregation protein SMC	Absent	Present
	ATP-dependent protease La	Absent	Present
	ubiquitin carboxyl-terminal hydrolase 8-like	Absent	Present
	UTP-glucose-1-phosphate uridylyltransferase	Absent	Present
	NIMA-related protein kinase	Absent	Present
	SRSF protein kinase 3	Absent	Present
	DNA polymerase delta subunit 1	Absent	Present
	methionine aminopeptidase 2b	Absent	Present
	methionine aminopeptidase 1d	Absent	Present
	Trehalose-phosphatase, variant 5	Absent	Present
	N-acetylserotonin O-methyltransferase -like	Absent	Present
	peptidase M16 family protein	Absent	Present
	Glycosyltransferase, GTA type	Absent	Present

(All proteins were sorted according to the method used by Gentekaki *et al.*)

Proteromonas peroxisomal Genes

Table 19 below shows the predicted peroxisomal proteins found in *Proteromonas* and their respective functions in other organisms. These predicted peroxisomal proteins were only found in *Proteromonas* and not *Blastocystis,* hence the lack of a comparison as with the previous tables.

Table 19. Predicted peroxisomal proteins in *Proteromonas.* The predicted peroxisomal proteins in *Proteromonas* with their respective cellular localisation and their associated function.

Organelle	Protein	Localisation	Function
Peroxisome	Pex 4	Cytosol, peroxisomal membrane	Docking receptor
	Pex 10	Peroxisomal membrane	Docking receptor
	Pex 19	Cytosol	Peroxisome membrane biogenesis
	Pex 23	Peroxisomal membrane	Regulates perxisome size/number

3.2 Immunofluorescence of fixed *Proteromonas* cells

Prior to this investigation there was no protocol for fixing *Proteromonas* had been published, therefore we had to base ours off of other protocols. The protocol used was for fixation was one initially used for fixing *Blastocystis* developed by Tsaousis *et al.* 2010. Figure 15 below is the result of this fixation, showing the typical teardrop shape of *Proteromonas*. While not too clear, the mitochondrion can be seen as a more intense red closely associated with the nucleus.

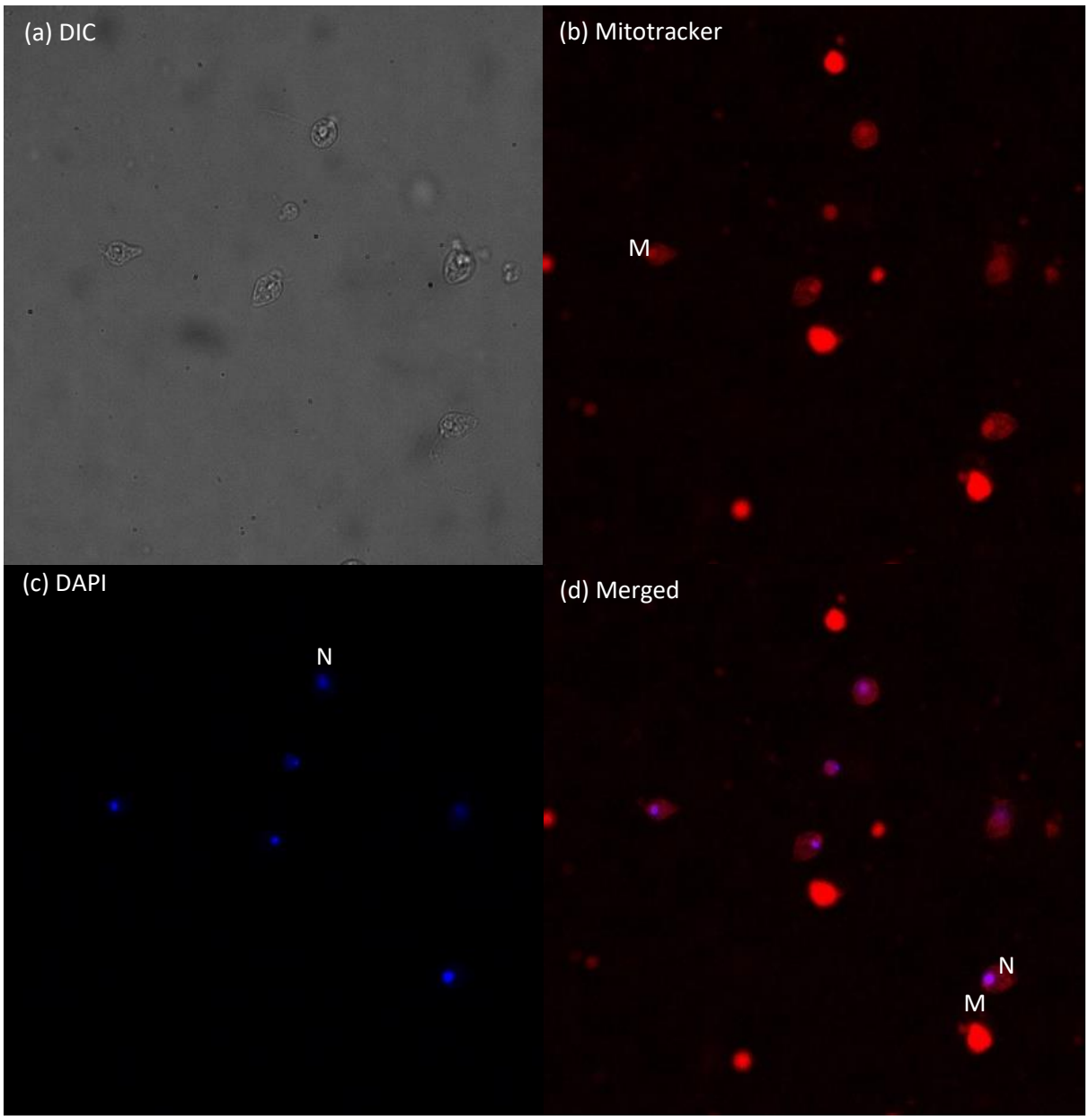

Figure 15. Fixation of *Proteromonas lacertae.* *Proteromonas* was fixed in 3.7% formaldehyde. The mitochondrion was stained with (b) Mitotracker™ (red) and the (c) nucleus stained with DAPI (blue). N, nucleus; M, mitochondrion.

3.3 Immunofluorescence of specific *Proteromonas* proteins

Figure 16 shows the cytoplasmic localisation of the Fe-S cluster maturation protein SufCB in *Proteromonas* using an anti-SufCB antibody (green). This protein, like *Blastocystis,* is shown to not to localise within the mitochondria, but (Figure 16) within the cytoplasm. There is some degree of non-specificity with the antibody, as it also coats the flagellum. This could potentially be cell debris that has been targeted by the antibody. SufCB was not initially found bioinformatically due to its location within the cytoplasm, and it was not looked for using PCR of cDNA or western blotting of protein extract, and was only found using an antibody and confocal microscopy.

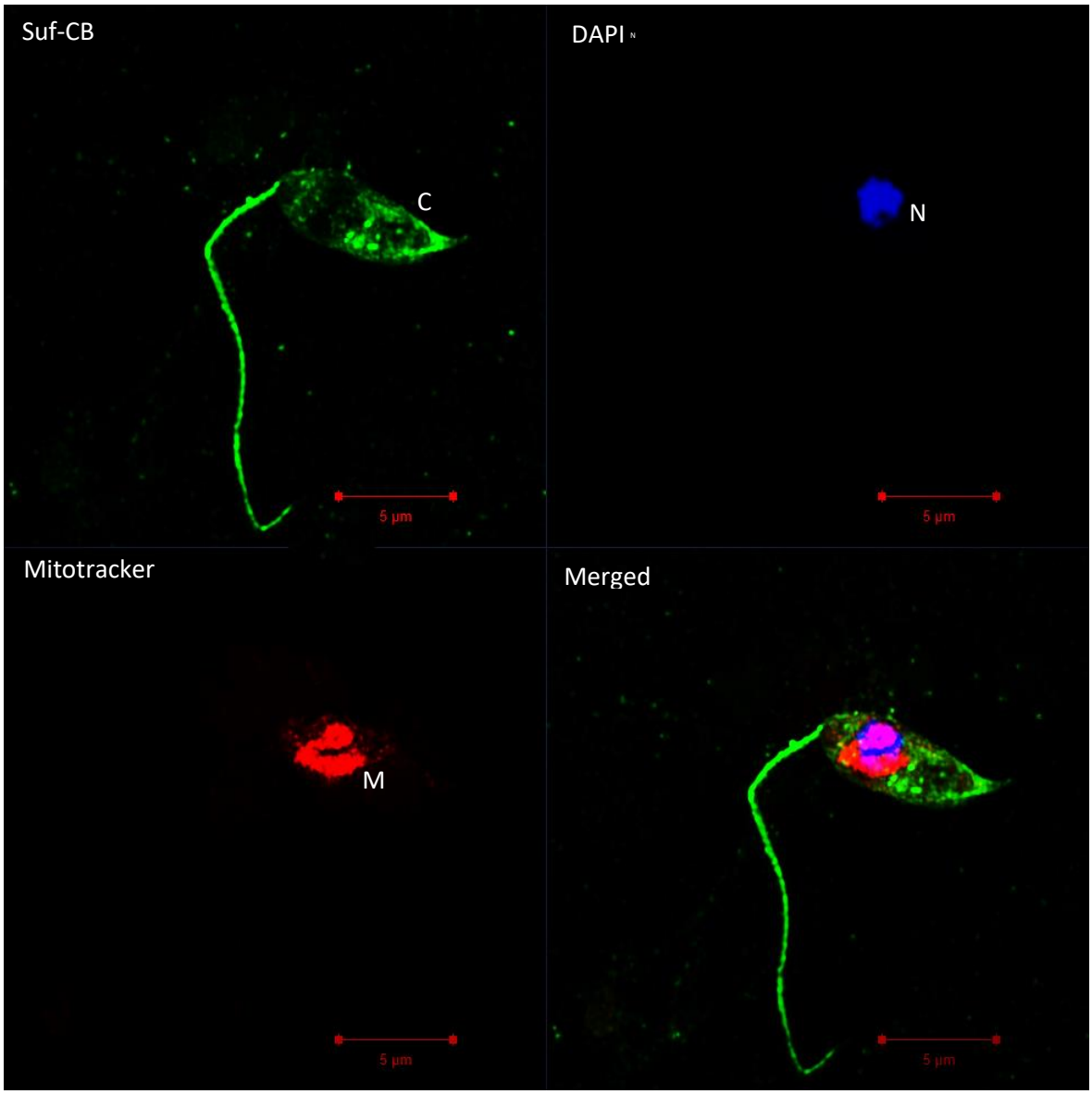

Figure 16. Cellular localisation of SufCB in *Proteromonas*. *Proteromonas* was fixed using 3.7% formaldehyde. The mitochondrion was stained with mitotracker™ (red), the nucleus stained with DAPI (blue) and a rat anti-SufCB antibody (green), 1:250, was used. Visualised using confocal microscopy. C, cytoplasm; N, nucleus; M, mitochondrion.

Figure 17 shows the identification of the peroxisome associated protein Pex19. It was initially identified through bioinformatics, and then through PCR of cDNA using primers made specifically against predicted *Proteromonas* Pex19 sequence (A) resulting in a band roughly 700bp in size. Following protein extraction, a western blot (B) was performed using an anti-Pex19 antibody, generating a band ~50kDa in size.

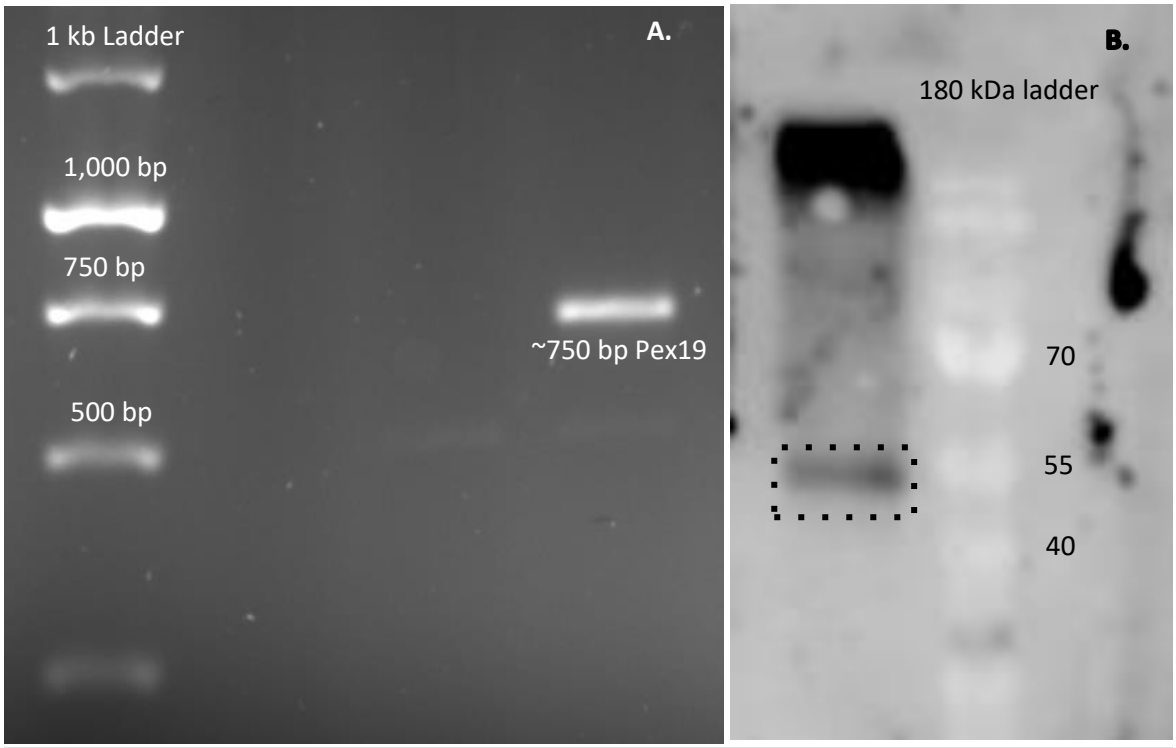

Figure 17. A. DNA agarose gel following PCR amplification of Pex19. **B.** Western blot of Pex19 using anti-Pex19 antibody, 1:400.

Figure 18 shows localisation of Pex19 in the cytoplasm of *Proteromonas*. As described, pex19 was first identified through bioinformatics and the presence proven through PCR of *Proteromonas* cDNA and western blotting using anti-Pex19 antibody (Figure 17 A and B). The presence of this protein and its location were finally confirmed through the fixation of *Proteromonas* and the targeting of Pex19 using antibodies.

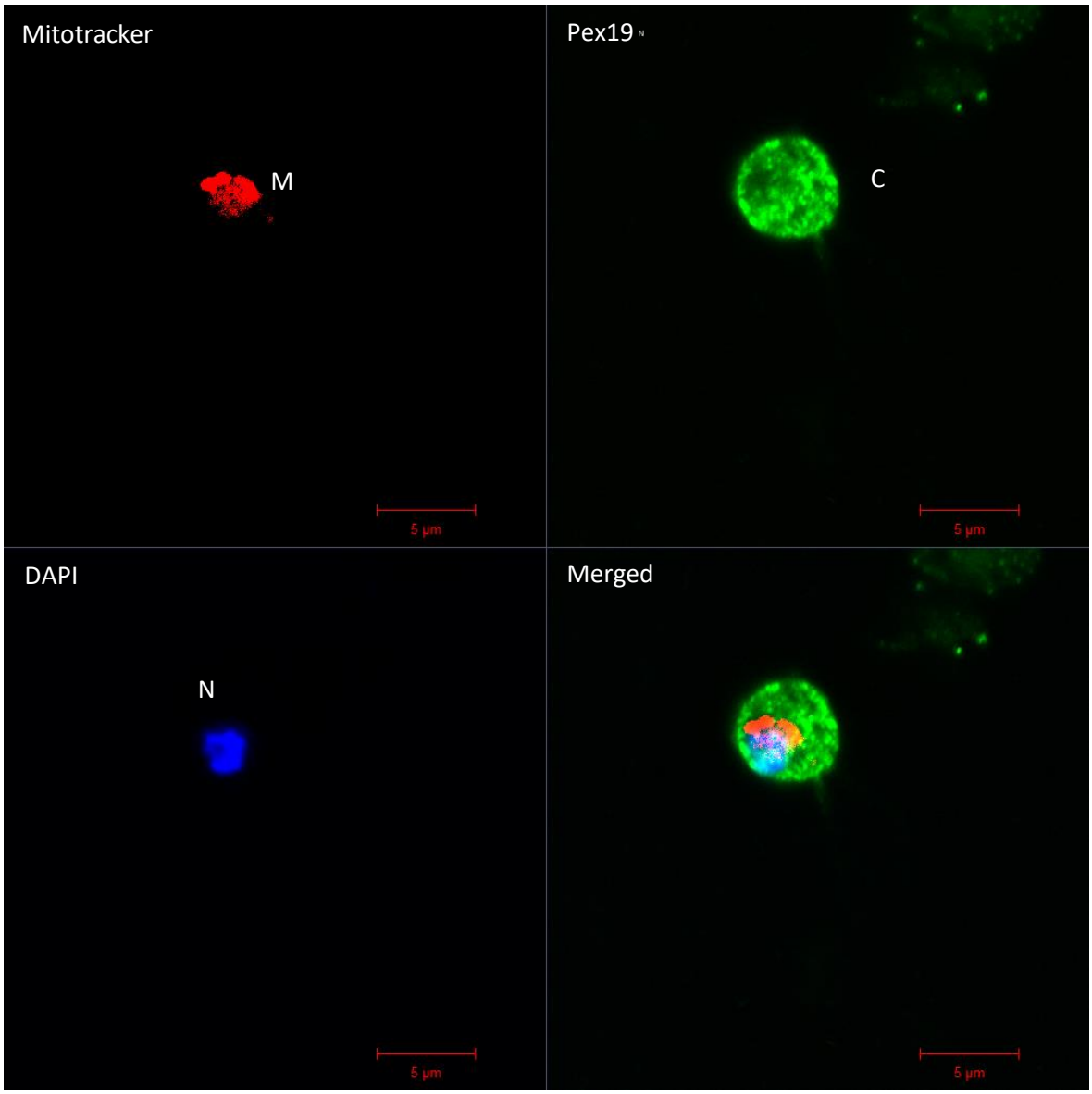

Figure 18. Cellular localisation of Pex19 in *Proteromonas.* Localisation of cytoplasmic peroxisome protein Pex19, following fixation in 3.7% formaldehyde, using anti-Pex19 antibody (green) 1:200. The mitochondrion was stained with Mitotracker™ (red) and the nucleus stained with DAPI (blue). C, cytoplasm; M, mitochondrion; N,nucleus

Figure 19 below shows the identification of alternative oxidase following (A) the PCR of *Proteromonas* cDNA using primers specifically targeted to predicted AOX sequences. The resultant band was, as expected, roughly 1,000 bp in size. Following protein extraction, a western blot (C) using a mouse anti-AOX, 1:1,000, antibody was performed, resulting in a band 35 kDa in size.

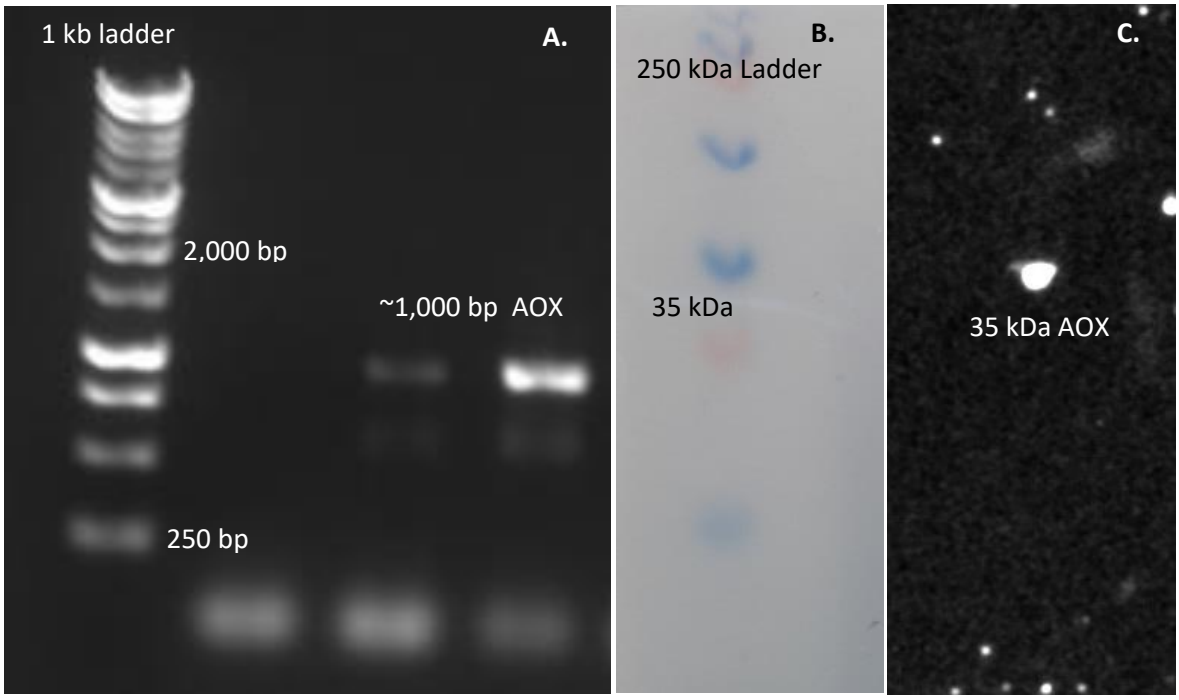

Figure 19. Identification of AOX in *Proteromonas.* **A.** DNA agarose gel following PCR amplification of AOX from *Proteromonas* cDNA. **B.** Thermo Fisher PageRuler Plus protein ladder used to indicate the size of the band on the Western blot (C). **C.** Western blot of AOX using 1:1,000 anti-AOX antibody.

Figures 20 and 21 both show the localisation of AOX within the *Proteromonas* mitochondrion. The mouse anti-AOX antibody was used in conjunction with mitotracker™ which shows red and green respectively, and as expected. Both co-localise forming the yellow colour observed in the merged panels of figures 20 and 21, confirming the presence of AOX within the mitochondrion of *Proteromonas*. AOX was first identified through bioinformatics, proven to be present in *Proteromonas* using PCR of cDNA and western blot of protein extract and confirmed to be mitochondrial through localisation and confocal microscopy.

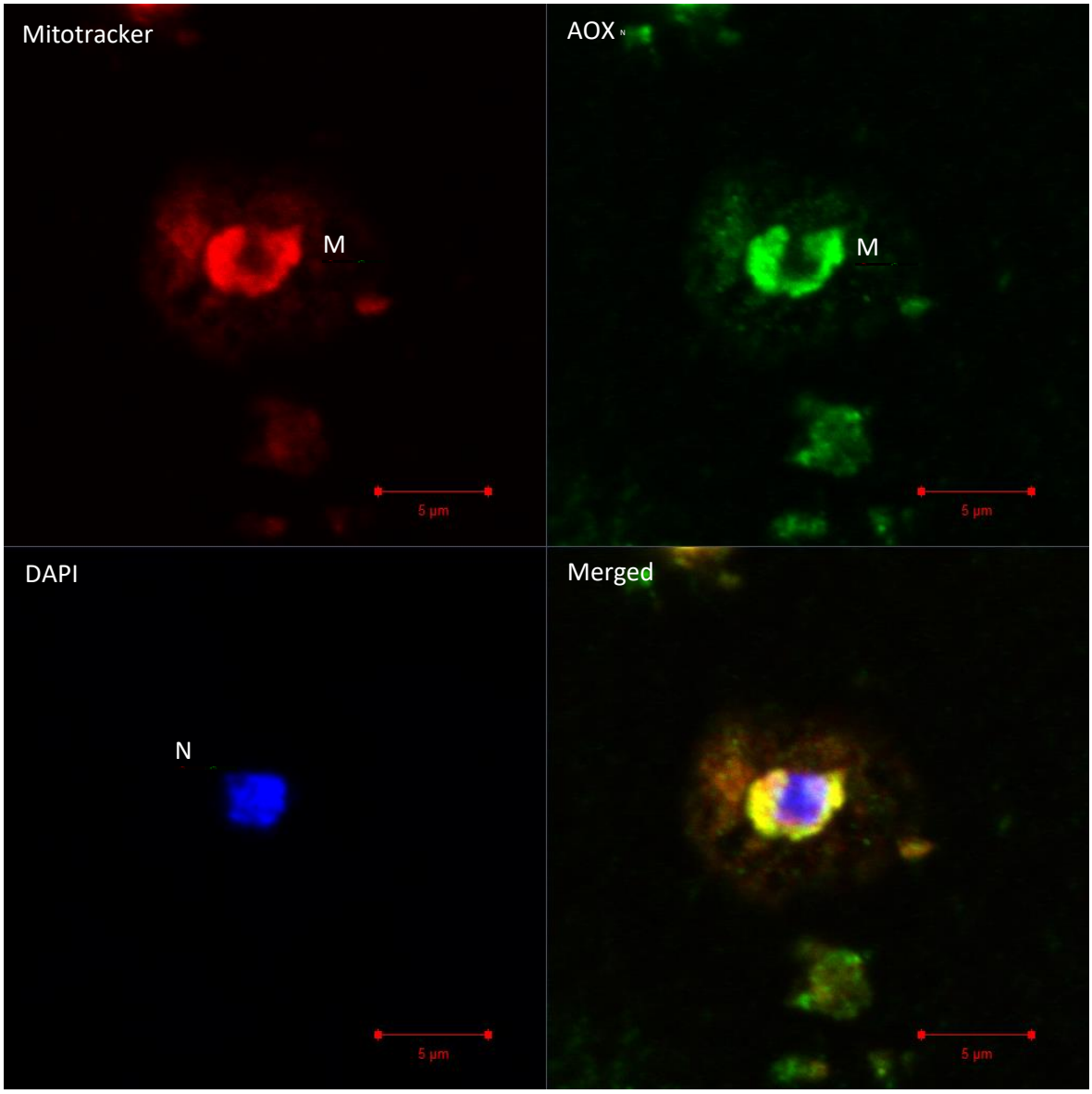

Figure 20. Mitochondrial localisation of Alternative Oxidase in *Proteromonas.* Localisation of mitochondrial AOX using mouse anti-AOX antibody (green) 1:250. The mitochondrion was stained with Mitotracker™ (red) and the nucleus stained with DAPI (blue). N, nucleus; M, mitochondrion.

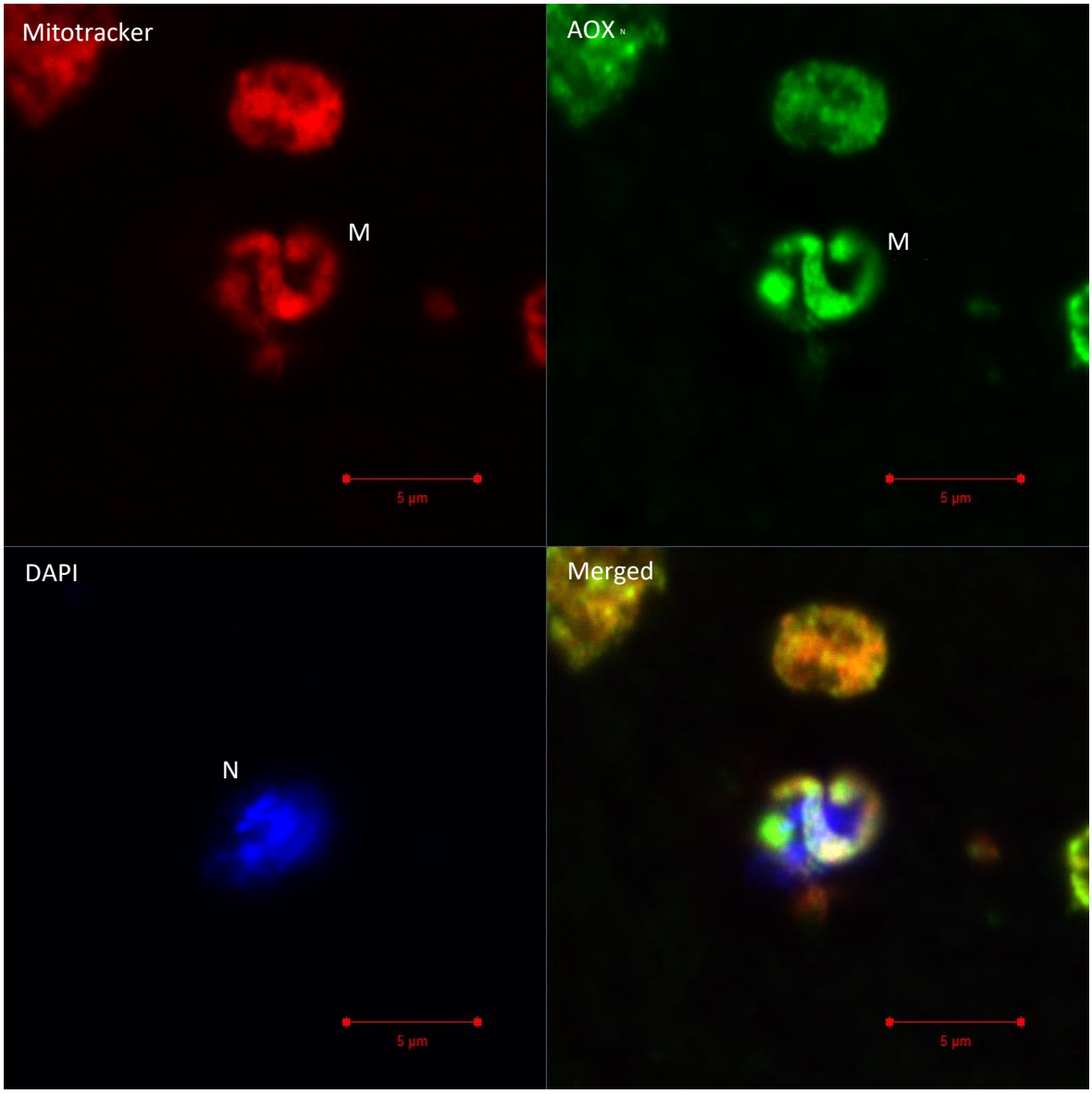

Figure 21. Mitochondrial localisation of Alternative Oxidase in *Proteromonas.* Localisation of mitochondrial AOX using mouse anti-AOX antibody (green) 1:250. The mitochondrion was stained with Mitotracker™ (red) and the nucleus stained with DAPI (blue). N, nucleus; M, mitochondrion.

3.4 Investigation of Protein Metabolic Activity

It is known that the electron transport chain in *Blastocystis* uses only complexes I, II and an AOX. Using the Agilent XFp mitofuel flex test, our aim was to investigate the effect of AOX inhibitors on *Proteromonas*, and to determine whether or not *Proteromonas* had complexes III, IV and V or just AOX. If the cells continued to die due to AOX inhibition and oxygen consumption drops, then theoretically only AOX is present and if it recovers or does not change, then complex IV is a part of *Proteromonas* ETC.

The graph below, Figure 22, shows SDHA and AOX activity, and the effect of their inhibition on *Proteromonas*. This was tested through the injection of the SDHA inhibitor TTFA and the AOX inhibitor SHAM. TTFA addition has a less significant effect on *Proteromonas*, and their decline in oxygen consumption is much slower than those injected with SHAM first then TTFA, in which cell death occurs rather dramatically. The AOX inhibitor SHAM acts to block electron transfer, preventing the reduction of oxygen to water in the final step of oxidative phosphorylation, which is why its inhibition results in the drop in oxygen consumption. SDHA activity drops in the presence of TTFA due to it inhibiting quinone docking, preventing electron transfer.

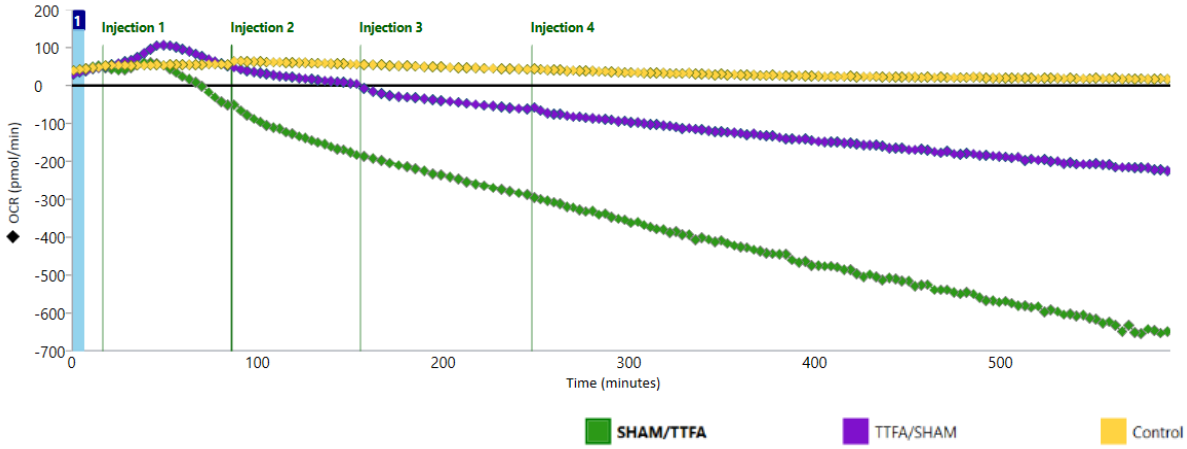

Figure 22. The activity of SDHA and AOX in the presence of inhibitors. The effect of both TTFA and SHAM on the activity of SDHA and AOX respectively in *Proteromonas.* Abbreviations: SDHA, succinate dehydrogenase subunit A; TTFA, 2-thenoyltrifluoroacetone; SHAM, Salicylhydroxamic acid.

As with Figure 23 below, SHAM blocks electron transfer, preventing the reduction of oxygen to water in the final step of oxidative phosphorylation, which is why its inhibition results in the drop in oxygen consumption. The graph below, Figure 23, shows that, at low concentrations, both 0.5 and 1.0 mM of SHAM, the AOX functions relatively unhindered, but beyond that, with the injection of 2.5 and 5.0 mM SHAM, AOX stops functioning as it should and O_2 consumption decreases.

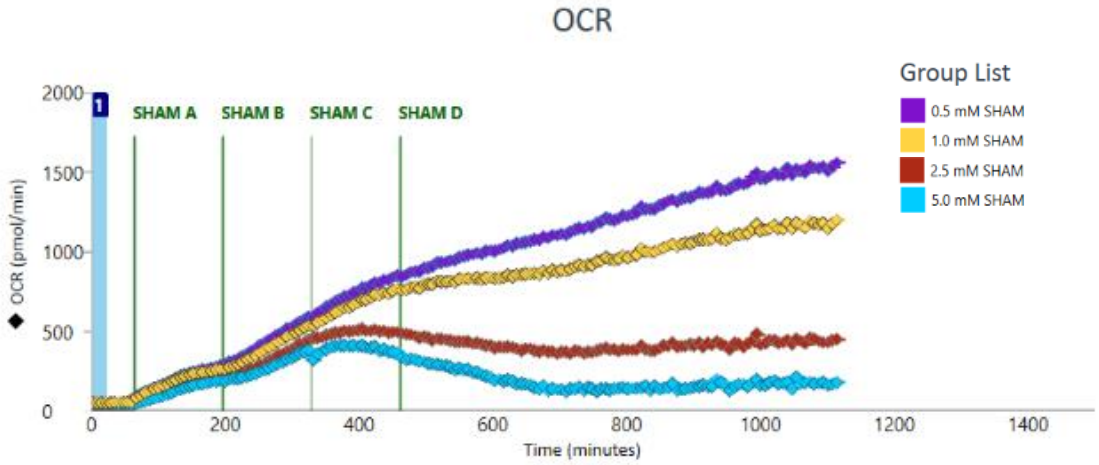

Figure 23. The activity of AOX in the presence of varying inhibitor concentrations. The presence and activity of alternative oxidase was investigated using the Agilent XFp analyser. This was done using varied concentrations of the alternative oxidase inhibitor Salicylhydroxamic acid (SHAM).

3.5 Investigating mitochondrial structure using TEM

Following the initial fixation of *Proteromonas* and observation under immunofluorescence microscopy, it was noticed that the mitochondrion and nucleus had a very close association with one another and that the mitochondria seemed to 'hug' the nucleus forming a 'pocket' for the nucleus to sit inside. Upon examination of TEM images by Graham Clark, it was also noticed that there was a form of connection between these two organelles, and where this connection occurred, the membrane was somewhat more fluid. What this connection was and why it was there was a mystery, and required further investigation to see whether or not this was a one-time occurrence or if we could see this in multiple cells. In order to look this closely, the cells were fixed in preparation for Transmission electron microscopy and observed under the TEM microscope at varying magnifications (4K, 8K, 15K and 25K).

What we observed following fixation and microscopy, was that this connection occurred in multiple different cells, and where this connection occurred, the membranes of the two organelles were usually somewhat fluid, as there was little to no definition in the membranes. In figures 24 A, B, D and F, we can see this connection between the organelles, whereas in C, we cannot. This reuqired a more in-depth look at these organelles, requiring serial sectioning to see whether it occurs at random points between the nucleus and mitochondrion as opposed to being fixed to on location.

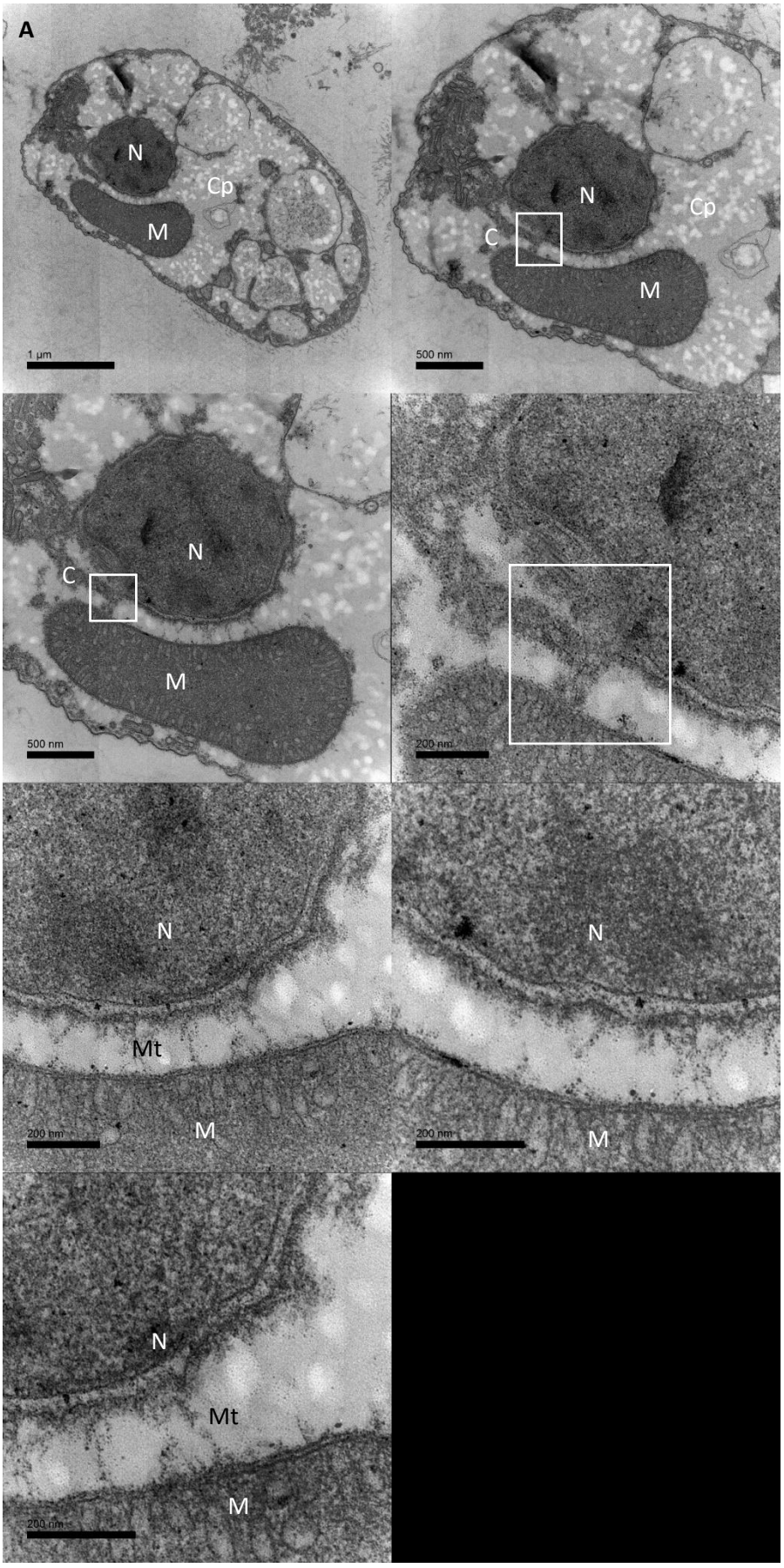
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Figure 24 A. Transmission electron microscopy of *Proteromonas* organelles. Multiple sections were investigated under TEM to identify whether the mitochondrion and nucleus made contact with one another in multiple cells. They were fixed, stained, cut into 70 nm sections and observed under TEM at 4K, 8K, 15K and 25K magnification. The connection occurs at multiple points here, although at only one of these points are the membranes fluid, these smaller connections where the membranes are not fluid could potentially be microtubules. N, nucleus; M, mitochondrion; C, connection, Cp, cytoplasm; Mt, Microtubules (possibly).

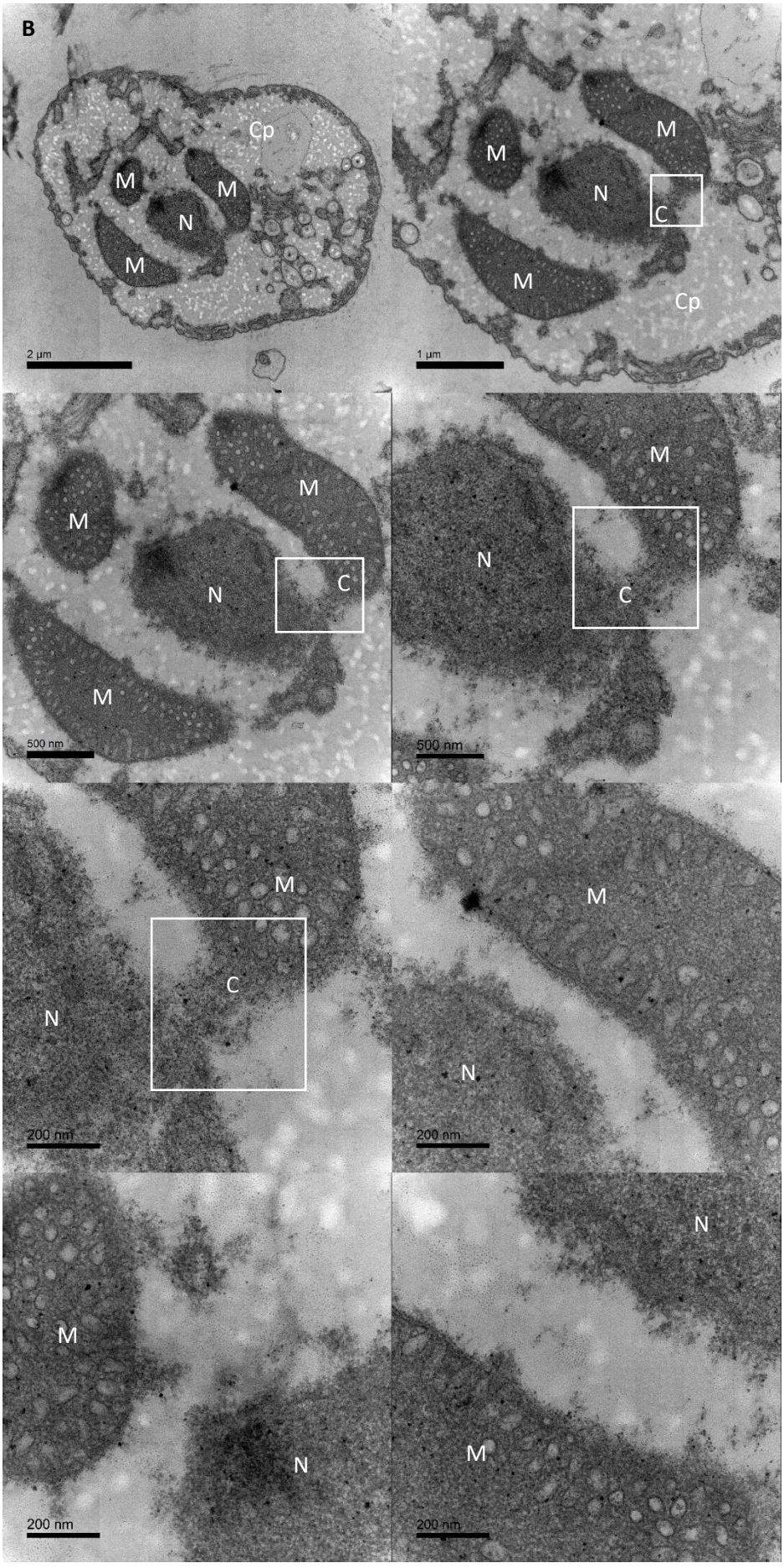
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Figure 24 B. Transmission electron microscopy of *Proteromonas* organelles. Multiple sections were investigated under TEM to identify whether the mitochondrion and nucleus made contact with one another in multiple cells. The larger connections are seen here, yet the smaller ones, suspected to be microtubules, are not. They were fixed, stained, cut into 70 nm sections and observed under TEM at 4K, 8K, 15K and 25K magnification. N, nucleus; M, mitochondrion; C, connection, Cp, cytoplasm.

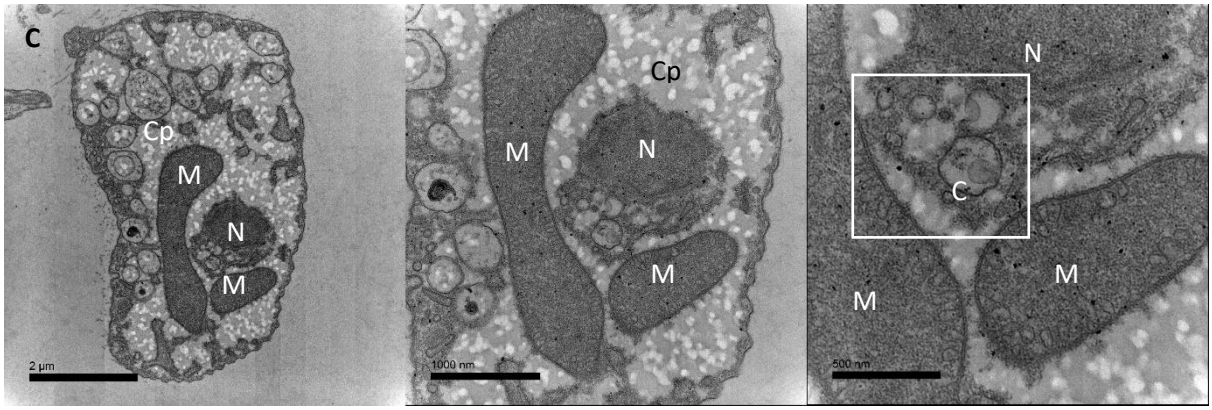


Figure 24 C. Transmission electron microscopy of *Proteromonas* organelles. Multiple sections were investigated under TEM to identify whether the mitochondrion and nucleus made contact with one another in multiple cells. They were fixed, stained, cut into 70 nm sections and observed under TEM at 4K, 8K, 15K and 25K magnification. While the nucleus and the mitochondrion appear to be touching in some way, where this contact occurs lacks to observed membrane fluidity typical of these connections. N, nucleus; M, mitochondrion; C, connection, Cp, cytoplasm.

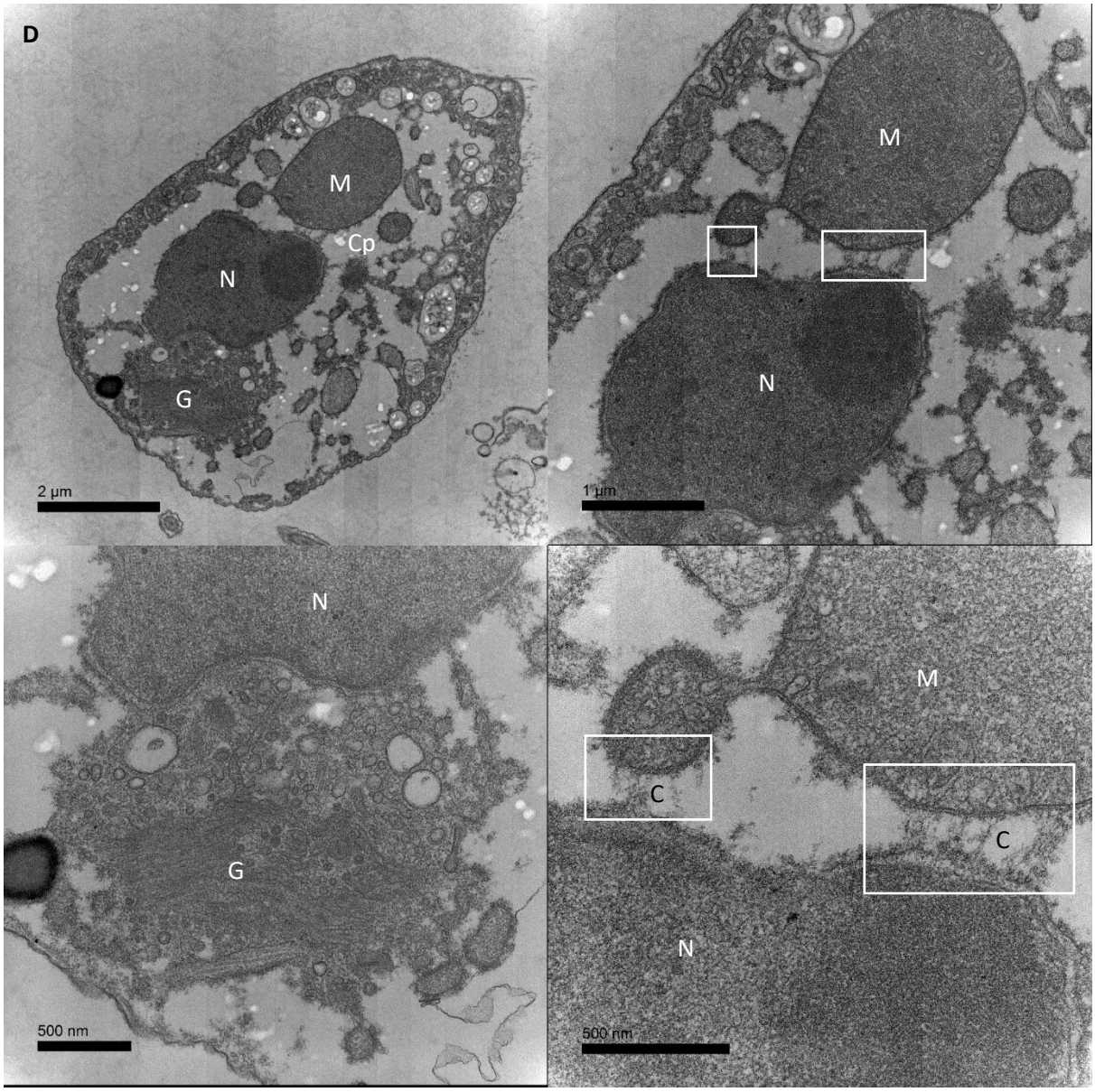
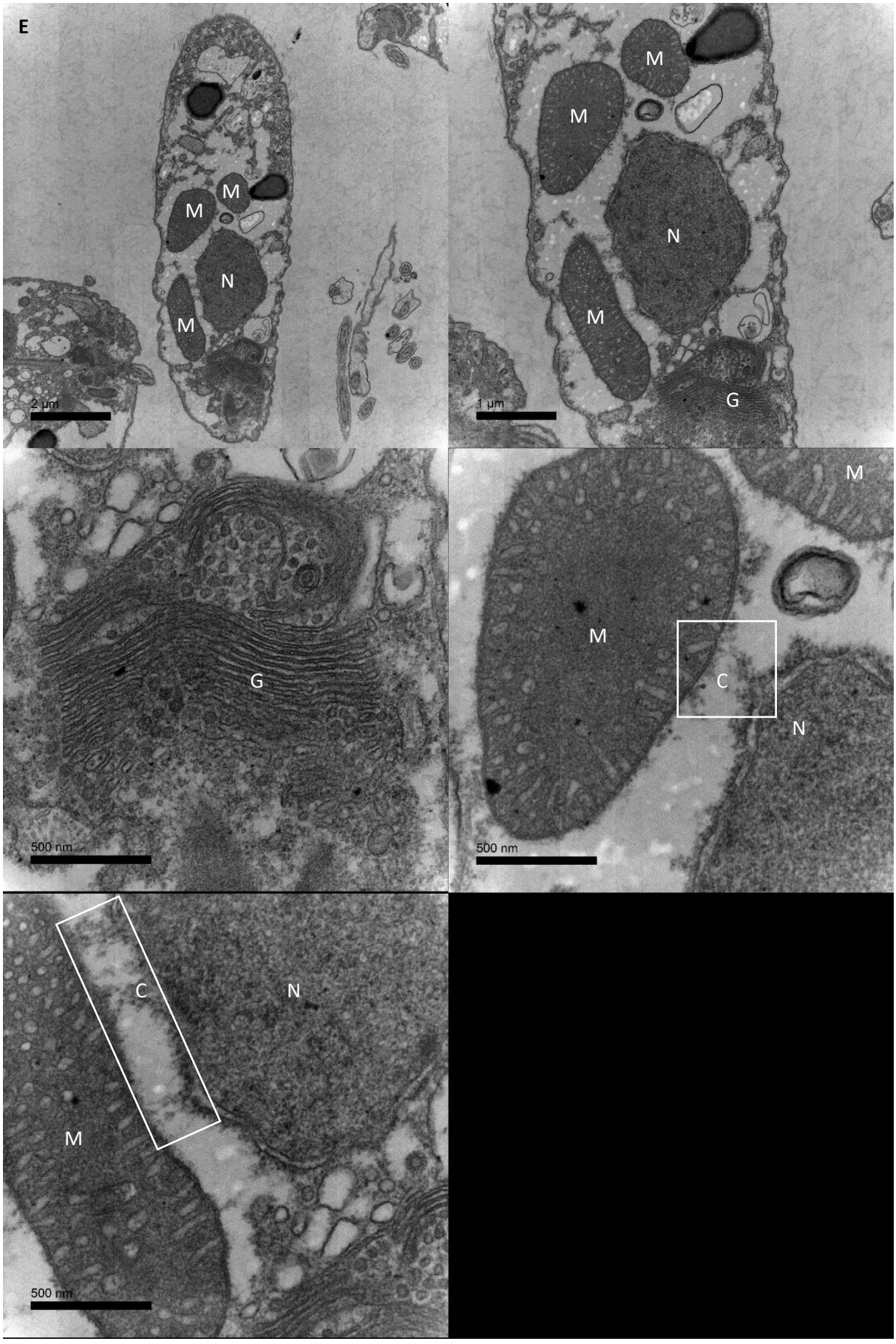


Figure 24 D. Transmission electron microscopy of *Proteromonas* organelles. Multiple sections were investigated under TEM to identify whether the mitochondrion and nucleus made contact with one another in multiple cells. They were fixed, stained, cut into 70 nm sections and observed under TEM at 4K, 8K, 15K and 25K magnification. N, nucleus; M, mitochondrion; C, connection, Cp, cytoplasm; G, golgi.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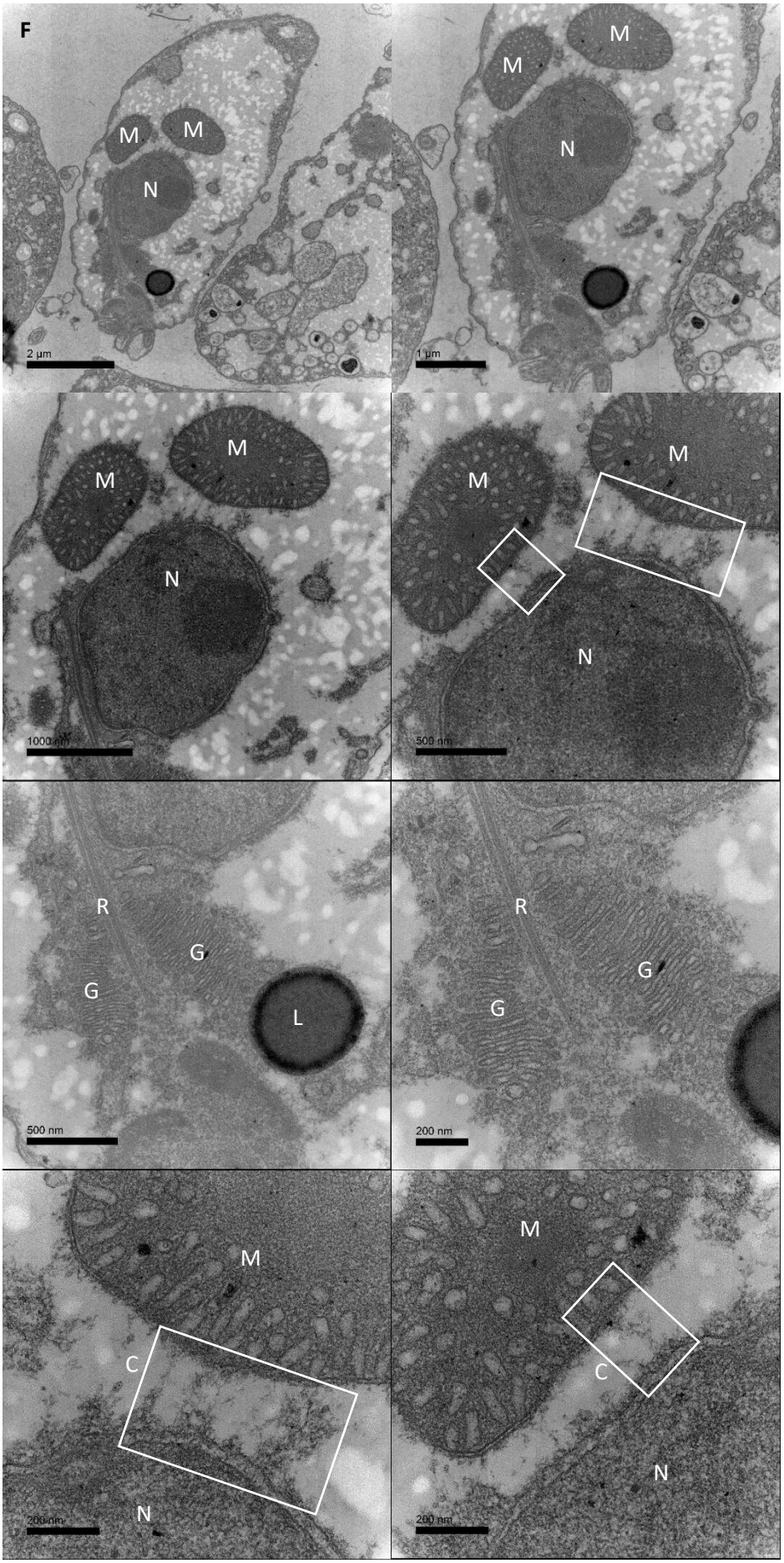
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Figure 24 F. Transmission electron microscopy of *Proteromonas* organelles. Multiple sections were investigated under TEM to identify whether the mitochondrion and nucleus made contact with one another in multiple cells. They were fixed, stained, cut into 70 nm sections and observed under TEM at 4K, 8K, 15K and 25K magnification. As with the other micrographs, these connections are present and are also potentially microtubules. N, nucleus; M, mitochondrion; C, connection, Cp, cytoplasm; R, rhizoplast and G, golgi; L, lipid droplet.

3.51 Serial Sectioning

Following standard TEM, the connection between the nucleus and mitochondrion was observed in the majority of the cells. This led us to believe that there was some significance to the closeness of these two organelles. It was not, however, observed in all cells. This connection seemed to be fairly important due to its presence in most of the cells, so to investigate whether it was present at one point or in multiple we needed to carry out serial sectioning of cells prepared for TEM, creating slices of 70 nm thickness. The advantage of serial sectioning is that we can visualise multiple points throughout the cell, and see whether this connection is present.

Serial sectioning of *Proteromonas* (below) has shown that this connection is seemingly always present in the cell, although not as we expected, as at this point of contact the membranes are not always fluid. As well as being observed in all sectioned cells, the connection can occur at multiple points. Finally, serial sectioning has given us more insight into the structure of the mitochondrion, and how it does not resemble the canonical mitochondria found in text books. Instead of the standard 'bean'-like shape, the mitochondrion of *Proteromonas* has many different lobes, best shown in figures 25 D, E and F, and that the mitochondrion always forms around the nucleus.

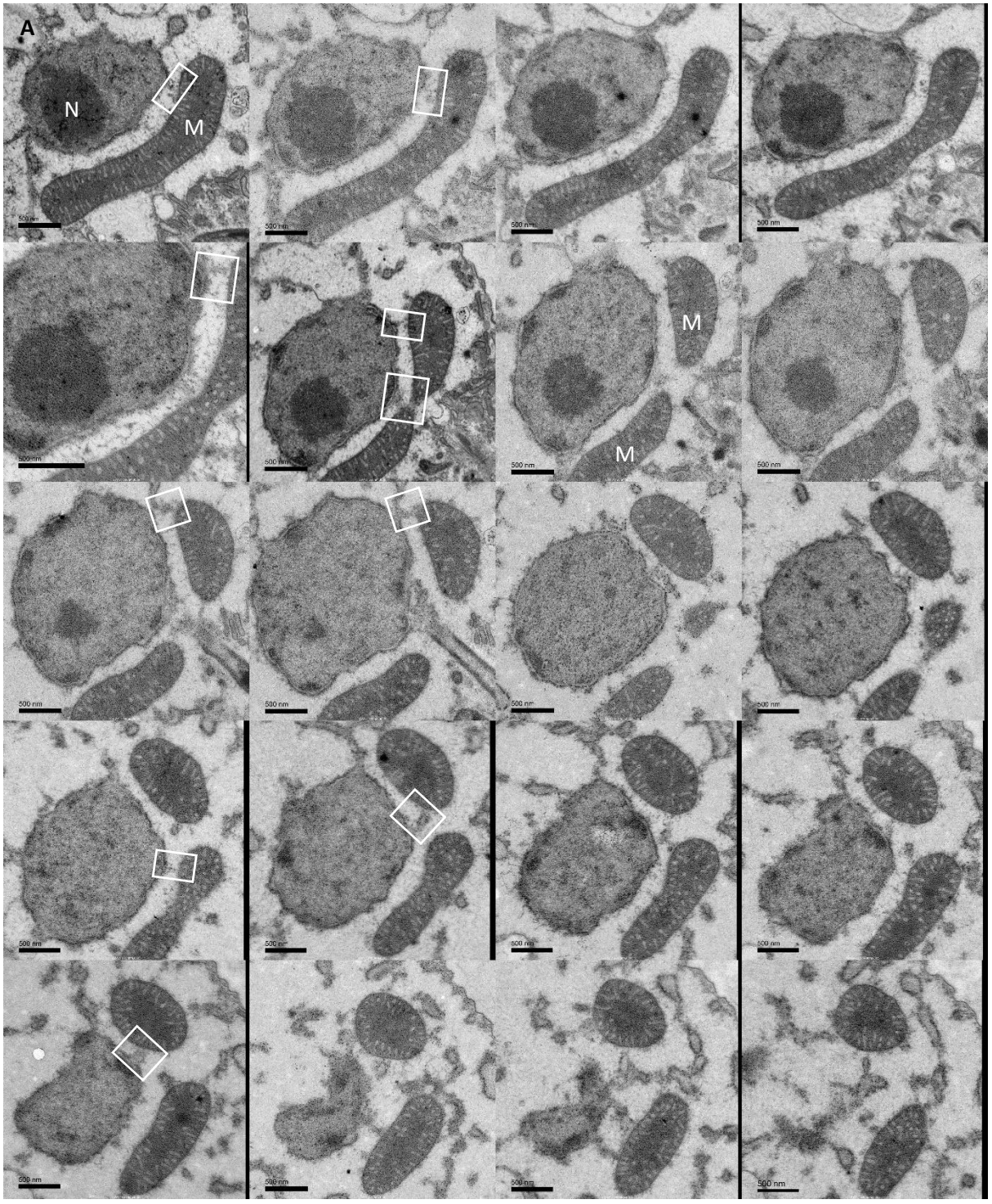

Figure 25 A. Serial sections of *Proteromonas*. Serial sections of *Proteromonas* used to investigate whether or not connections between mitochondrion and nucleus occurred at multiple points. They were fixed, stained, cut into 70 nm sections and observed under TEM 15K magnification. At certain points, connections (possibly microtubules) can be seen between the nucleus and the mitochondrion, and the nucleus clearly sites within a pocket formed by the mitochondrion. N, nucleus; M, mitochondrion; R, rhizoplast. The observed connections are shown in the white boxes.

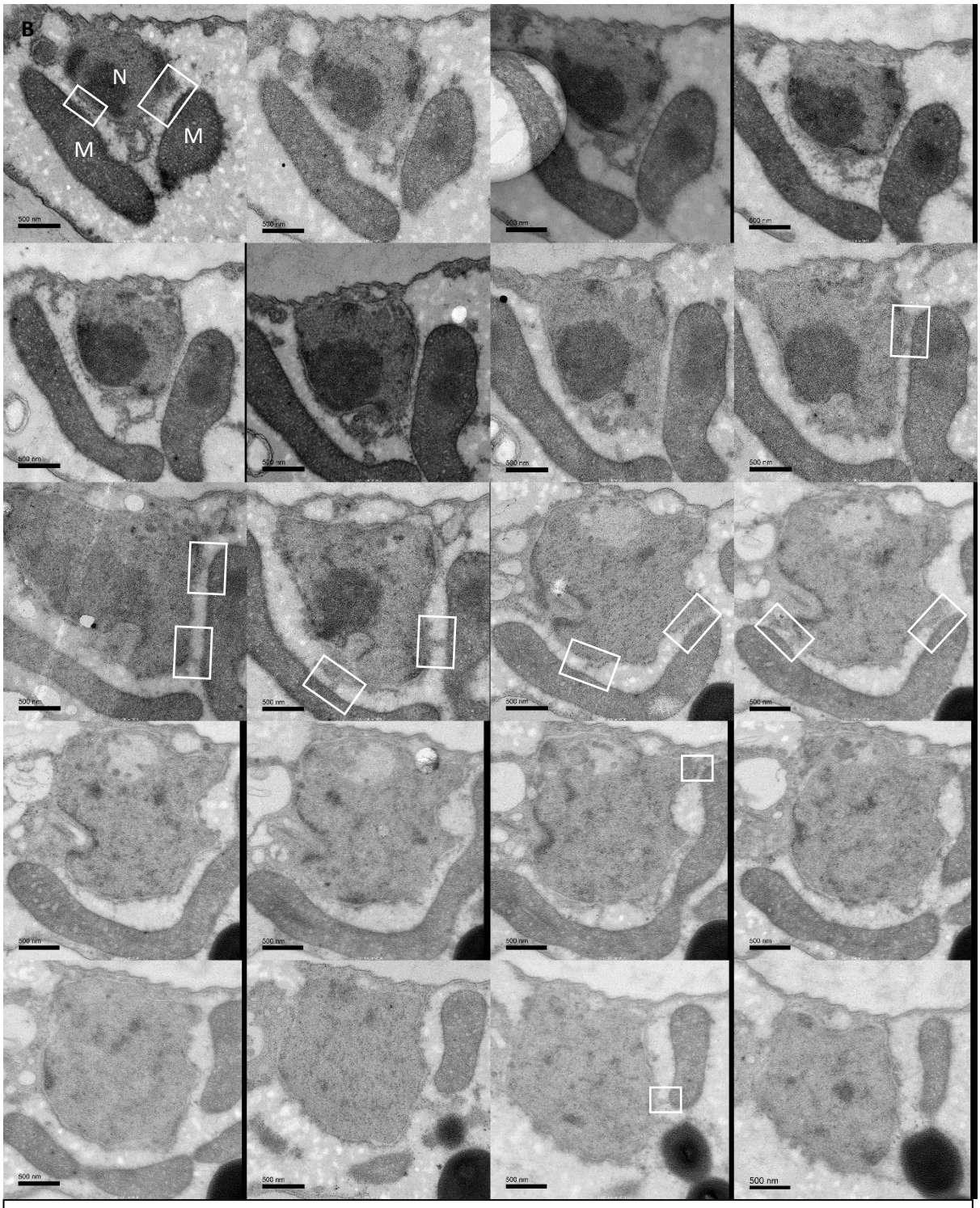

Figure 25 B. Serial sections of *Proteromonas*. Serial sections of *Proteromonas* used to investigate whether or not connection between mitochondrion and nucleus occurred at multiple points. They were fixed, stained, cut into 70 nm sections and observed under TEM 15K magnification. At certain points, connections (possibly microtubules) can be seen between the nucleus and the mitochondrion, and the nucleus clearly sites within a pocket formed by the mitochondrion. N, nucleus; M, mitochondrion. All possible connections here are shown in the white boxes.

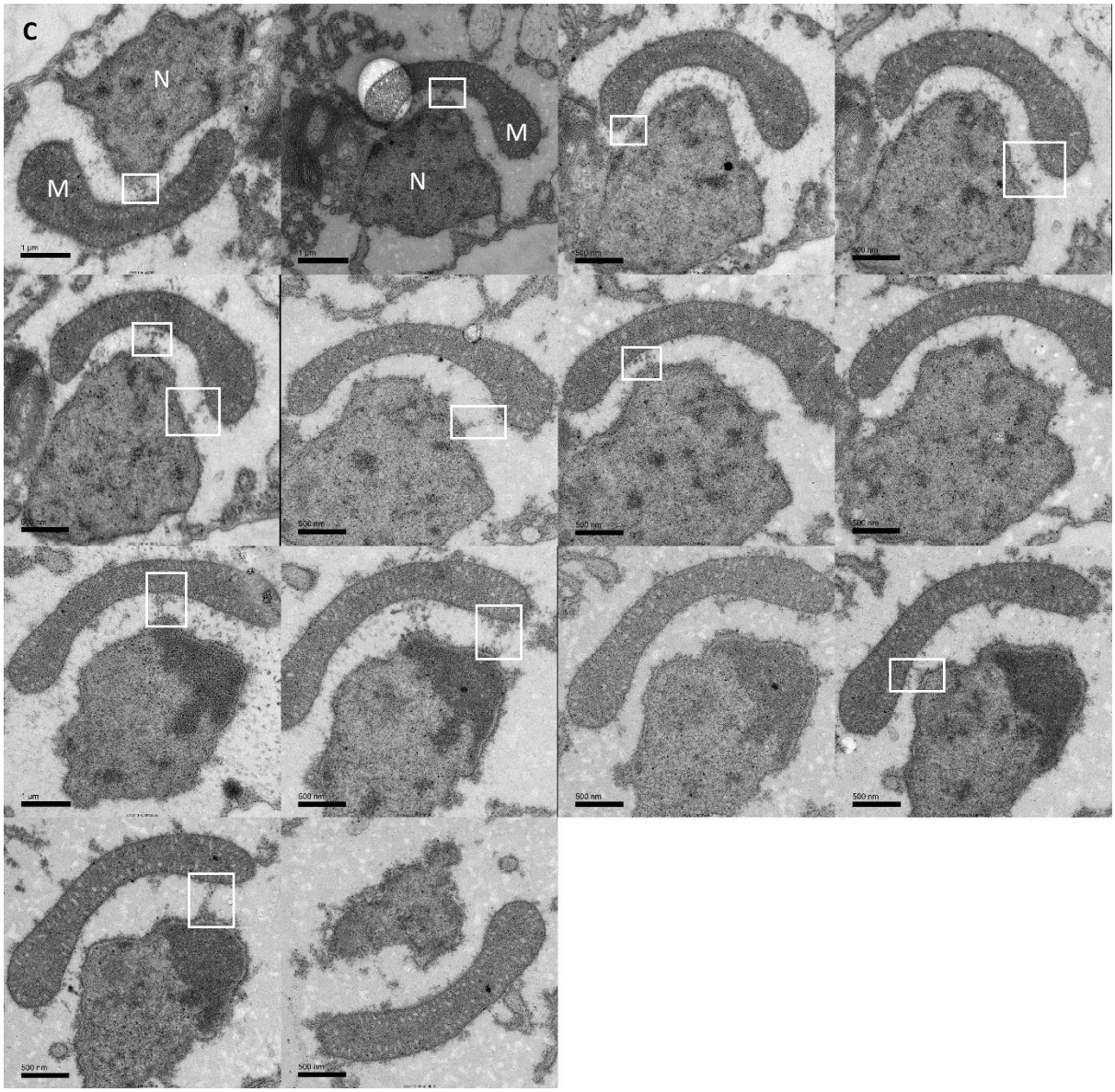

Figure 25 C. Serial sections of *Proteromonas*. Serial sections of *Proteromonas* used to investigate whether or not connection between mitochondrion and nucleus occurred at multiple points. They were fixed, stained, cut into 70 nm sections and observed under TEM 15K magnification. At certain points, connections (possibly microtubules) can be seen between the nucleus and the mitochondrion, and the nucleus clearly sites within a pocket formed by the mitochondrion. N, nucleus; M, mitochondrion. These observed connections are shown in the white boxes.

For many different proteins, confocal microscopy is not enough due to some antibodies lacking complete specificity, and some antibodies can bind to structures such as flagella etc. therefore, in order to confirm the protein presence a higher resolution is necessary such as immune EM, under which the structures bound by antibodies can be observed in more detail.

4.0 Discussion

4.1 General Analysis

From the beginning of this investigation, the main objective was to characterise the biochemical contents of the *Proteromonas* mitochondrion and compare it with *Blastocystis* (Table 1-18). This was carried out using bioinformatic analysis of the predicted proteome of *Proteromonas*, and was processed using BLASTP, a protein database. Over time, the results were collected and used to construct potential metabolic pathways within the mitochondrion, and in comparing it to *Blastocystis*, would allow us to see just how similar or different these organisms are biochemically, and gain some insight into the evolutionary history within their clade. Confirmation of their cellular location was performed through the use of TargetP 1.1, Mitoprot and Mitominer 4.0, although, given how the data within these databases concerns mainly yeast and plants, all were used in order to gain a more accurate idea as to whether these proteins could be mitochondrial.

Through analysis of this data, we can come to two main conclusions: The first is that, biochemically, *Proteromonas* and *Blastocystis* are very similar, sharing roughly 80% of their total mitochondrial protein content. Their similarity seems odd, given they differ drastically in two main ways. The first is the difference in their organellar morphologies, *Proteromonas* possessing a single, very large mitochondrion and *Blastocystis* with multiple, much smaller MROs, and their cellular morphologies, as *Blastocystis* has lost all the characteristic features of Stramenopiles. These features have been suggested to have been lost over time by *Blastocystis*, in a process called secondary loss, which is suggested to be a morphological reversion, in which the organism takes on a more primitive appearance, and parasites are especially susceptible to such changes (Clark. 1999). This loss of Stramenopile characteristics could potentially be a result of its habitat within larger organisms, but if this is the case, and this secondary loss of its flagella, mastigonemes and other features are a result of living within larger organism, then why does *Proteromonas* still resemble a Stramenopile? Is it's colonisation of reptiles a more recent event, and over time, will we see it turn into something more resembling *Blastocystis* or is the increased motility provided by flagella more vital to *Proteromonas* than it was to *Blastocystis*? Questions for which there are, at this moment in time, no answers. The second difference is that these organisms inhabit quite different environments, with *Proteromonas* living within the hindgut of cold blooded lizards and *Blastocystis* inhabiting the gastrointestinal tract of various different animals.

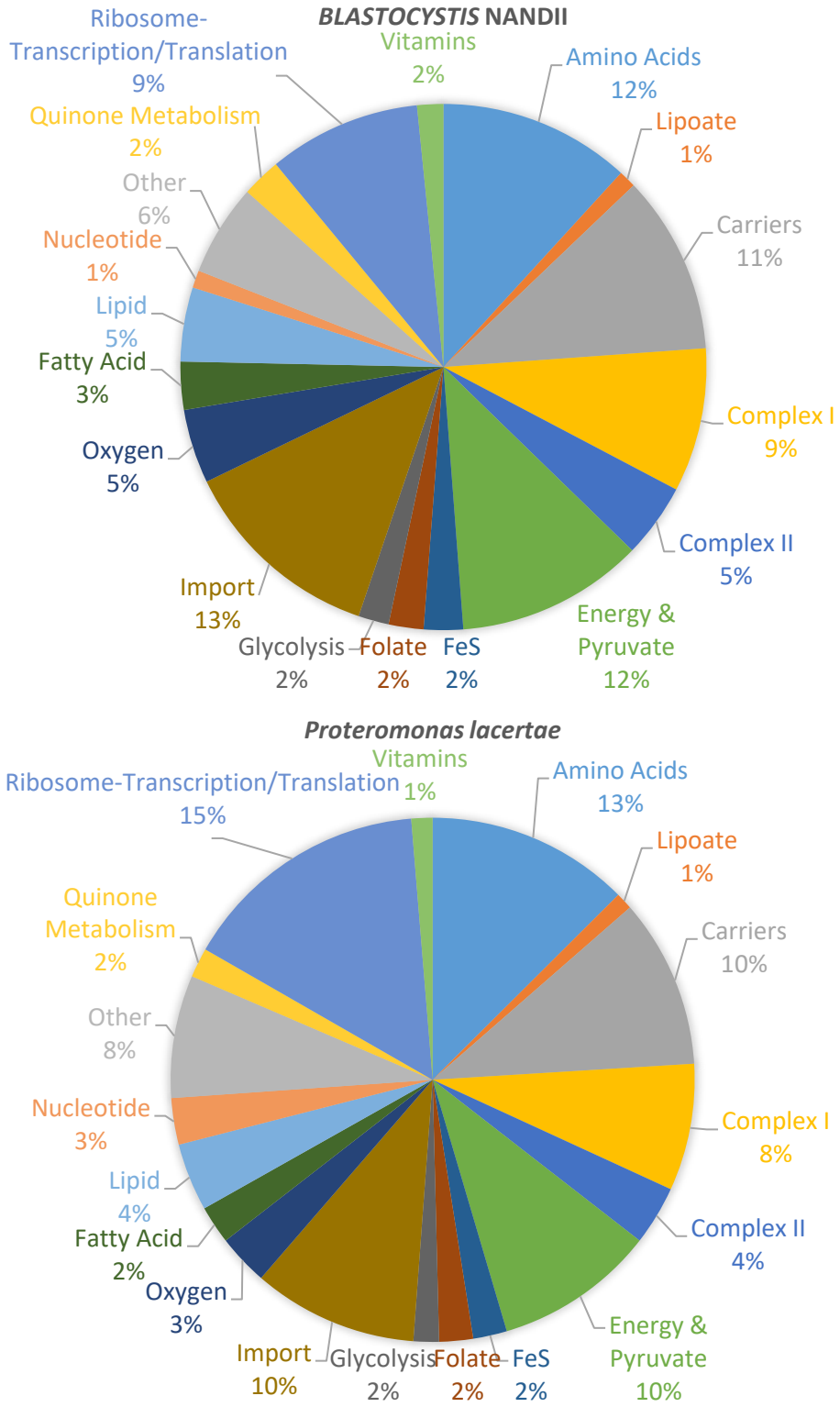

Figure 26. A comparison of protein percentages in *Blastocystis* and *Proteromonas*. A comparison between *Blastocystis* and *Proteromonas* with regards to the percentage of their total proteins each biochemical group makes up.

Of their total mitochondrial proteins, *Blastocystis* has been predicted to have 283 and we have predicted, following predicted sequence analysis, *Proteromonas* has 303, with a total of 229 shared proteins following comparative analysis. The main groups that set these two organisms apart is the import system for *Blastocystis*, while the numbers of proteins are not too different, the absence of

proteins from *Proteromonas* is what makes this result somewhat more significant. For *Proteromonas*, it is the ribosomal transcription and translation system, having 58 proteins, compared to 34 in *Blastocystis*. Given how the majority of their mitochondrial proteins are shared, 75% of *Proteromonas* proteins are shared by Blastocystis and 81% of *Blastocystis* proteins are shared *Proteromonas* (percentages differ as both organisms have slightly differing amounts of proteins (Figure 10 and 26)), it can be assumed that most of their biochemical pathways are shared as well (Figure 28), there are a few major differences, which will be discussed in detail later on.

4.2 The protein import System of *Proteromonas lacertae*

The mitochondrial protein import system is an integral part of the mitochondrial membranes, as it allows for the delivery of necessary proteins from the cytosol into either the membranes themselves, the intermembrane space or the matrix. In comparison to the mammalian import system, it is known that the import system of *Blastocystis* is quite significantly reduced, lacking many Tom proteins and certain SAM associated proteins (Gentekaki *et al.* 2017), yet, in spite of this reduction, *Blastocystis* still possesses some of the more vital import proteins, *Proteromonas* however, seems to be lacking. Unlike *Blastocystis*, *Proteromonas* has not been found to possess any proteins associated with the mitochondrial outer membrane import machinery, it lacks the pore, Tom40, it lacks Tom70, SAM50 and any other TOM or SAM associated proteins, which leaves us with the question: How does the mitochondria import proteins from the cytosol if there is no import machinery on the outer membrane? A difficult question to answer, since no other organism has been reported in literature to lack these proteins, and if they do lack import proteins, then they still typically possess the pores and the major components, therefore it is impossible to know for certain just how this affects the mitochondria and its delivery of proteins from the cytosol. While it cannot be said for certain why *Proteromonas* has lost the need for these outer membrane import proteins, looking at other mitochondrial features would allow us to speculate just why it has lost the need for protein import. As well as lacking TOM proteins, the mitochondrion also lacks many different inner membrane import proteins. It lacks Tiny Tims 8, 9, 10 and 13 which would be unnecessary if there was no outer membrane import as their main function is to act as chaperones, transporting proteins from Tom40 to Tim22 in *Blastocystis. Proteromonas* is also without a Tim22, but has a Tim23 and associated proteins (Tim44, Tim50, Pam18 but not Pam16 or Tim17) which allow the formation of a functional Tim complex, permitting the import of either proteins of RNA through the mitochondrial inner membrane. It has also been found that *Proteromonas* also has both an Oxa1 and IMP, both of which are essential in inserting proteins into the inner membrane.

When looking at the structure of the *Proteromonas* mitochondrion, it is shown to have a very close association with the nucleus (Figure 20 and 21) and, not only are they in close proximity to one another, but the mitochondrion seems to either fold or wrap around the nucleus, forming a

"pocket" of sorts that the nucleus resides inside of (Figures 16, 20 and 25 A, B, C). Following localisation of AOX using a mouse anti-AOX antibody, we can see how some mitochondrion not only form a pocket for the nucleus, but can coil around it as well (Figure 21). The need for this close association is not too clear. There are other organisms that have MROs close to their nucleus, such as *Malawimonas*, an Excavate, but the reason for this is not explained (Heiss *et al*. 2018). Through the use of TEM, we have seen that at random points on the *Proteromonas* mitochondrion, there seems to be a form of connection (Figures 24A-F), whether this connection is made by the outer membranes is unknown, and where this connection forms, the outer membranes of both the nucleus and the mitochondrion appear to be more fluid, as there is a lack of a defined membrane. Due to the random nature of this connection, we carried out serial sectioning of *Proteromonas* cells (Figure 25 A, B, C), to see whether mitochondrion and nucleus make contact at multiple points or if, in some cells it does not form at all. We observed these connections within nearly all cells examined, although in every cell where the connection exists, there is not always a lack of definition in the membranes and it does not always occur at multiple points. We have a few theories as to why this connection exists. Perhaps, the mitochondrion does not need to import proteins from the cytosol, and forms this connection, whether permanent or via some form of docking, in order to import RNA directly from the nucleus. Another feature that supports this is the ribosomal content of the mitochondrion. In comparison to *Blastocystis*, *Proteromonas* has nearly 80% more ribosomal proteins, which could be due to an increased need to translate more RNA into proteins, as well as this, it also over 40% more tRNAs than *Blastocystis*, again, potentially due to the increased need for protein translation.

While these features seem to support the absence of a complete import system, it is impossible to say for certain whether these observed features (the strange membrane fluidity, the ribosomes and tRNAs) are linked in any way and whether or not these are all adaptations that have occurred to make the acquisition of RNA from the nucleus more efficient. In order to gain more insight into these features, further experiments investigating them would have to be carried out, such as tagging certain mitochondrial outer membrane proteins, or tag the RNA itself. The ribosomes could also be investigated, to see whether they are travelling back and forth from the mitochondrion to the nucleus in order to collect RNA, as it is not definite that this observed connection is the mitochondrial and/or nuclear membrane, and could be a mass of shuttles transporting RNA. Another potential reason for the connections both large and small (given the slightly smaller connections between the organelles) could be microtubules. Given how the mitochondrion always seems to curve around the nucleus, perhaps there are microtubules acting as a tethering system to keep the organelles close. As well as this, they could serve as bridges, allowing the transfer of material form one organelle to the other.

4.3 The Electron Transport Chain

The electron transport chain in mammals and other aerobic organisms is a pathway responsible for the generation of a large amount of mitochondrial ATP, present within the mitochondrial inner membrane. Through the use of NADH and $FADH_2$, electrons are pumped into the intermembrane space, which will eventually pass through an ATP synthase, which will form ATP through the use of ADP+Pi. In mammals, the electron transport chain consists of five main complexes, complex I (NADH dehydrogenase) is the first complex, which removes H^+ from NADH to form NAD^+, after which an electron passes into the intermembrane space and so does a total of four H^+. The second complex, complex II, is succinate dehydrogenase. This protein does not have any part to play in the donation of protons into the intermembrane space, but in its conversion of $FADH_2$ to FAD^+, it donates electrons to ubiqinone, and along with the electrons donated by complex I, they are transferred to complex IV. Complex III, ubiquinol-cytochrome C oxidoreductase is a site of electron transfer from one quinone to another and, like complex I, is also responsible for donating $4H^+$ into the intermembrane space. The final complex, complex IV or cytochrome C oxidoreductase, catalyses the transfer of the electrons on the quinone to O_2, which acts as the terminal electron acceptor in

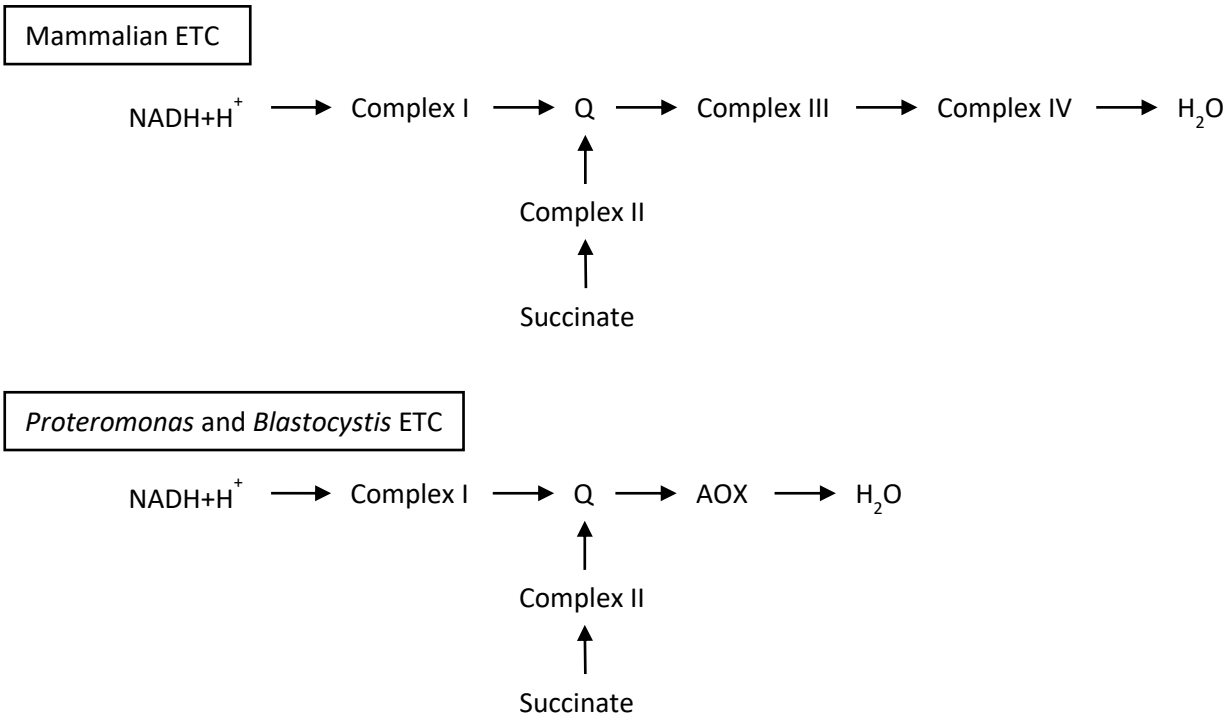

Figure 27. Electron transfer pathway in both mammalian ETC and *Proteromonas* and *Blastocystis* ETC. Simplified electron flow in the electron transport chains of mammals, *Proteromonas* and *Blastocystis* is similar, with major differences being due to the absence of complexes III and IV in *Proteromonas* and *Blastocystis*.

aerobic organisms, forming H_2O. Complex IV also donates $2H^+$ to the IMS. The $10H^+$ donated in this chain then pass through ATP synthase, forming 1 molecule per $3H^+$.

Anaerobic organisms can possess mitochondria, but there will be a few major differences, such as the terminal electron acceptor, since oxygen isn't always present, and whether the anaerobic mitochondria do or do not produce H^+. Hydrogenosomes are anaerobic mitochondria-related organelles present in a variety of anaerobic organisms such as trypanosomes, and are more reduced than canonical mitochondria, typically lacking features such as a genome or an electron transport chain. The MROs of *Blastocystis* fir somewhere in between a mitochondria and a hydrogenosome, since they are reduced by comparison to the mitochondria, but is more complex than a hydrogenosome in that it has a genome and an electron transport chain, albeit an incomplete one.

The electron transport chains of both *Blastocystis* and *Proteromonas* are nearly identical in composition (Figure 11). Complex I, NADH dehydrogenase, of both organisms is similar, in that *Proteromonas* has the same subunits as *Blastocystis* although *Blastocystis* has two more subunits at 11 compared to the 9 subunits in the *Proteromonas* complex. The second complex shared by the two organisms is succinate dehydrogenase is identical in both, and as a result, it is assumed that their activity is both the same, however, whether this complex functions as a succinate dehydrogenase or fumarate reductase is still unknown.

Blastocystis lacks the final three complexes in it electron transport chain, and in their place sits an alternative oxidase, a protein which functions similarly to complex IV, transferring electrons to the terminal electron acceptor, but is typically found in organisms that need to cope with oxygen stress, such as lose living in low oxygen environments. The presence of AOX was something that could be tested for in order to see whether the mitochondrial adaptations occurred prior to or following the diversification of *Proteromonas* and *Blastocystis*. The protein was first found through bioinformatics analysis, and from there was located using PCR of *Proteromonas* cDNA. The activity was tested, and therefore the presence of AOX, by investigating oxygen consumption over time in the presence of the AOX inhibitor SHAM (salicylhydroxamic acid), which acts by preventing the transfer of electrons from AOX to the terminal electron acceptor. *Proteromonas* is anaerobic, not obligately, living in an environment of low oxygen and as such can still use oxygen as its terminal electron acceptor. In the absence of SHAM, the cells live and function as normal, there is little to no loss in oxygen consumption and there is very little change when 0.5 mM and 1 mM SHAM is added, although we still see a small decrease in activity (Figure 23). It would appear that, following the addition of both 2.5 mM and 5 mM SHAM, there was a significant decrease in oxygen consumption, which was most likely a result of cells beginning to die. Theoretically, what would be seen if *Proteromonas* has a complex IV, would be either no change in oxygen consumption, since they function similarly and there would be no need to switch to AOX, or O_2 consumption would either stabilise or return to

normal levels, yet neither of these occurred, suggesting that there is no cytochrome C oxidoreductase activity, and *Proteromonas* is using exclusively AOX as the terminal electron acceptor. The electron transport chain of *Proteromonas*, to briefly summarise, is nearly identical to *Blastocystis* as it has a complex I, II and AOX but no complexes III, IV or V were found.

4.4 Glycolysis

The glycolytic proteins of *Blastocystis* are split into two main compartments, with the enzymes of the preparatory phase being located within the cytosol and the enzymes of the payoff phase, the main energy production stage, being located within the mitochondria (Bartulos *et al.*2018). *Proteromonas* possesses all of the same glycolysis enzymes as *Blastocystis*, with all of the enzymes present in the payoff phase having mitochondrial targeting sequences, as they did with *Blastocystis* and all of them having scores of between 0.87 and 0.98, indicating there is a high probability that these enzymes are located within the mitochondrion of *Proteromonas*. It can be suggested that *Proteromonas* also has the complete set of glycolytic proteins, due to that fact that only mitochondrial proteins were being investigated, none were found or recorded, although it seems unlikely that all preparatory phase enzymes would be missing. To identify whether *Proteromonas* has TPI-GAPDH, we had to run sequences for *Blstocystis*, *Phaedactophylum*, *Phytopthora infestans*, *Saccharina latissimi* found in "Mitochondrial targeting of glycolysis in a major lineage of eukaryotes" (Bartulos *et al.*2018) against the predicted sequences of *Proteromonas* and BLASTP those results. From the top hits in BLAST, it was found that, while *Proteromonas* has both TPI and GAPDH, it does not seem to have the fusion. GAPDH, due to the mitochondrial targeting sequence and score of between 0.98 and 1, can be suggested to be in the mitochondrion of *Proteromonas*, TPI however cannot, as it has no MTS and has a score no higher than 0.25, which is low. Whether this protein is cytosolic and GAPDH is mitochondrial is a possibility ad since TPI is part of the preparatory phase and GAPDH is part of the payoff phase, TPI not being mitochondrial supports the claim that the payoff phase proteins are mitochondrial (Figure 13).

4.5 Tricarboxylic Acid Cycle

The TCA cycle of *Blastocystis* is a reduced version of the standard mammalian one, possessing only half of the catalytic enzymes and therefore, required steps. *Proteromonas*, as with many of their metabolic functions, does not differ here either, in fact, their citric acid cycles are identical in protein composition. The same issue exists with *Proteromonas* as does with *Blastocystis*, in that the exact direction this cycle acts in is as of yet unknown and could act in a forward manner with oxaloacetate being the end product, or reverse with succinate being the end product. The method by which pyruvate is imported into the mitochondria of both *Proteromonas* and *Blastocystis* remains a mystery, as the main protein responsible for this is simply called mitochondrial pyruvate

carrier (Vanderperre *et al.* 2015) of the inner membrane, and none have been found in either organism, or any other pyruvate importer for that matter. Following arrival of pyruvate into the mitochondrion, the cycle (Figure 12) is the same in both *Proteromonas* and *Blastocystis*, they both have pyruvate:ferredoxin oxidoreductase, which converts the pyruvate into acetyl-CoA and CO_2 through oxidative decarboxylation (Furdui and Ragsdale, 2000). As well as having PFO, *Proteromonas* has, as previously mentioned, succinate dehydrogenase (succinate to fumarate), fumarate hydratase (fumarate to malate), malate dehydrogenase (malate to oxaloacetate) and pyruvate carboxylase (to convert pyruvate to oxaloacetate). Phosphoenolpyruvate carboxykinase, an enzyme that converts oxaloacetate into phosphoenolpyruvate, which can then be converted back to pyruvate by pyruvate kinase (however, this enzyme is cytosolic and was not found while looking for exclusively mitochondrial proteins).They both also have multiple subunits for the pyruvate dehydrogenase complex, both having PDH $E_1\alpha$ and $E_1\beta$, E_2, although no E_3 subunit was found in *Proteromonas* bioinformatically, either we did not find it, or the PDH of *Proteromonas* only has three subunits as opposed to four. Other than this difference, the TCA cycle of the two organisms appears to be the same.

4.6 Fe-S cluster biosynthesis

Fe-S cluster biosynthesis is an essential pathway within both the cytosol and MROs of nearly all eukaryotes and the iron sulphur clusters themselves are involved in various different pathways within the cell. *Proteromonas* and *Blastocystis* are no different, and in their mitochondrion and MROs respectively exists Fe-S cluster biosynthesis machinery. The first component of both the *Blastocystis* and *Proteromonas* machinery is NIfU-like protein, which is responsible for both the fixation of nitrogen and the maturation of Fe-S clusters (Hwang *et al.* 1996). Cysteine desulphurase and Frataxin are also both present in *Blastocystis*, yet in *Proteromonas*, no Frataxin has been identified. Frataxin is an iron chaperone, which delivers iron to the cluster being synthesised. An absence of frataxin suggests that iron transport to the Fe-S cluster would either be limited or stopped entirely, however, it has been suggested that glutaredoxin, which is found in the *Proteromonas* mitochondrion, is also an ionic iron and iron-sulphur cluster chaperone in the cytosol (Frey *et al.* 2016), and could presumably have the same function in the mitochondrion, although this is merely speculation. Frataxin not being found in Proteromonas could indicate that it is non-essential, and there are other proteins within the mitochondrion that are able to compensate. The other suggestion is that frataxin could possibly be present in the mitochondrion of *Proteromonas*, only it was not found through prediction or bioinformatics. Erv1, a FAD dependant sulfhydryl oxidase found in the intermembrane space, aids in both the import of cysteine rich proteins and the maturation of iron sulphur clusters (Ozer *et al.* 2015). Scaffold protein IscA1 is also a part of the *Proteromonas* ISC machinery, and while its exact function is not exactly clear, it is thought to

aid in the maturation of Fe-S clusters as well (Beilschmidt *et al.* 2017). *Proteromonas*, like *Blastocystis*, is also in possession of the fused SufCB protein, which was found through immunolocalisation and, also similarly to *Blastocystis,* is shown to localise within the cytosol (Figure 13) and due to its presence, it can be assumed that SufCB is also involved in Fe-S cluster maturation.

Iron sulphur cluster biogenesis machinery seems to be another feature of the mitochondrion that *Proteromonas* shares with *Blastocystis*, as the protein makeup of the two systems is nearly identical, with the only major difference being that frataxin has been found in *Blastocystis*, but not in *Proteromonas*. If *Proteromonas* has a protein that is able to compensate for frataxin is unknown, or whether frataxin is actually absent and was just not found through sequence predication has yet to be confirmed.

4.7 Peroxisomes

While the main focus of this investigation was the mitochondrion of *Proteromonas*, it was not the sole focus, we were also looking at whether or not peroxisomes are present within *Proteromonas*, since *Blastocystis* has been found to be without them.

Stramenopiles are a large and very diverse group of organisms, yet in spite of this diversity many of them seem to have peroxisomes, and peroxins such as Pex 1, 2, 5 and 10 are conserved within them (Gabaldon, Ginger and Michels, 2015). Five of the Stramenopiles shown with peroxisomes are parasitic, yet they are plant parasites, in comparison to apicomplexans, some of which are notorious animal parasites such as *Cryptosporidium parvum* and *Plasmodium falciparum* (Schlüter *et al.* 2006) and Excavates such as *Trichomonas vaginalis* have been shown to be without these conserved peroxins, and it can be suggested that they too do not have peroxisomes (Gabaldon, Ginger and Michels, 2015). It was shown that, due to not having these conserved proteins that *Blastocystis* lacks peroxisomes, a ubiquitous organelle responsible for ROS management, fatty acid oxidation and many other functions. Without peroxisomes, it could be assumed that organisms either lose these functions or relocate them to other compartments within the cell, such as the mitochondria or the cytosol. In terms of maintaining ROS levels, *Blastocystis* has an AOX, another protein which acts to prevent ROS over production (Zarsky and Tachezy, 2015). Also, parasitic organisms could simply use host metabolic pathways such as those involved in fatty-acid metabolism.

Pex19, a cytosolic peroxisome protein (Figure 18) that binds to multiple different peroxisomal membrane proteins, directing them to the appropriate place within the cell and organising the peroxisomes and when interaction with Pex16p, aids in peroxisomal membrane biogenesis. Pex19 was the target in finding peroxisomes in *Proteromonas*, and using an anti-Pex19 antibody, it was found using both fixation and confocal microscopy and western blotting. The initial result seemed unusual at first, as the 700bp band and sequence length indicated the protein should be roughly

26kDa in size, but it was found that Pex19 is actually dimeric (Hadden et al. 2006), explaining why the resulting band on the blot was double the size. The presence of Pex19 indicates that *Proteromonas* does indeed possess peroxisomes due to the importance of this specific peroxin. Pex19 has been shown in the cytosol of *Proteromonas*, and confirms that unlike *Blastocystis*, that *Proteromonas* is in possession of peroxisomes, suggesting that this is another feature *Blastocystis* has lost via secondary loss. In order to confirm whether *Proteromonas* has peroxisomes, they would need to be looked at in a higher resolution through immune EM.

Proteromonas has also been predicted to possess three other peroxisomal genes (Table 19.), these genes being for Pex4, a cytosolic and peroxisomal membrane protein which acts as a docking receptor; Pex10, a peroxisomal membrane protein which also acts as a docking receptor and Pex23, a peroxisomal membrane protein that regulates both the size and number of peroxisomes. Alongside Pex19, investigating these proteins would allow either the presence or absence of peroxisomes to be confirmed. Confirmation using these is necessary as the Pex19 data is weak at best, as the blot is not too clear and the microscopy data simply shows that our antibody bound to something. So, in order to improve the data on peroxisomal presence in *Proteromonas* these other proteins would need to be found as well.

5.0 Conclusions

Although they are closely related, *Blastocystis* and *Proteromonas* appear to be two very different organisms. When comparing their morphology, there is little to no resemblance, with *Proteromonas* having retained the features of other Stramenopiles, and *Blastocystis* having lost them in a process known as secondary loss. As expected, the internal layout differs about as much as the external, given then shape of the cells, however, the internal contents of the cells bear some, albeit not much, similarity to one another. They both have a single nucleus, and other standard organelles such as golgi, ER etc. While *Blastocystis* possesses an enormous vacuole, *Proteromonas* has multiple in the anterior portion of the cell, meaning that, similarly to *Blastocystis*, the cell is largely empty space and the function of these vacuoles is unknown. Their mitochondria are drastically different in terms of morphology, with *Blastocystis* having multiple, smaller, MROs and *Proteromonas* with one, very large mitochondrion that can be lobed and seems to always fold around the nucleus, forming a pocket for the nucleus. Surprisingly, they are nearly identical in terms of their mitochondrial biochemistry, sharing roughly 90% of their mitochondrial proteins and from this it can be assumed that their biochemical pathways are similar of not the same, differing only when specific proteins are not present. They possess the same ETC layout, the same TCA cycle proteins, similar pyruvate metabolism proteins and proteins involved in quinone metabolism etc. A simple summary of the pathways in the *Proteromonas* mitochondrion is shown in Figure 29.

The most significant difference between the two is the import system, which as previously discussed, appears to be missing in the *Proteromonas* mitochondrial outer membrane. The mitochondrial inner membrane proteins are similar in their organisation, with *Proteromonas* still being slightly more reduced than *Blastocystis*. How *Proteromonas* imports proteins is something that can be speculated, but at the moment the real mechanism is unknown. It could import RNA itself through a form of docking with the nuclear outer membrane, or at certain positions could be fused. Chaperones, transporters or ribosomes could move the RNA from the nucleus to the mitochondrion or RNA could be jettisoned towards the mitochondria. All that is known is that at certain positions on the mitochondrial outer membrane connections, whether temporary or permanent have been seen and that *Proteromonas* lacks TOM proteins. Another mentioned explanation which could also support the association between the organelles and the absence of import was microtubules, which could act as tethers to keep the organelles close and as bridges to assist in the transport of material from the nucleus to the mitochondria and vice versa.

Another feature discovered is the presence of peroxisomes, where *Blastocystis* has none, through the characterisation and localisation of the Pex19 protein, a major peroxin involved in peroxisome membrane biogenesis and docking.

In summary, despite their significant morphological differences, the biochemical make-up of their mitochondria is nearly identical, and it can be suggested that the mitochondrial adaptations occurred prior to these two organisms diversifying, with their separate alterations coming later on. The same can be said for peroxisomes, that they were lost by *Blastocystis* after this point in a form of metabolic streamlining. Their reason for peroxisomes being retained by *Proteromonas* could possibly be due to their role in coping with ROS production. It is known that AOX plays a role in preventing oxidative damage, and perhaps, due to *Proteromonas* favouring somewhat anoxic conditions, peroxisomes could also play a major role in maintaining ROS production and preventing or reducing the damage they cause.

Future Works

Potential future works following this investigation include the localisation of multiple different proteins in order to confirm their presence within the mitochondrion of *Proteromonas*. For instance, the payoff phase enzymes of glycolysis, the presence of MTS is enough to theorise the proteins belong in the mitochondrion, but to confirm this, they would need to be investigated under fluorescence/confocal microscopy or under TEM. In order to localise with greater specificity, the production of monoclonal antibodies is necessary, therefore successful protein expression should be attempted. As well as protein localisation, biochemical characterisation of proteins such as succinate dehydrogenase, by looking at its activity and whether it acts as a fumarate reductase or a succinate dehydrogenase in order to see which way to TCA cycle goes, whether it acts in reverse or in the same way as mammalian TCA cycle. The contact between the membranes of mitochondrion and nucleus should be investigated, in order to elucidate what exactly this connection is, by tagging RNA, ribosomes etc. As well as this, the use of antibodies specific to microtubule proteins could be used in order to investigate whether they are responsible for the mitochondrial shape and closeness to the nucleus as well as the connections. Use of immune EM to generate higher resolution images of antibody binding to specific proteins, such as Pex19 or SufCB, to confirm they are binding correctly due to polyclonal antibodies having some degree of non-specificity. Finally, now that *Proteromonas* and *Blastocystis* mitochondrial proteins have been compared to one another, they could both potentially be compared to *Cafeteria roenbergensis,* the next organism within their clade (Figure 3), to see whether features such as the import system were lost just by *Proteromonas* or by both *Proteromonas* and *Blastocystis* and somehow reacquired by *Blastocystis* later on. Finally, another series of experiments to attempt would be to see the effect of protein insertion, mutation and deletion through the use of the CRISPR/Cas9 or RNA transfection system.

Figure 29 below shows a simple summary of the major biochemical pathways within the mitochondrion of *Proteromonas*.

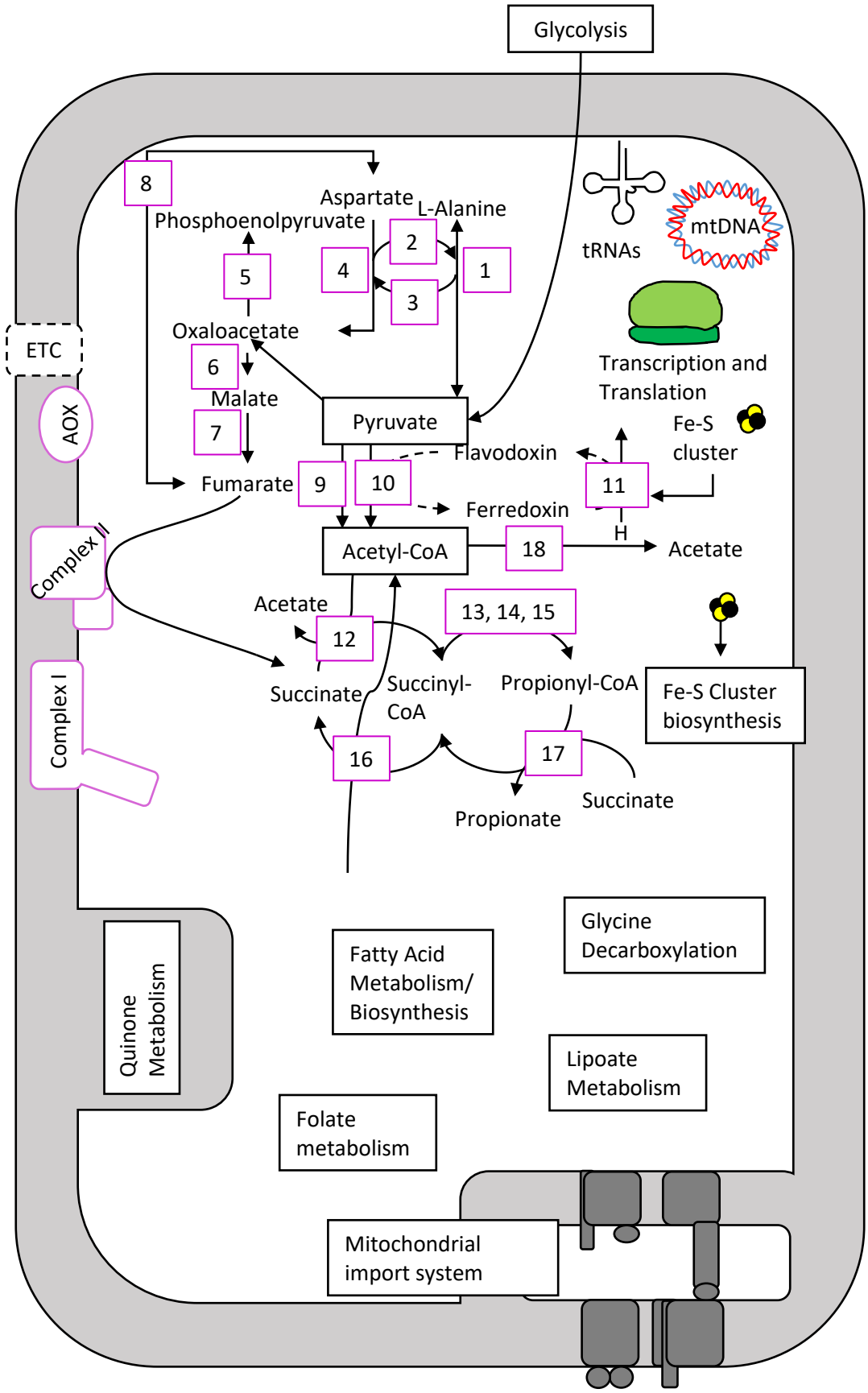

Figure 28. Major biochemical pathways in *Proteromonas* mitochondrion. A summary of the biochemical pathways in the mitochondrion of *Proteromonas.*

Table 19. Figure 28 proteins	
No.	**protein**
1	Alanine aminotransferase
2	α-ketoglutarate
3	L-Glutamate
4	Aspartate aminotransferase
5	Phosphoenolpyruvate carboxykinase
6	Malate dehydrogenase
7	Fumarate hydratase
8	Aspartate ammonia lyase
9	Pyruvate dehydrogenase
10	Pyruvate:ferredoxin oxidoreductase
11	Fe-Fe Hydrogenase
12	Acetate:succinate CoA transferase
13	Methylmalonyl-CoA mutase
14	Methylmalonyl-CoA epimerase
15	Propionyl-CoA carboxylase β
16	Succinyl-CoA synthetase
17	Acetate:succinate CoA transferase
18	rSAM-HydE

References

Bártulos, C., Rogers, M., Williams, T., Gentekaki, E., Brinkmann, H., Cerff, R., Liaud, M., Hehl, A., Yarlett, N., Gruber, A., Kroth, P. and van der Giezen, M. (2018). Mitochondrial glycolysis in a major lineage of eukaryotes. *Genome Biology and Evolution*.

Beilschmidt, L. *et al.* (2017). ISCA1 is essential for mitochondrial Fe4S4 biogenesis in vivo. *Nature Communications*, 8, p.15124.

Berg, J., Tymoczko, J., Gatto, G. and Stryer, L. (2002). *Biochemistry*. 5th ed. New York: W.H.Freeman and Co., pp.Chapter 18.3.1.

Bolender, N. *et al.* (2008). Multiple pathways for sorting mitochondrial precursor proteins. *EMBO reports*, 9(1), pp.42-49.

Braymer, J. and Lill, R. (2017). Iron–sulfur cluster biogenesis and trafficking in mitochondria. Journal of Biological Chemistry, 292(31), pp.12754-12763.

Brugerolle, G. and Bardele, C. (1988). Cortical cytoskeleton of the flagellate Proteromonas lacertae: Interrelation between microtubules, membrane and somatonemes. *Protoplasma*, 142(1), pp.46-54.

Cardol, P. (2011). Mitochondrial NADH:ubiquinone oxidoreductase (complex I) in eukaryotes: A highly conserved subunit composition highlighted by mining of protein databases. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1807(11), pp.1390-1397.

Clark, C. (1999). The Effect of Secondary Loss on Our Views of Eukaryotic Evolution. The Biological Bulletin, 196(3), pp.385-388.

Dellibovi-Ragheb, T., Gisselberg, J. and Prigge, S. (2013). Parasites FeS Up: Iron-Sulfur Cluster Biogenesis in Eukaryotic Pathogens. PLoS Pathogens, 9(4), p.e1003227.

Denoeud, F. *et al.* (2011). Genome sequence of the stramenopile Blastocystis, a human anaerobic parasite. *Genome Biology*, 12(3), p.R29.

Dolezal, P. *et al.* (2005). Giardia mitosomes and trichomonad hydrogenosomes share a common mode of protein targeting. *Proceedings of the National Academy of Sciences*, 102(31), pp.10924-10929.

Dunn, Jr. *et al.* (2005). Pexophagy: The Selective Autophagy of Peroxisomes. Autophagy, 1(2), pp.75-83.

Dyall, S., Brown, M. and Johnson, P. (2004). Ancient Invasions: From Endosymbionts to Organelles. *Science*, 304(5668), pp.253-257.

Efremov, R., Baradaran, R. and Sazanov, L. (2010). The architecture of respiratory complex I. *Nature*, 465(7297), pp.441-445.

Elthon, T., Nickels, R. and McIntosh, L. (1989). Monoclonal Antibodies to the Alternative Oxidase of Higher Plant Mitochondria. PLANT PHYSIOLOGY, 89(4), pp.1311-1317.

Embley, T. *et al.* (2003). Hydrogenosomes, Mitochondria and Early Eukaryotic Evolution. *IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)*, 55(7), pp.387-395.

Endo, T., Yamano, K. and Kawano, S. (2011). Structural insight into the mitochondrial protein import system. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1808(3), pp.955-970.

Fransen, M. (2012). Peroxisome Dynamics: Molecular Players, Mechanisms, and (Dys)functions. ISRN Cell Biology, 2012, pp.1-24.

Fransen, M. *et al*. (2012). Role of peroxisomes in ROS/RNS-metabolism: Implications for human disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, 1822(9), pp.1363-1373.

Frey, A., Palenchar, D., Wildemann, J. and Philpott, C. (2016). A Glutaredoxin·BolA Complex Serves as an Iron-Sulfur Cluster Chaperone for the Cytosolic Cluster Assembly Machinery. *Journal of Biological Chemistry*, 291(43), pp.22344-22356.

Furdui, C. and Ragsdale, S. (2000). The Role of Pyruvate Ferredoxin Oxidoreductase in Pyruvate Synthesis during Autotrophic Growth by the Wood-Ljungdahl Pathway. *Journal of Biological Chemistry*, 275(37), pp.28494-28499.

Gabaldón, T. and Huynen, M. (2004). Shaping the mitochondrial proteome. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1659(2-3), pp.212-220.

Gabaldón, T. and Pittis, A. (2015). Origin and evolution of metabolic sub-cellular compartmentalization in eukaryotes. *Biochimie*, 119, pp.262-268.

Gabaldón, T., Ginger, M. and Michels, P. (2016). Peroxisomes in parasitic protists. *Molecular and Biochemical Parasitology*, 209(1-2), pp.35-45.

Gentekaki, E. *et al.* (2017). Extreme genome diversity in the hyper-prevalent parasitic eukaryote Blastocystis. *PLOS Biology*, 15(9), p.e2003769.

Goodman, J. (2005). Structure, Function and Biogenesis of Peroxisomes. Encyclopedia of Molecular Cell Biology and Molecular Medicine, 13(2), pp.609-626.

Gray, M. (2015). Mosaic nature of the mitochondrial proteome: Implications for the origin and evolution of mitochondria. *Proceedings of the National Academy of Sciences*, 112(33), pp.10133-10138.

Hadden, D., Phillipson, B., Johnston, K., Brown, L., Manfield, I., El-Shami, M., Sparkes, I. and Baker, A. (2006). ArabidopsisPEX19 is a dimeric protein that binds the peroxin PEX10. *Molecular Membrane Biology*, 23(4), pp.325-336.

Hamblin, K., Standley, D., Rogers, M., Stechmann, A., Roger, A., Maytum, R. and van der Giezen, M. (2008). Localization and nucleotide specificity ofBlastocystissuccinyl-CoA synthetase. *Molecular Microbiology*, 68(6), pp.1395-1405.

Heiss, A. *et al.* (2018). Combined morphological and phylogenomic re-examination of malawimonads, a critical taxon for inferring the evolutionary history of eukaryotes. *Royal Society Open Science*, 5(4), p.171707.

Hildenbeutel, M. *et al.* (2012). The Membrane Insertase Oxa1 Is Required for Efficient Import of Carrier Proteins into Mitochondria. *Journal of Molecular Biology*, 423(4), pp.590-599.

Hwang, D., Dempsey, A., Tan, K. and Liew, C. (1996). A modular domain of NifU, a nitrogen fixation cluster protein, is highly conserved in evolution. *Journal of Molecular Evolution*, 43(5), pp.536-540.

Karnkowska, *et al.* (2016). A Eukaryote without a Mitochondrial Organelle. *Current Biology*, 26(10), pp.1274-1284.

Kulawiak, B. *et al.* (2013). The mitochondrial protein import machinery has multiple connections to the respiratory chain. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, 1827(5), pp.612-626.

Lantsman, Y., Tan, K., Morada, M. and Yarlett, N. (2008). Biochemical characterization of a mitochondrial-like organelle from Blastocystis sp. subtype 7. *Microbiology*, 154(9), pp.2757-2766.

Leipe, D. *et al.* (1996). 16S-like rDNA sequences from Developayella elegans, Labyrinthuloides haliotidis, and Proteromonas lacertae confirm that the stramenopiles are a primarily heterotrophic group. *European Journal of Protistology*, 32(4), pp.449-458.

McDonald, A. and Vanlerberghe, G. (2004). Branched Mitochondrial Electron Transport in the Animalia: Presence of Alternative Oxidase in Several Animal Phyla. *IUBMB Life (International Union of Biochemistry and Molecular Biology: Life)*, 56(6), pp.333-341.

McDonald, A., Vanlerberghe, G. and Staples, J. (2009). Alternative oxidase in animals: unique characteristics and taxonomic distribution. *Journal of Experimental Biology*, 212(16), pp.2627-2634.

Modica-Napolitano, J. and Singh, K. (2002). Mitochondria as targets for detection and treatment of cancer. *Expert Reviews in Molecular Medicine*, 4(09).

Muller, M. *et al.* (2012). Biochemistry and Evolution of Anaerobic Energy Metabolism in Eukaryotes. *Microbiology and Molecular Biology Reviews*, 76(2), pp.444-495.

Nelson, D., Cox, M. and Lehninger, A. (2013). Lehninger principles of biochemistry. 6th ed. Basingstoke: Macmillan, pp.544-588 (glycolysis), 633-656 (TCA cycle), 731-754 (ETC).

Ozer, H. *et al.* (2015). Cytosolic Fe-S Cluster Protein Maturation and Iron Regulation Are Independent of the Mitochondrial Erv1/Mia40 Import System. *Journal of Biological Chemistry*, 290(46), pp.27829-27840.

Pennisi, R. *et al.* (2016). Molecular Evolution of Alternative Oxidase Proteins: A Phylogenetic and Structure Modeling Approach. Journal of Molecular Evolution, 82(4-5), pp.207-218.

Perez-Brocal, V., Shahar-Golan, R. and Clark, G. (2010). A Linear Molecule with Two Large Inverted Repeats: The Mitochondrial Genome of the Stramenopile Proteromonas lacertae. *Genome Biology and Evolution*, 2(0), pp.257-266.

Perry, R., Mast, F. and Rachubinski, R. (2009). Endoplasmic Reticulum-Associated Secretory Proteins Sec20p, Sec39p, and Dsl1p Are Involved in Peroxisome Biogenesis. Eukaryotic Cell, 8(6), pp.830-843.

Pyrih, J. *et al.* (2016). Minimal cytosolic iron-sulfur cluster assembly machinery of Giardia intestinalisis partially associated with mitosomes. Molecular Microbiology, 102(4), pp.701-714.

Richly, E., Chinnery, P. and Leister, D. (2003). Evolutionary diversification of mitochondrial proteomes: implications for human disease. *Trends in Genetics*, 19(7), pp.356-362.

Roger, A., Muñoz-Gómez, S. and Kamikawa, R. (2017). The Origin and Diversification of Mitochondria. *Current Biology*, 27(21), pp.R1177-R1192.

Rutter, J., Winge, D. and Schiffman, J. (2010). Succinate dehydrogenase – Assembly, regulation and role in human disease. *Mitochondrion*, 10(4), pp.393-401.

Schatz, G. (1996). The Protein Import System of Mitochondria. *Journal of Biological Chemistry*, 271(50), pp.31763-31766.

Schlüter, A. *et al.* (2006). The Evolutionary Origin of Peroxisomes: An ER-Peroxisome Connection. *Molecular Biology and Evolution*, 23(4), pp.838-845.

Silberman, J., Sogin, M., Leipe, D. and Clark, C. (1996). Human parasite finds taxonomic home. Nature, 380(6573), pp.398-398.

Stechmann, A. *et al.* (2008). Organelles in Blastocystis that Blur the Distinction between Mitochondria and Hydrogenosomes. *Current Biology*, 18(8), pp.580-585.

Tan, K. (2008). New Insights on Classification, Identification, and Clinical Relevance of Blastocystis spp. *Clinical Microbiology Reviews*, 21(4), pp.639-665.

Tielens, A., Rotte, C., van Hellemond, J. and Martin, W. (2002). Mitochondria as we don't know them. Trends in Biochemical Sciences, 27(11), pp.564-572.

Timmis, J. *et al.* (2004). Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nature Reviews Genetics*, 5(2), pp.123-135.

Tsaousis, A. *et al.* (2008). A novel route for ATP acquisition by the remnant mitochondria of Encephalitozoon cuniculi. Nature, 453(7194), pp.553-556.

Tsaousis, A. *et al.* (2010). A Functional Tom70 in the Human Parasite Blastocystis sp.: Implications for the Evolution of the Mitochondrial Import Apparatus. *Molecular Biology and Evolution*, 28(1), pp.781-791.

Tsaousis, A. *et al.* (2010). *Anaerobic Parasitic Protozoa- Genomics and Molecular Biology*. Chapter 9: The Blastocystis Mitochondrion-like Organelles. Norfolk: Caister Academic Press, pp.205-215.

Tsaousis, A. *et al.* (2012). Evolution of Fe/S cluster biogenesis in the anaerobic parasite Blastocystis. Proceedings of the National Academy of Sciences, 109(26), pp.10426-10431.

Van Vranken, J. *et al.* (2014). Protein-mediated assembly of succinate dehydrogenase and its cofactors. *Critical Reviews in Biochemistry and Molecular Biology*, 50(2), pp.168-180.

Vanderperre, B., Bender, T., Kunji, E. and Martinou, J. (2015). Mitochondrial pyruvate import and its effects on homeostasis. *Current Opinion in Cell Biology*, 33, pp.35-41.

Vanlerberghe, G. (2013). Alternative Oxidase: A Mitochondrial Respiratory Pathway to Maintain Metabolic and Signaling Homeostasis during Abiotic and Biotic Stress in Plants. *International Journal of Molecular Sciences*, 14(4), pp.6805-6847.

Vishwakarma, A. *et al.* (2015). Importance of the alternative oxidase (AOX) pathway in regulating cellular redox and ROS homeostasis to optimize photosynthesis during restriction of the cytochrome oxidase pathway in Arabidopsis thaliana. *Annals of Botany*, 116(4), pp.555-569.

Wanders, R., Waterham, H. and Ferdinandusse, S. (2016). Metabolic Interplay between Peroxisomes and Other Subcellular Organelles Including Mitochondria and the Endoplasmic Reticulum. Frontiers in Cell and Developmental Biology, 3.

Wang, Z. and Wu, M. (2014). Phylogenomic Reconstruction Indicates Mitochondrial Ancestor Was an Energy Parasite. *PLoS ONE*, 9(10), p.e110685.

Warnock, D., Fahy, E. and Taylor, S. (2004). Identification of protein associations in organelles, using mass spectrometry-based proteomics. *Mass Spectrometry Reviews*, 23(4), pp.259-280.

Wiedemann, N. and Pfanner, N. (2017). Mitochondrial Machineries for Protein Import and Assembly. *Annual Review of Biochemistry*, 86(1), pp.685-714.

Žárský, V. and Tachezy, J. (2015). Evolutionary loss of peroxisomes – not limited to parasites. *Biology Direct*, 10(1).

Zhang, X. *et al.* (2011). Ultrastructural insights into morphology and reproductive mode of Blastocystis hominis. *Parasitology Research*, 110(3), pp.1165-1172.