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Isolation of intact plastids of the secondary alga *Chromera velia* and treatment of the alga with rifampicin

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Annotation:

Photosynthetic organism *Chromera velia* was discovered recently. Thanks to the fact that it is harmless algae but close relative to apicomplexan parasites it could be used as a helpful-tool for basic research. Apicoplast serves as a target of some antimalarial agents, so my first task was to isolate pure fraction of plastid organelle. And next to apply antibiotic rifampicin at the same doses that are working in *Plasmodium falciparum* to see whether it would be possible to use *C. velia* as a model organism in first screening of new antimarial drugs.

Poděkování:
Ráda bych poděkovala v první řadě školiteli, Mírovi Oborníkovi za šanci pracovat na takovém projektu a za obrovskou pomoc a trpělivost při realizaci. Dále své rodině, která mě vše umožnila. A nakonec všem, kteří mě pomohli radou i praktickou pomocí: členům a kamarádům z laboratoří Molekulární taxonomie a Molekulární biologie prvoků za cenné rady a neutuchající dobrou náladu! Dále Martinovi Lukešovi, za rady a ochotu, se kterou mi velmi pomohl s isolací plastidů a gradientovou centrifugací, Lachymu a týmu prof. O. Prášilovi, kteří mě umožnili pracovat v Třeboni. Členům laboratoře Transmisní elektronové mikroskopie, za pomoc a rady při zpracování vzorků, hlavně Léně. A nakonec Marti z laboratoře Franty Marce za pomoc a trpělivost u fluorescenčního mikroskopu.

Prohlašuji, že svoji diplomovou práci jsem vypracoval/a samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury.

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INTRODUCTION

1. TREE OF EUKARYOTES

Current understanding of eukaryotic diversity leads to classification of eukaryotes into six hypothetic supergroups: Opisthokonta, Amoebozoa, Rhizaria, Excavata, Chromalveolata, and Archaeplastida (Yoon et al. 2008; Reeb et al. 2009). These six putative supergroups show complex and their histories are not easy to read. The molecular phylogenetic support for these supergroups is highly variable (Parfrey et al. 2006). It can vary from moderate to strong support depending on different phylogenetic analyses, gene and taxon sampling (Yoon et al. 2008). Early molecular analyses have often suffered from either a broad taxon sampling using only single-gene data or have used multigene data with a limited sample of taxa (Baldauf 2003; Keeling 2005; Yoon et al. 2008). New analyses with taxon-rich data indicate that this six-supergroups hypothesis is likely premature (Yoon et al. 2008). The knowledge of the microbial lineages is the key to relationships among Eukaryotes, because the vast majority of eukaryotic diversity is hidden in the protist world.

In this thesis the supergroup Chromalveolata represents the target of interest. The Chromalveolate hypothesis was initially proposed by Cavalier-Smith (Cavalier-Smith 2002) and is defined by a single secondary endosymbiosis in which putative common ancestor of the whole supergroup engulfed a red alga that became a plastid, that was independently lost in some lineages. Chromalveolates consist of six phyla, traditionally divided into two subgroups: Alveolata (Ciliophora, Dinozoa, and Apicomplexa) and Chromista (Haptophyta, Cryptophyceae, and Stramenopiles). However this traditional classification has changed; phylogenetic analyses of nuclear proteins showed that the Stramenopiles form a group with Alveolata, and the cryptophytes and haptophytes stay together as a monophyletic group (Hackett et al. 2007; Patron et al. 2007), and surprisingly Rhizaria clade are positioned within Chromalveolata (Hackett et al. 2007). Recent multigene nuclear non-plastid targeted protein analyses place chromalveolates into two major clades the SAR (Stramenopiles, Alveolata, and Rhizaria) and Cryptophyceae + Haptophyta (Reeb et al. 2009), and plus several new phyla that have been provisionally placed as sister groups to existing chromalveolate members. Due to several different molecular phylogenetic analyses a new clade – Hacrobia was established. It unites Cryptophyceae and Haptophyta, plus some lineages, namely: telonemids, katablepharids, centrohelids and perhabs biliphites. The Katablepharidophyta (freshwater and marine heterotrophic flagellates) are a sister group to cryptophytes (Okamoto et al. 2009) and

the genus Telonema (a group of marine heterotrophic protists) are likely related to Cryptophyceae and/or Haptophyta (Shalchian-Tabrizi et al. 2006; Okamoto et al. 2009; Reeb et al. 2009).

2. ALVEOLATA

Alveolata are a diverse group of protists, most single- and multigene analyses support their monophyly and sister group relationship to Stramenopiles. They consist of three large distinctive heterogeneous subgroups: Ciliophora, Dinozoa, and Apicomplexa (Hoppenrath and Leander 2009) and several minor lineages (Leander and Keeling 2004). Dinozoa and Apicomplexa – collectively known as the Myzozoa (Cavalier-Smith 1993), are sister groups, whereas the ciliates diverged near the origin of Alveolata (Leander and Keeling 2004). Perkinsids (including *Perkinsus* and *Parvilucifera*) are a group of intracelular parasites that form a sister lineage to dinoflagellates (Hoppenrath and Leander 2009) while colpodellids and the phylum Chromerida including *Chromera velia*, are more closely related to apicomplexans (Moore et al. 2008; Hoppenrath and Leander 2009; Reeb et al. 2009).

Members of the group Alveolata share only little in terms of morphology, but few unique features such as a specific pattern of cortical alveoli subtending the plasma membrane and presumptive pinocytotic structures called micropores (Leander and Keeling 2004). Another feature recently identified to be common to all alveolates (but not on morphological level) are alveolins – a family of proteins with a unique repeating motif, which are associated with alveoli (Sven et al. 2008).

2.1. ALVEOLATA SUBGROUPS – CHARACTERISATION

2.1.1. CILIOPHORA

Ciliophora is a group of protists characterized by the hair-like cilia (ciliature apparatus), which are shorter than flagella and are usually present in large numbers all over the surface. Unlike other eukaryotes, ciliates have two different nuclei - a diploid micronucleus, and a large polyploid macronucleus; the former is a germ-line reserve, and the latter controls physiological and biochemical functions of the cell. The life cycle is complex and comprises of conjunction as a sexual process and asexual reproduction. Most members

are cosmopolitan free-living organisms that can be found almost in any liquid environment, only relatively few representatives are parasitic. Most heterotrophic ciliates feed on smaller organisms, such as bacteria and algae, and detritus by „mouth“. Some are mouthless and feed by absorption, while others are predatory and eat other microbial prey. Last but not least there are also ciliate species known to harbour symbiotic bacteria or alga (Hausmann and Bradbury 1996; Lynn 2007).

2.1.2. DINOZOA

The **dinoflagellates** are protists with two different flagella. The major variety of dinoflagellate species belongs to marine plankton, but they can be found in fresh water habitats as well. About half of all dinoflagellates are photosynthetic, the rest lives as predators or parasites (Lee 2008). Some photosynthetic species are symbiotic such as for instance *Symbiodiniun* spp. (zooxanthellas), playing an important role in the biology of coral reefs. In this symbiosis the host cells exchange inorganic waste metabolites for organic nutrient produced by dinoflagellate photosynthesis. The dinoflagellate genus *Symbiodinium* is divided into eight clades, the genetic diversity within *Symbiodinium* spp. likely correlate to the range of physiological properties in the host-symbiont assemblages from less to more beneficial to the coral host. The symbiotic interactions with *Symbiodinium* spp. includes marine invertebrates from four phyla (Cnidaria: corals, jellyfish, anemones, zoanthids; Mollusca: snails and clams; Platyhelminthes: flatworms; Porifera: sponges) and the single-celled protist Foraminifera (Stat et al. 2006; Stat et al. 2008).

A typical dinoflagellate cell is separated by girdle - cingulum into two parts: a hypocone and an epicone. Close to the surface there is a layer of plates forming typical armour for the cell. The number and arrangement of thecal plates is characteristic for the particular genus. There is a longitudinal sulcus running vertically to the girdle. The two flagella emerge through the thecal plates in the area where the girdle and sulcus meet. The longitudinal flagellum project out from the cell, whereas the transverse flagellum is wave-like and lying flat/close at the girdle (van den Hoek et al. 1995; Lee 2008).

Most dinoflagellates have a specific nucleus, called a dinokaryon, with unique features: the chromosomes are permanently condensed, even the dividing chromosomes remain highly condensed, and organised without histones which were lost. RNA acts to maintain the chromosome structure, which is arrangement in stacked rows of arches. The type of mitosis evolved with the mitotic spindle outside the nuclear membrane (Costas and Goyanes 2005).

Most of dinoflagellate plastids originated from a secondary endosymbiosis with a red alga. These chloroplasts are surrounded by three membranes and contain chlorophyll *a* and *c*, and peridinine as the major photosynthetic pigments (Lee 2008). However there is also fraction of dinoflagellates with red tertiary plastids and dinoflagellates possessing secondary green plastid (Keeling 2004). About half of photosynthetic dinoflagellates that have been examined by electron microscopy have a pyrenoid in their plastid (more about dinoflagellate plastid – see Complex Plastids) (Lee 2008).

2.1.3. APICOMPLEXA

All members of the **Apicomplexa** are parasitic, some of them are extremely important since they cause disease of both humans and animals. The most devastating pathogen to human is *Plasmodium* sp., causing malaria - that is responsible for killing millions of people in the developing world. Other species cause numbers of substantial infection of medical importance such as toxoplasmosis (*Toxoplasma gondii*) and cryptosporidiosis (*Cryptosporidium* spp.). And also those apicomplexans such as *Eimeria*, *Babesia* and *Theileria* are of high veterinary relevance and are responsible for heavy losses in domestic and wild animals (Schmidt et al. 2005).

Apicomplexa are traditionally defined by possessing an apical complex located at the anterior apex of the cell. The apical complex is distinguishable only in the electron microscope and groups set of vesicular structures important for host cell invasion - one or more polar rings, a conoid, rhoptries, micronemes and subpellicular microtubules. At least one stage of the life cycle of apicomplexans has flattened subpellicular vesicles (Schmidt et al. 2005). All Apicomplexa are parasitic except Colpodellida – freshwater predatory flagellates on other protists. It is known that colpodellids do not ingest prey cells but rather “suck” their contents partially or completely like some dinoflagellates, such a way of feeding is called myzocytosis. The cell posses three-membrane pellicle, micropores, subpellicular microtubules, open conoid, rhoptries, micronemes, extrusive organelles (trichocysts), and mitochondria with tubular cristae. The known colpodellids species are equipped with two flagella (Mylnikov 2009).

Another unique feature is the apicoplast – reduced plastid, that lacks the photosynthetic function but is irreplaceable for maintain other important metabolic pathways. This organelle is also mentioned with respect to an effect of the delayed death response (Waller and McFadden. 2005).

DELAYED DEATH EFFECT BOX 1

This phenomenon is related to the action of some antibiotics on apicoplast and subsequently on the organism and its life cycle. Characteristically, the drugs do not exert any visible effect in the first intracellular cycle, but during the second cycle the parasites are killed after the invasion of the new host cell.

In *Toxoplasma* parasites drug effects are observed only in the second infectious cycle. The apicoplast is enable to segregate properly and the growing and replication of the apicoplast-deficient parasites within the second parasitophorous vacuoles is dramatically slowed and they eventually die (Fichera et al. 1995; He et al. 2001).

In *Plasmodium* sp. the death of antibiotic-treated daughter parasites occurred during the course of their new infection in a second host cell (during erythrocyte stage). The segregation of the apicoplast is not disrupted, but the apicoplasts are abnormal and their function is thus altered. Parasites failure to complete the cytokinesis in the second cycle and thus die (Dahl and Rosenthal 2007; Goodman et al. 2007).

APICOMPLEXANS OF VETERINARY AND MEDICAL IMPORTANCE BOX 2

PARASITE	HOSTS
Plasmodium	primates, birds, rodents, reptiles
Babesia	cattle, humans, mice, dogs, cats, horses, sheep
Theileria	cattle, sheep, horses
Gregarina	various invertebrates
Cryptosporidium	humans, and other mammals, birds, reptiles
Eimeria	poultry, humans, various herbivores
Neospora	cattle, dogs
Toxoplasma	cats, and other mammals including humans, birds
Sarcocystis	reptiles, various mammals, birds

***Plasmodium* spp.** are unicellular organisms that exhibit series of morphological transformation during their multistage life cycle involving two hosts (a mosquito and a vertebrate host). The parasite life cycle (is general for all malaria causing parasites) within the vector-mosquito begins when gametocytes are taken up in an infected blood meal; after forming gametes and fertilisation, the resulting zygote differentiates into a motile ookinete

that traverses the midgut and transforms into an oocyst. The oocyst is an asexually replicating form, which produces sporozoites. Sporozoites migrate to salivary glands, where they mature and are stored, ready for transmission to the mammalian host upon the next blood meal. After injection into a vertebrate host sporozoites are brought by circular system to the liver and invade hepatocytes. Then within a cell the parasite undergoes asexual replication known as schizogony. This process culminates in the production of merozoites that are released into the bloodstream and attack erythrocytes. Inside the invaded erythrocyte the merozoite transforms to a form referred as trophozoite. The stage of trophozoite multiplies further, again asexually, periodically breaking out of its hosts to invade fresh red blood cells. Within the host cell the apicomplexan is taken up in the parasitophorous vacuole, the rhoptries and micronemes are emptied into the space between the parasite plasma membrane and parasitophorous vacuole membrane. Several such amplification cycles occur (Baldacci and Menard 2004; Lasonder et al. 2008). The blood stage is responsible for manifestation of the malaria disease, but there are many factors that can affect the outcome for example: polymorphisms in genes encoding β-globin (White 1997; Wellems et al. 2009). And also not all bites by infected mosquitoes lead to manifestation of malaria – most children exposed to such bites have no apparent disease outcome, also possible is infection with no symptoms, infection with symptoms, and finally only in few cases the patients suffer from severe malaria (Wellems et al. 2009).

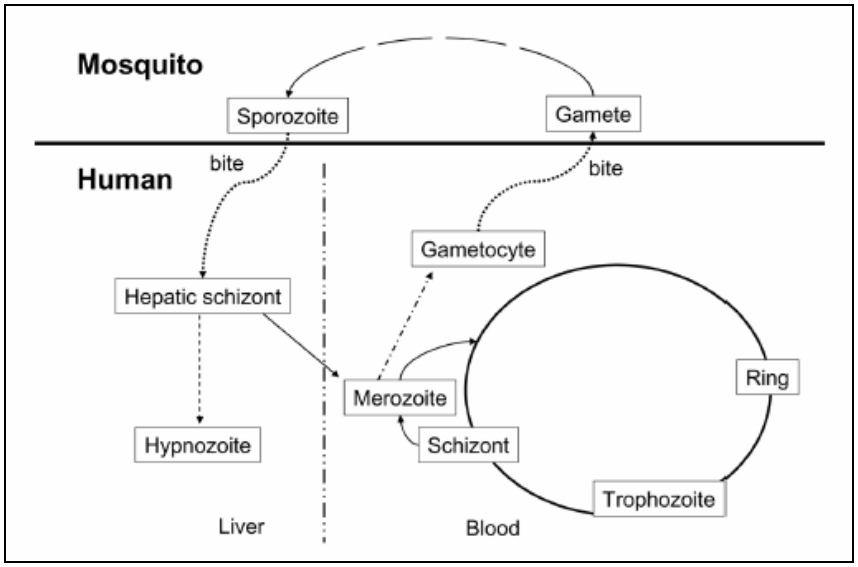

Figure 1. Life cycle of malaria parasites (Schlitzer 2008)

3. CHROMERIDA

The taxon *Chromera velia* is a photosynthetic alga, but also a very close relative to parasitic apicomplexans (Moore et al. 2008; Oborník et al. 2009; Janouškovec et al. 2010). For very first time this alga was isolated from a coral *Plesiastrea versipora* (Moore et al. 2008) and although it resembles symbiotic dinoflagellates (genus *Symbiodinium*), results of the phylogenetic analyses of nuclear genes revealed its close relationship to the phylum Apicomplexa (Moore et al. 2008; Oborník et al. 2009; Janouškovec et al. 2010).

C. velia can grow in a simple cultivation medium (Moore 2008) under wide range of condition, the lowest temperature limit is about 10ºC and the culture flourishes at temperatures between 22 and 31ºC. In the majority of cases the alga is present as an immobile oval-shaped brownish cell – layer at the bottom of a flask, or either forms random clumps of the coccoid cells (Oborník et al. 2011).

There seem to be three life stages of *C. velia*: coccoid, cystic and flagellate. The immotile coccoid stage is predominant in all cultures, the size of a single cell ranges from 5.1 to 9.5 μm. The single cell can go through a binary division to form two daughter cells that are enclosed by a thin cell wall. Consequently the daughter cells can be released and undergo another round of division or they stay enveloped in the coccoidal wall and the second round of division results in a tightly packed four-cell cyst. The three cells are arranged in the same parallel so they are observable in the same focal layer and the fourth one is visible in another focal layer (Oborník et al. 2011).

The alternative stage to immotile coccoid-cells is high speed moving bi-flagellate, which is equipped with two heterodynamic flagella. The primary oval cell elongates to bean-shaped bi-flagellate, with length ranging from 4.9 to 7.3 μm and width ranging from 2.7 to 4.8 μm. The way of motion was reconstructed from video sequences and the move forward is in zig zag manner, changing direction every 250 to 375 ms. The transformation process is also very fast, within few minutes, and was observed for one/two/four-cellular coccoids. After few hours the flagellates return back to a single coccoid, which can further divide, or die (Oborník et al. 2011).

In low light conditions the flagellates emerge at lower rate below 1% (stationary culture). In exponential culture (12:12 / day:night period, light intensity of 3.15 $W.m^{-2}$, and at 26 ºC) the exflagellation starts growing on the day 7, reaching peak on the day 11, and then slowly decrease. When the culture is illuminated ten times higher (35.8 $W.m^{-2}$), there is an

increasing trend of number of motile cells, and they appear significantly earlier – the peak comes on the day 5. Under conditions (12:12 - day:night period, light intensity of 3.15 $W.m^{-2}$, and at 26ºC) and monitored at the day of exflagellation peak - the rapid transformation into motile stages appears 2 hours after illumination, within another 2 hours culminates and thereafter slows down, and when the dark period starts only coccoids are present. That indicates there is likely correlation between the emergence of flagellates and the 24hr light/dark cycle (Oborník et al. 2009). What also effects the motile-immotile shift are nutrient levels and salinity, they tested the trend of motile transformation in salinity (20, 40, 60, and 80g/L), and f-medium concentration (5f, f, f/2, f/4). The highest percentage of motile forms were detected for salinity of 20g/L (mean = 39.8%) and for f-medium concentration f/4 and f/2 (mean = 16.8% and 16.4%) with salinity 40g/L. The transition was inhibited in 5f level (Guo et al. 2010).

The ultrastructure is only recently being investigated. The surface of a cyst is covered with a structured cell wall – composed of a central electron-lucent layer between outer/inner layers. Similar structure has a coccoid wall, but with a thicker inner layer and vesicles sprouting off the plasma membrane. To mention with a respect to alveolates there are flattened alveoli at the subsurface of the plasma membrane supported by microtubules, and also the micropores, but their distribution varies between different stages – coccoid cells and flagellates. The easy recognizable organelles are central-positioned nucleus and a single sausage-like plastid. The plastid is bounded by four membranes and fills up large content of the cell. The arrangement of thylakoid lamellae, which are in stacks of three, resembles the organization in plastids of peridinin dinoflagellates. There is present chlorophyll *a* but what differs from photosynthetic alveolates is the absence of chlorophyll *c*. The additional pigments are violaxanthin, a novel carotenoid (most likely isomer of isofucoxanthin), and β,β-carotene as a minor component. In cells that are in log-phase culture the plastid is the dominant structure, whereas in cells from stationary culture large portion of inner space is fulfilled with numerous of granules containing storage compounds. Other recognisable organelles that are evident are Golgy apparatus and membraned vesicules of uncertain identity. Mitochondrion is likely one with tubular crists (Oborník et al. 2011). What was originally considered to be a mitochondrion (Moore et al. 2008) might be a novel organelle – chromerosome at its early stage. In small number of cells it develops into a stage that resembles finger-shaped protrusion. Its function is absolutely not known yet, but may be homologous to trichocysts of ciliates and dinoflagellates (Oborník et al. 2011).

4. OTHER PHOTOSYNTHETIC CHROMALVEOLATES

CRYPTOPHYCEAE are both marine and fresh water organisms small in size, the cell is flagellated with two unequal flagella. Most of them have a single plastid with a pyrenoid. The plastid is surrounded by four membranes. In between the two pairs of membranes are starch grains and a nucleomorph. Thylakoids are grouped in pairs in arrangement that is characteristic for the Cryptophyceae group. The pigments are: chlorophylls *a* and *c*2, carotenoids, phycobilins, and xanthophylls (Lee 2008). Nucleomorph is a remnant nucleus of a red algal endosymbiont. Its genome in cryptophytes varies in size between 450-845 kb and is spread over three chromosomes – this seems to be universal characteristic for cryptophytes and also chlorarachniophytes algae (Lane and Archibald 2006). Some representatives are not photosynthetic, but are heterotrophic. Colourless *Chilomonas* has a non-photosynthetic plastid and a nucleomorph. Another colourless cryptophyte is *Goniomonas* that completely lacks a plastid. The ecology of the group is influenced by the fact that these algae are light sensitive. To avoid high levels of irradiance, they often form deepest living populations in oligotrophic lakes or can survive under snow and ice cover (Lee 2008).

HAPTOPHYTA are a group of algae commonly equipped with a haptonema between two smooth flagella. A haptonema is a filamentous accessory but thinner and with different structure and properties than flagella. The cells are usually covered with scales – in many cases the scales are calcified (Coccolitophora). In each cell there are usually two elongated plastids with thylakoids aggregated in bands of three (Lee 2008).

HETEROKONTA are a large phylum containing ecologically important algal groups – diatoms, brown algae, and chrysophytes. They are present in freshwater, marine and terrestrial habitats. The diversity among hetorokonts is striking. The size of the heterokont algal cell can range from giant multicellular seaweeds to tiny unicellular species. The plastid is bounded by four membranes and the photosynthetic pigments are chlorophylls *a* and *c*1, *c*2, and accessory pigment fucoxanthin. Motile stages are equipped with two unequal flagella (Lee 2008).

5. ENDOSYMBIOSES

Endosymbiosis is extremely important process that is essential for the eukaryotic evolution and appearance of organelles. The organelle is a differentiated structure within a eukaryotic cell that performs a specific function. The endosymbiotic origin of plastid was first introduced by Mereschkowski in 1905 (english translation) – but ignored, till few decades later was popularized by prof. Lynn Margulis (1967, On the Origin of Mitosing Cells). The result of this symbiotic relation is the symbiotic origin of semiautonomous organelles – mitochondrion and plastid. A mitochondrion evolved from an α-proteobacteria in contrast to a primary plastid which is an enslaved cyanobacterial endosymbiont (Gray 1999; Keeling 2004; Keeling 2010).

It is difficult to draw a line and distinguish between an organelle and an endosymbiont. A common view is that, unlike endosymbionts, organelles have transferred genes to their host and are dependent on targeting system to re-import their protein products. According another definition one might argue that an endosymbiont becomes an organelle when its host controls its division and segregation, even without genetic integration (Keeling and Archibald 2008).

5.1. PRIMARY ENDOSYMBIOSIS

In the **primary endosymbiosis** a phagotrophic eukaryote (already containing mitochondrion) engulfed and integrated photoautotrophic prokaryote, which evolved into a form of primary plastid. The key feature to specify the type of passed endosymbiosis is the number of membranes surrounding the plastid; primary plastids are bounded by two membranes that are homologous to the inner and outer membranes of the engulfed cyanobacteria (Keeling 2004; Gould et al. 2008; Archibald 2009; Keeling 2010). Next thing to mention - concern the genome of cyanobacterial endosymbiont, which undergoes a massive gene loss followed by a transfer of most of the endosymbiont remnant genes to the eukaryotic nucleus. The protein products necessary for plastid machinery, now encoded in the host nucleus, are transported to the organelle thanks to the transit peptide placed at the N-terminus of the protein. The transit peptide specifically interacts with the plastid protein import machinery (protein complexes TIC and TOC – translocon inner/outer chloroplast membrane) (Bruce 2001; Jarvis and Soll 2001; Gould et al. 2008).

It is mostly believed that primary endosymbiosis occurred once in the evolution. There are three lineages with primary plastids known so far: Glaucophyta, Rhodophyceae, and

Chloroplastida (including land plants) (Keeling 2004; Keeling 2010). However these groups still account for only a fraction of the diversity of plastid-bearing eukaryotes on Earth.

Other possible case of independent primary endosymbiosis is the algae *Paulinella chromatophora* (Cercozoa, euglihphid amoeba) and its cyanobacterial endosymbiont. Most eugliphids are non-photosynthetic heterotrophs. Each *P. chromatophora* cell contains two kidney-shaped cyanobacterial endosymbionts (called chromatophores) that allows the amoeba to live without heterotrophic feeding. The division of endosymbiont is synchronized with that of the host cell so each daughter *P. chromatophora* retains again two symbionts. The cyanobacterial endosymbiont is member of *Synechococcus*/*Prochlorococcus* lineage (Yoon et al. 2006; Yoon et al. 2009). The chromatophore genome is 1Mbp in size and encodes 867 genes (Nowack et al. 2008), the elimination of cyanobacterial genome concerns functional classes of genes and the whole pathways, it seems that there was no gene transfer to the host nucleus and so there is no protein targeting (Keeling 2010).

5.2. SECONDARY ENDOSYMBIOSIS

Plastids of some algal groups have originated via **secondary endosymbiosis**, when a primary alga was engulfed by a eukaryote and evolved into a secondary plastid. These plastids are surrounded by three or four membranes. Compared with two membranes surrounding the primary plastid, the additional membranes of the secondary plastid are the result of the phagocytotic process: the outermost membrane is homologous to the phagotrophic membrane of the host; it is a part of endomembrane system of the secondary host cell. The second outermost membrane corresponds to the plasma membrane of the engulfed eukaryotic alga. And the two inner membranes correspond to the two membranes of the primary plastid (Archibald 2009; Keeling 2010). Such a complex event was accompanied by huge endosymbiont genome remodelling; again most genes were simply lost, but some of both eukaryotic and prokaryotic origins were transferred to the nucleus of the secondary host. The primary alga and its nucleus are mostly degenerated as whole, but in Chlorarachniophyta and Cryptophyceae the nucleus is retained in a highly divergent form called a nucleomorph possessing highly reduced eukaryotic genome (Archibald 2007). The nucleomorph genomes of the cryptomonad, *Guillardia theta* and of the chlorarachniophyte, *Bigelowiella natans* have been entirely sequenced. The two genomes are similar in size, 551 and 373 kb, respectively, and in their basic architecture (Lane and Archibald 2006). Plastid targeted proteins encoded in the secondary host nucleus are directed to the place of action by a bipartite targeting

presequence composed of a ER signal-transit peptide followed by a transit peptide (Gould 2008). Surprising event has been described in a plastid genome of peridinine containing dinoflagellates, which was broken up into minicircles, typically about 2-3kbp in size. The minicircles encode about 13 genes, mostly there is only one gene encoded on a single minicircle; or two genes or just fragments of genes, or no identifiable coding regions at all (Zhang et al. 1999; Howe et al. 2002; Howe et al. 2008).

Secondary plastids of green algal origin are found in euglenids, chlorarachniophytes and in a small group of chromalveolate algae (see later). Secondary plastids of red algal origin have been found in chromalveolate groups. It was hypothesized that the whole supergroup Chromalveolata is a result of a single endosymbiotic event (Cavalier-Smith 1999; Harper et al. 2005; Janouškovec et al 2009), however this scenario is not fully accepted and other versions are possible (Falkowski et at. 2004; Bodyl 2005). The other hypothesized scenarios how the c-chlorophyll plastids spread across photosynthetic chromalveolate groups may be more complex: independent serial secondary endosymbioses or serial tertiary endosymbioses model in which plastids emerge in the major lineages independently and spread horizontally (Baurain et al. 2010).

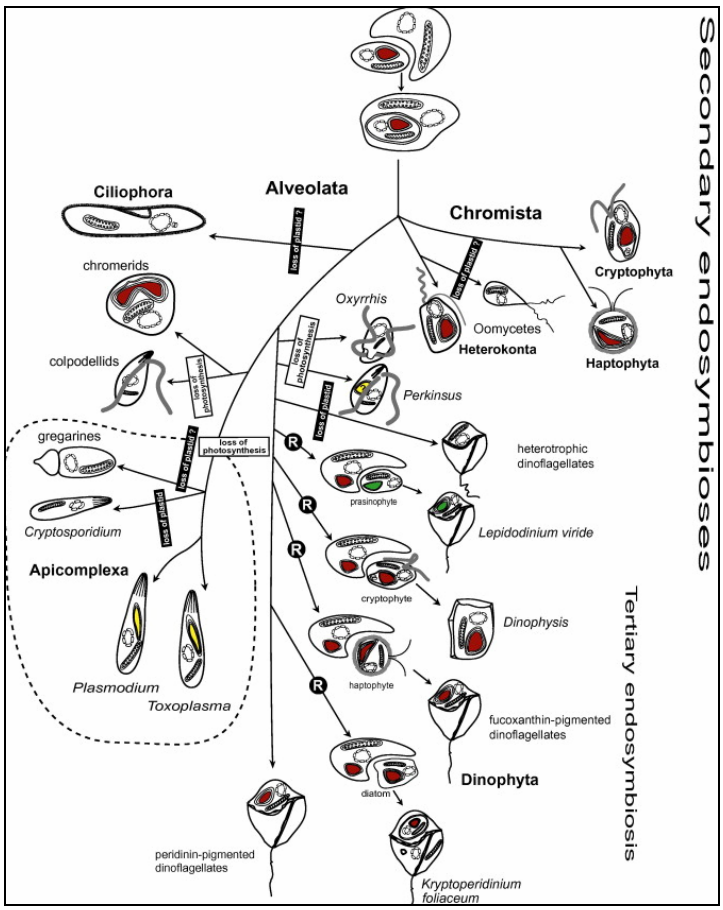

Figure 2. (Oborník et al. 2009), R in a black circle means plastid replacement

5.3. COMPLEX PLASTIDS IN CHROMALVEOLATES

5.3.1. PHOTOSYNTHETIC CHROMALVEOLATES

Plastids of most photosynthetic dinoflagellates are surrounded by three membranes and contain chlorophylls *a* and *c2* as the major photosynthetic pigments and characteristic carotenoid - peridinin. Peridinin serves as a chemotaxonomic marker for dinoflagellates since it does not occur in any other phytoplankton group (Frassanito et al. 2006). These peridinin-plastids originated from secondary endosymbiosis with a red alga (Cavalier-Smith 1999; Harper et al. 2005; Lee 2008; Janouškovec et al. 2010). Next to this majority there is small number of dinoflagellates that have derived plastid from **tertiary endosymbiosis**. Instead of peridinin as a major carotenoid these algae have hexafucoxanthin and pigments typical of algal-endosymbiont (Hansen et al. 2000). The examples of tertiary endosymbiosis are: *Karenia brevis*, *K. mikimotoi* and *Karlodinium micrum* (endosymbiont – Haptophyta); and *Kryptoperidinium foliaceum*, *Durinskia baltica* (endosymbiont – diatoms) (Imanian and Keeling 2007), toxic *Dinophysis acuminata* (endosymbiont – Cryptophyceae) (Morden and Sherwood 2002; Yoon et al. 2002). In case of system: diatom endosymbiont and dinoflagellate host (*D. daltica* and *K. foliaceum*) the complex cell suggests an early stage of integration. The endosymbiont is separated from its host by a single membrane and retains plastids, mitochondria, a large nucleus, and many eukaryotic organelles and structures. Also the complete plastid genomes have been recently sequenced, the size is reported to be *D. daltica* (116470 bp) and *K. foliaceum* (140426 bp) and the genomes are circular molecules. The data suggests that these two genomes share similar gene content and genome organization as is found in the free-living pennate diatom *Phaeodactylum tricornutum* (Imanian et al. 2010).

Other examples interesting from perspectives of plastid diversity are plastids in algae *Lepidodinium chlorophorum* and *Lepidodinium viride*. These dinoflagellates replaced their original secondary plastid by engulfing alga containing primary green plastid – **serial secondary endosymbiosis** (Keeling 2004; Bodyl 2005). **Kleptoplastids** is a term used for short-term plastids "stolen" from an eaten prey containing a chloroplast (Keeling 2004). The time of preservation of the kleptoplastids in dinoflagellates depends greatly on the species involved and the conditions under which they grow for example: in *Gymnodinium 'gracilentum'* 1 - 2 days, *Dinophysis fortii* 40 days, *Dinophysis caudata* 2 months (source of kleptoplastids cryptophyte algae). The dinoflagellates *D. fortii* and *D. caudata* cannot get the

kleptoplastids directly, and obtain it from the ciliate *Myrionecta rubra* that feed on cryptophytes (Kim and Archibald 2010)

5.3.2. APICOPLAST

Till the advent of electron microscopy, the potentials of observation of tiny apicoplexan parasites were limited. In the 1960s using light and electron microbiology the scientists discovered organelle-like structure, different from mitochondria but with close association to it. They called it "spherical body". The breakout came with the molecular methods, isolation of extrachromosomal DNA. Anyway it took another few years than it was definitely confirmed there was the 35 kb plastid organelle and its localization (McFadden 1996; Waller and McFadden 2005). The apicoplast is clearly a remnant plastid of secondary endosymbiotic origin. According to the origin of plastid in *C. velia* – the photosynthetic sister group to Apicomplexa, the algal endosymbiont of apicoplast was a red algae (Oborník et al. 2009). Since the conventional chemotherapies like chloroquine and sulphadox-pyrimethamine has lost its efficiency owing to resistance, there is need to find as many drug targets as possible. The cyanobacterial ancestry of the apicoplast, and the fact that some biochemical pathways differ from human metabolism, offers new prospects for drug development against *P. falciparum*.

In apicomplexan parasites the apicoplast was described for main lineages, but has not been detected in *Cryptosporidium* spp. (Zhu et al. 2000) and gregarines (Toso and Omoto 2007). Number of membranes in some members were identified as four (*Toxoplasma* and other coccidians), and in *Plasmodium* spp. there were debate whether three or four membranes (Hopkins et al. 1999). Unfortunately *P. falciparum* is notorious for poor ultrastructural preservation by chemical fixation, so the electron microscopy is not in this case that helpful (Waller and McFadden 2005).

As well as plastids of algae and plants apicoplasts are semi-autonomous with their own genome and expression machinery. The size of the apicoplast genome is 35 kb, and encodes less than 50 proteins, many genes (about 900) of the endosymbiont have been transferred to the host nucleus, and the number of remnant genes is about a dozen. Moreover apicoplasts import numerous proteins encoded by nuclear genes. These nuclear genes are largely derived from the endosymbiont through a process of intracellular gene relocation. The exact role of a plastid in parasites is uncertain but is essential to the parasites. Predicted apicoplast proteome has been assembled, and putative pathways indicate the synthesis of lipids, heme, isoprenoids, and iron-sulphur clusters as possibilities (Lim and McFadden 2010).

In *Plasmodium* spp. each parasite contains only one apicoplast that appears in close association to the single mitochondrion (van Dooren et al 2005). The shape of the apicoplast changes during the life cycle, but for most of the asexual parasite life cycle it persists in spherical conformation (Waller and McFadden 2005).

6. MALARIA DISEASE AND FIGHTING AGAINST

Malaria affects huge number of people worldwide and is one of the most devastating parasitosis: up to 250 million clinical cases, mainly children, emerge each year leading to nearly 1 million deaths; an estimated 863 000 deaths occurred in 2008, 89% of those were in Africa (WHO report). The disease is caused by the genus of parasites called *Plasmodium*. Four *Plasmodium* species predominantly infect humans: *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. However this picture has recently changed with the emergence of *P. knowlesi*, a natural parasite of macaque monkeys, found in human populations in Southeast Asia (Cox-Sing et al. 2008; Wellems et al. 2009).

Symptoms of malaria include fever, shivering, pain in the joints, headache and nausea. They usually appear between 10^{th} and 15^{th} days after the bite of an infected mosquito. If not treated, malaria can cause severe illness and may be fatal. *P. falciparum* and *P. vivax* are the most common as causative agents. The most serious kind of malaria is caused by the *P. falciparum* parasite, and can become deadly within two days (Wellems et al. 2009).

There are many anti-bacterial drugs that inhibit growth of parasites by targeting their bacterium–derived endosymbiotic organelles, the mitochondria and the plastid (apicoplast) (Goodman et al. 2007). In many regions of the world the parasites have developed resistance to a number of malaria medicines. Especially the increasing spread of drug-resistant *P. falciparum* is a world wide problem for the chemotherapy of malaria, because it makes widely used antimalarials chloroquine and sulfadoxine/pyrimethamine practically useless. That's why it is important to learn more about the target of action and modality – the organelles.

DRUG RESISTANCE BOX 3

is defined as the ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within tolerance of the subject.

Currently used antimalarials stem from 7 drug classes: 4-Aminoquinolines, Arylaminoalcohols, 8- Aminoquinolines, Artemisinines, Antifolates, Inhibitors of respiratory

chain, and Antibiotics (Schlitzer 2008). The antibiotics and some other drugs are mostly used in combination.

The antibiotic **Rifampicin** has antimalarial activity both *in vitro* and *in vivo* (Strath et al. 1993; Hou et al. 2004). At clinically relevant concentrations, it kills *P. falciparum* strains quickly, preventing them from initiating cell division (Dahl and Rosenthal 2007). Rifampicin inhibits DNA-dependent RNA polymerase (apicoplast-encoded RpoB gene) activity by forming a stable complex with the enzyme. It thus suppresses the initiation of RNA synthesis leading to a suppression of RNA synthesis and cell death (http://www.drugbank.ca/).

SOME OTHER DRUGS WITH ANTIMALARIAL ACTIVITY BOX 4

Ciprofloxacin targets the apicoplast genome, inhibiting DNA replication. The bactericidal action of ciprofloxacin results from inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, and recombination (Fichera and Roos 1997)

The mechanism of specific plasmodicidal action of **chloroquine** is not completely certain. Like other quinoline derivatives, it is thought to inhibit heme polymerase activity. This results in accumulation of free heme, which is toxic to the parasites (http://www.drugbank.ca/).

Minocycline is tetracycline; it can pass directly through the lipid bilayer or passively diffuse through porin channels in the bacterial membrane. It binds to the 30S ribosomal subunit, preventing the binding of tRNA to the mRNA-ribosome complex and interferes (inhibit) with protein synthesis (Lin et al. 2002).

Thiostrepton binds to plastid rRNA, but not to the mitochondrial and cytoplasmic rRNA. Thiostrepton inhibits translation of apicoplast encoded TufA and interacts specifically with the apicoplast ribosome. It impaired the plastid protein synthesis, resulting in inhibition of the transcription of the rpoB/C gene (McConkey et al. 1997).

SUMMARY AND AIMS OF MY PROJECT

Chromera velia is a close relative to apicomplexan parasites that cause many severe illnesses. Thanks to the fact that this organism is a harmless photosynthetic algae that can be easily cultivated it is supposed to be possible to use *C. velia* as a useful test-tool for basic research in developing new malarial drugs against *Plasmodium* spp.

The apicoplast of *Plasmodium* spp. serves as a target of action of some drugs used against malaria. For any further studies on chromeran plastid, including plastid proteomics, it would be helpful to obtains intact plastids. My first task was to isolate and purify the secondary plastid from *C. velia*. I also tested susceptibility of *C. velia* to rifampicin treatment at doses used for *P. falciparum*.

MATERIALS AND METHODS

7.1. *Chromera velia* – CULTURE CONDITIONS

C. velia was cultured in culture flasks with f/2 medium, salinity of 20g per L (sea salt). The medium was prepared with distilled water and filtered through a 0.22μm filter. Cultures were incubated at 26 – 27ºC, under a 12:12 hour or 16:8 hour – light:dark cycle, and also constant light.

7.2. PLASTID ISOLATION

For plastid isolation I used cultures which grew under constant light at 26ºC. The cells were harvested by centrifugation for 20 minutes, 6,000 RCF, 4ºC. I resuspended and rinsed the pellet with a brush in 20 – 40mL of hypotonic buffer (180mM sucrose, 5mM KCl, 5mM EDTA, 20mM TRIS, 5mM $MgCl_2$, 7.6 pH), then the pellet was again centrifuged at 4,500 RCF for 10 minutes, 4ºC. I repeated the washing step 2 more times. I resuspended the final pellet with a brush in 25mL (for Multiflex breaking), or 4mL (for sonication breaking) of hypotonic buffer. For breaking the cells I used two different techniques: Multiflex, pressure 1,500 PSI for 5 – 10 minutes, and sonication. During sonication breaking the material was kept on ice to avoid overheating. I sonicated the cells for 30 seconds, power 20 and cycle 5. I did the sonication steps 4 times, and 8 times, during every new sonication step the cells were kept on ice for 1 minute. I collected the broken cells by centrifugation for 10 minutes at 500 RCF, 4ºC. I obtained a pellet and a brownish supernatant. The remainder supernatant was also centrifuged but at 45,000 RCF for 25 minutes, 4ºC. Both pellets – from 500 RCF and from 45,000 RCF centrifugation were resuspended in 2mL buffer (0.3M sucrose, 25mM Hepes, 1mM $MgCl_2$) and loaded on gradient.

I have tried sucrose and percoll gradient. For sucrose gradient I used different volumes of layers of 0.5M-0.8M-1.3M-1.5M-1.8M-2M sucrose. I used a gradient buffer (5mM Hepes, 10mM EDTA, pH 7.5) as a base for dissolving the 2mM sucrose, and thereafter for diluting the 2mM sucrose to the rest of molar mass needed. The total volume of sucrose gradient was 36mL and a single layer differs from 2mL to 10mL. The optimal stratification was 2mL of 2M sucrose, 8mL of 1.8M sucrose, 10mL of 1.3M sucrose, 8mL of 0.8M sucrose and 8mL of

0.5M sucrose. The gradient was laid on from the smallest molar mass from the bottom of the tube. For the percoll gradient I used from 2 – 12mL of 30-40-50-60-80% percoll stratification. The gradient was centrifuged in a swing-out rotor in the ultracentrifuge (Beckman) at 25,000 RCF for 1 hour, 4ºC.

The single layers were taken by a pipette and used for direct observation under the light and fluorescence microscope; the photographs were taken by a digital camera. For longer storage the obtained layers were diluted in buffer without sucrose (25mM Hepes, 1mM $MgCl_2$, pH 7.5) and centrifuged for 20 minutes at 45,000 RCF, 4ºC. The supernatant was discarded and the pellet was washed again in buffer (25mM Hepes, 1mM $MgCl_2$, pH 7.5) and centrifuged in a table centrifuge, 14,000 RCF, 20 minutes, 4 ºC, the final pellet was resuspended in the same buffer as used in previous steps and stored in -20ºC, or embedded in 2.5% glutaraldehyde in 0.1M phosphate buffer for TEM.

7.3. TRANSMISSION ELECTRON MICROSCOPY (TEM)

For transmission electron microscopy the material was fixed in 2.5% glutaraldehyde (in 0.1M phosphate buffer) at 4ºC for at least overnight or more days. Then according to the protocol I collected the material by centrifugation - 3,000 RCF, 4ºC, and the supernatant was discarded. I added the washing buffer (4 % glucose in 0.1M phosphate buffer) to the pellet, let the sample shacked for 15 minutes, centrifuged for 5 minutes (14,000 RCF) and discarded the supernatant. The washing step was repeated 2 more times. The last washing step was centrifuged with swing-out rotor, 3,000 RCF, 10 minutes, 4ºC. The supernatant was drained off as much as was possible and the obtained pellet was fast cast in a prewarm resin (60ºC) and moved into a fridge for few minutes to let the resin polymerised. The sample in a resin was cut into small pieces and put into an eppendorf tube with washing buffer and shacked for 15 minutes. After this I took off some washing buffer and add 4% solution of OsO_4 to final dilution 1:3 (4% OsO_4 : washing buffer). Then I let the sample shake for 2 hours. After 2 hours the solution with osmium was drained off and new washing buffer was added, next followed again washing steps (with shacking for 15 minutes) for 3 times. Thereafter the final washing step I added to the sample 30% acetone, let it shake for 15 minutes and discarded the solution. The same as described for 30% acetone I did with 50%-70%-80%-90%-95%-100% acetone in order from 50% to 100% acetone. It is possible to leave the sample in 70% acetone in 4ºC for the next day to continue. After series of acetone washing I prepared resin : 100% acetone dilution in ratio 1:2, 1:1, 2:1, these were added to the sample in order described and

let it shake for 1 hour. Finally I added pure resin and left the samples in exicator overnight. The next day I placed a single piece of sample in polymerised agar into a plastic form and filled it with resin. The form was put into a thermostat (60ºC) for 2 days. These samples are ready for ultrathin sectioning and TEM.

For TEM microscopy I prepared the cells of *C. velia* in resin - Spurr and Epon, and I also used fractions from gradient – the single layers from sucrose gradient of cells broken by Multiflex, and cells broken by sonication. The samples from cells broken by Multiflex were put into both Spurr and Epon. I prepared the samples from cells broken by sonication according to the protocol described above; plus after the samples were embedded in agar and cut into small pieces I microwaved (80W, microwave oven) the samples in water bath for 30 second before every shaking step. The resin I used for these samples was Spurr.

7.4. ENZYMATIC TREATMENT

I prepared enzyme digestion solution (McLeod et al. 2008): lysing enzyme from *Trichoderma* (Sigma) 0.5%, cellulase 1%, mannitol 0.4M, $CaCl_2$ 10mM, KCl 20mM, $MgS0_4$ 20mM (pH 5.7) in distilled water, and f/2 medium. I harvested 50mL of culture of *C. velia* and had the cells digested in 50mL of enzyme digestion solution under shaking conditions, 33ºC. After 4, 8 hours, and overnight of incubating I harvested the culture and washed the cells in PBS, and resuspended in breaking buffer (mannitol 0.3M, EDTA 5mM, $MgCl_2$ 5mM, KCl, MOPS 20mM, 7.5 pH). Thereafter the cells were broken by Multiflex for 5 minutes (1,500 PSI), and microscoped.

7.5. FLUORESCENCE MICROSCOPY AND DNA STAINING

For fluorescence DNA staining I used three types of dyes: DAPI (4',6-diamidino-2-phenylindole), SYBR Green I and Propidium Iodide. For DAPI staining I used the culture of *C. velia*, which I put onto the underlying glass, and let the culture dry off, then I dropped DAPI (VECTASHIELD) onto the material, and the whole sample was covered with the top glass. I dried off the redundant liquid with a tissue and observed the section in fluorescence microscope; the pictures were taken by a digital camera. I used different fixation - I added 10µL of 38% formaldehyde to 100µL of cell culture, 10µL of 75% methanol to 100µL of cell

culture, and 10µL of Lugol solution to 100µL of cell culture. Thereafter I continued with DAPI staining as described above.

SYBR Green I staining (Vítová et al. 2005) - the 15mL of culture were incubate with 15µL of SYBR Green for 14 hours, and more than 48 hours under culture conditions but in dark. The 20mL of culture were incubated with 10µL, and also 1µL of SYBR Green I in room temperature and dark for 1 hour. Then I observed the samples in fluorescence microscope (ZEISS).

The Propidium Iodide staining - I followed the protocol for oocyst. I prepared 3 x 100µL of culture, one of them were treated for 2 minutes in 75ºC, and one for few hours in -20ºC. To each sample I added 10µL of Propidium Iodide (1mg/mL in 0.1M PBS), kept in room temperature for 30 minutes, and I washed the samples in PBS buffer, then I microscoped the samples.

7.6. LIGHT CONDITIONS

Flasks with 50mL of *C. velia* were cultured in thermoboxes (26ºC) with different light intensity: 20mol/m^2s, 40mol/m^2s, 95mol/m^2s (Photo/radiometer). The day:night period was 16:8 hours starting at 6 o'clock in the morning. I harvested the cells and diluted them in f/2 medium of salinity 20g/L, and 40g/L to initial density of $1x10^6$ cells/mL (Guo et al. 2010). I observed the cultures in an inverted light microscope at intervals 8-9 o'clock in the morning, 1-2 o'clock and 4-5 o'clock in the afternoon.

7.7. RIFAMPICIN APPLICATION

I used 40mL of *C. velia* in flasks with f/2 medium, salinity 20g/L, the culture conditions were 26ºC, shaking, and day-night cycle 12-12 hours. Young culture was diluted with fresh medium to density not to be over $1.5x10^6$ cells/mL. I determined the concentration using Burker Chamber, the 50µL of culture was fixed with 50µL of 1% formaldehyde, and then I counted the number of cells in 25 large squares of Burker Chamber. Afterwards I applied Rifampicin (1mM stock solution in DMSO) at concentrations 2µM, 6µM, 20µM, 40µM, and control with no drug addition. I counted the cells in Burker Chamber once a day, for 7 days. The experiments were carried out in thermoboxes, with light 40mol/m^2s, and one

experiment was performed in incubator with light at 40mol/m^2s, and simultaneously in incubator with light at 95moL/m^2s.

RESULTS

8.1. BREAKING OF *C. VELIA* AND FOLLOWING SEPARATION ON GRADIENT

For breaking of the cells I have used techniques sonication and Multiflex breaking. The sucrose gradient (every layer was of 6mL volume, total volume 36mL) with cells that were broken by Multiflex displayed brownish bands in between the layers of 2M/1.8M sucrose, 1.8M/1.5M sucrose, 1.5M/1.3M sucrose, and 1.3M/0.8M sucrose. These bands were prepared for TEM. In further experiments the thickness of bands depended on proportional volumes of sucrose, if the layer of 2M sucrose had been reduced to 2mL and the other sucrose layers were between 6 – 10mL, the bands were distributed at interface of 1.8M/1.5M sucrose, 1.5M/1.3M sucrose, and 1.3M/0.8M sucrose. I have to say that every gradient looked a little bit different, even if the stratification was the same (Fig. 8, 9). In the bands there were present the whole cells of *C. velia*, probably damaged cells, putative plastids, some vesicles, and „spins" – which was apparently pieces of the cell wall. Even if I got only two thick bands on gradient, there was no band, which would have contained only plastids, but there was always mixture of compounds. Generally speaking in the lower bands there were more „spins", and the combination – putative plastids plus the whole cells were in all bands.

Breaking the cells of *C. velia* using sonication, and subsequent separation on sucrose gradient resulted in two thick bands, and upper wide pale-brown band. In gradient of composition 6mL per each layer, the two lower bands were at the interface of 2M/1.8M sucrose and 1.8M/1.5M sucrose. When the 2M sucrose layer was reduced to volume 2mL, and the 1.5M sucrose layer was omitted (so the stratification was: 2mL of 2M sucrose, 8mL of 1.8M sucrose, 10mL of 1.3M sucrose, 8mL of 0.8M sucrose, and 8mL of 0.5M sucrose), the two thick bands appeared at 1.8M/1.3M sucrose and slightly under the dividing line of 1.3M/0.8M sucrose (Fig. 6). Observing in the light microscope the upper wide band contained the cells, putative plastids, and „spins" but all the compounds at low number. The two thick lower bands were full of putative plastids (Fig. 11). There was no visual difference in putative plastids from these two bands. In the fluorescence microscope the plastids showed up the autofluorescence of plastid, under the filters Cy5 and Cy3 (Fig. 13).

I have used also percoll gradient for separating of broken cells. Onto the 80% (12mL) / 60% (12mL) / 40% (12mL) percoll I loaded 2mL of suspension of cells broken by Multiflex. After the centrifugation one thick brown band appeared above the 60%/40% percoll and

throughout the 60% percoll there were floating brown clusters (Fig 10). Observing under the light microscope - the whole cells and putative plastids were compounds of the brown band. I used also cells broken by sonication (8 rounds, 30 seconds, power 20, cycle 5). The gradient I prepared was: 80%(4mL)/60%(8mL)/50%(8mL)/40%(8mL)/30%(8mL) percoll. After the centrifugation step the resulting bands were at 80%/60% percoll and slightly above 40%/30% percoll, and again floating brown clumps were present between 60% - 40% layer, mostly in 50% percoll layer (Fig 7). When I observed the bands in light microscope – there were putative plastids in the lower band which was dark brown and thicker than the upper band where putative plastids were as well but in smaller amount.

For the separation on the gradients I used suspension of pellet obtained from centrifugation of broken cells (500 RCF, 10 minutes, 4ºC), the supernatant from this step was also pelleted (45,000 RCF, 25 minutes, 4ºC) and this high-pellet was also used for separation on gradients. The better results – thicker and brown bands were obtained using the pellet from 500 RCF centrifugation (low-speed pellet).

Enzymatic treatment of *C. velia* - I used the enzyme digestion solution used for production of protoplast from *Phytophora* spp., and *Pythium aphanidermatum* (Oomycetes). The *C. velia* treated in the enzymatic solution for different incubation time was next broken by Multiflex. When observed in light microscope before breaking step there were cells of *C. velia* and huge amount of bacterial contamination, and after breaking step there was great portion of the whole cells, some putative plastids, vesicles and other compounds from broken cells, and also many bacterial contamination.

8.2. TRANSMISSION ELECTRON MICROSCOPY AND FLUORESCENCE MICROSCOPY

I have prepared samples of cells of *C. velia*, samples from four bands (at 2M/1.8M - 1.8M/1.5M - 1.5M/1.3M - 1.3M/0.8M sucrose) from sucrose gradient separation (Multiflex breaking), samples from two bands (at 1.8M/1.3M and 1.3M/0.8M sucrose) from sucrose gradient separation (sonication breaking for 4 rounds) and the same for sucrose gradient (sonication breaking 8 rounds). I have observed the ultrathin sections under Transmission Electron Microscope; the samples in Spurr were in a little bit better condition than those embedded in Epon. But generally speaking the preservation of *C. velia* cells is very bad, there were many dropped out spots. In all four bands from sucrose gradient (Multiflex breaking) there were whole cells, small number of putative plastids, and “spins” (pieces of the cell wall)

– which were more curl. In all samples prepared from sucrose gradient separation, sonication breaking there were whole cells that differ only in the level in which they were damaged inside. What I call in this paragraph putative plastids were objects with thylakoid membranes throughout the inside compartment but surrounded with a cell wall.

The plastid of *C. velia* has its own autofluorescence, which is very strong and visible under filters Cy3 (excitation filter: 510-560nm), Cy5 (590-650nm) and also in others for example FITC and Fs 05. The dyeing with fluorescence dyes DAPI and SYBR Green I was not successful. Propidium Iodide seemed to partly dye some structures, but because it has emission in red field it is hard to distinguish between the red autofluorescence of plastid and flourescence of the dye.

8.3. LIGHT INTENSITY, SALINITY AND FLAGELLATES

The *C. velia* was cultured in common f/2 medium of salinity 35g/L and 20g/L and under light conditions 40mol/m^2s and under light conditions 95mol/m^2s. The exflagellation started on the 3rd day, the peak was within next 2-3 days. The number of flagellates (in the peak-days) in culture with f/2 medium, salinity 35g/L was lower then the amount of flagellates at the same time in culture with f/2 medium, salinity 20g/L. This trend I observed at both light conditions. When I compare the cultures in f/2 medium, salinity 20g/L but cultured under different light conditions, the one in higher light intensity has up to 80% of flagellates at its peak day whereas the other under low light intensity has at its peak day much smaller percentage of flagellates. The same I observed for cultures in f/2 medium, salinity 35g/L cultured under lower and higher light. I quantified the flagellates only by classification into classes 1%, 10%, 20-40%, 40-60% and 60-80% when observing in the microscope.

8.4. RIFAMPICIN APPLICATION

I used concentrations 2μM, 6μM, 20μM, 40μM of rifampicin, none of them stopped growing of the culture. The growth of *C. velia* was obviously inhibited by rifampicin, when compared to control culture (Graph 1.), however, this inhibition was not comparable to that observed in *Plasmodium*. Doses able to significantly decrease growth of *Plasmodium* (2.19 μM) where only slightly effective in *C. velia*. Roughly estimated, the effective treatment of *C. velia* was done using doses 10 times higher than in *Plasmodium*. I also observed the

flagellates in the cultures. The flagellates started to emerge on the 2nd or 3rd day in all cultures, within the next two days there was the highest percentage of flagellates in cultures with 2μM, 6μM rifampicin and in control with no rifampicin added, and then slowly decreased. In cultures with 20μM and 40μM rifampicin the highest percentage of flagellates appeared one or two days later than in control and cultures with 2μM, 6μM rifampicin, and then also the flagellates slowly decreased. The amount of flagellates at their highest performance were the same for control and 2μM, 6μM, 20μM and the culture with 40μM rifampicin had slightly lower number of motile cells than the rest cultures. These experiments were carried out under the light conditions 40mol/m^{2}s. One experiment with 6μM, 20μM, 40μM rifampicin was done in conditions with higher light intensity, 95mol/m^{2}s. I observed the first flagellates appeared in all cultures on the 2nd day. The highest amount of motile cells was present on the 4th day in cultures with 20μM, 40μM rifampicin, and in the culture with 6μM of rifampicin and control it was one day after. The highest percentage of flagellates were the same for all cultures – up to 80% (at their peak-day), except for culture with 40μM of rifampicin where the highest amount of flagellates was a little bit less when compared to other cultures.

DISCUSSION

BREAKING OF *C. VELIA* AND FOLLOWING SEPARATION ON GRADIENT

The breaking of *C. velia* and subsequent separation of organelles appeared to be a big problem, mainly due to its very thick and persistent cell wall. So my first step was to weaken the cell wall, I used the enzymatic solution for producing the protoplast of some species from the phylum Oomycetes (McLeod et al. 2008). Oomycota are members of Chromalveolata kingdom, SAR clade, so they are relatively closely related to Chromerida. The digestive enzyme solution contains common cellulase and lysing enzyme from *Trichoderma* (Sigma) – it altogether has β-glucanase, cellulase, protease, and chitinase activities. The digestive treatment didn´t seem to have any effect on the morphology of *C. velia*, when observed under the light microscope. Contrariwise there was a negative effect – the bacterial contamination, which grew up during the cultivation at 33ºC. Also I did not see any improvement when the treated culture was broken by Multiflex (5 minutes, 1,500 PSI). I stopped using the digestive enzymatic treatment, because first of all there were no visible „better results", plus there was the accumulation of bacteria contamination during cultivation which might have been problem in next use, and also because in my primary experiments I used plenty of cell material and the lysing enzyme is an expensive item for such a use.

There are many protocols for plastid isolation mostly for higher plants, but also algae. For my purpose I tried to combine protocols for *Gracilaria tenuistitipata* (Hagopian et al. 2002), *Cyanidioschyzon merolae* (Rhodophyceae) (Miyagishima et al. 1999), *Odontella sinensis* and *Coscinodiscus granii* (Bacillariophyta) (Wittpoth et al. 1998), and *Guillardia theta* (Cryptophyceae) (Wastl and Maier 2000). Yeda Press breaking was successfully applied on the cells of calcified diatoms (Wittpoth et al. 1998), so at the beginning I used Multiflex breaking (5 minutes, 1,500 PSI), and next also sonication breaking (3 rounds, impulse 80, power 40), common breaking buffer contains Hepes, EDTA, and mannitol/sorbitol/sucrose from concentrations 0.25M up to adjusted sorbitol concentration of 0.626M in protocol for diatoms (pH around 7.5). Next step was centrifugation (performed at 250 – 1,000 RCF for 5-10 minutes) and obtaining low-speed pellet and the supernatant, which was centrifuged again to get high-speed pellet (3,000 RCF for 5-15 minutes). The low-speed pellet should contain still intact cells and large cell debris and according to protocols is discarded, while the high-speed pellet should contain crude plastid fraction and is used for further separation on gradient. In my experiments: the low-speed pellet contains the rest of intact cells and also most of putative plastids. I have tried centrifugation at 250 RCF, and 350 RCF but sometimes

there were still clumps floating in the supernatant and the pellet was not stable, finally the optimal speed that fit is 500 RCF. Also the time and speed of gradient centrifugation vary from protocol to protocol: from 12 minutes, 11,000 RCF (percoll gradient) for *C. merolae* to 112,000 RCF for 1 hour (sucrose gradient) in protocol for *G. tenuistitipata*. In my first experiments I used stationary culture of *C. velia* rather than exponentional, Multiflex breaking (5 minutes, 1,500 PSI) and sonication breaking (3 rounds, impulse 80, power 40), and next percoll gradient separation for 12 minutes at 11,000 RCF (according to *C. merolae* protocol) with high speed pellet. The results were poor - the layer with sample did not separate at all, so in next steps I raised the speed and time for centrifugation and also tried to optimalize the conditions for breaking.

What seems to play very important role is the culture conditions, the culture should be young and in exponential phase, then using of hypotonic buffer for washing and breaking the harvested cells. The hypotonic buffer seems to make the cells swell so they are readily to burst. The Multiflex breaking is not sufficient, because there is still amount of intact cells which do not separate in gradient from putative plastids, so there is no crude plastid band. The sonication breaking was finally not sufficient as well. As a product of sucrose gradient separation there were two bands full of putative plastids. The putative plastids are half-moon shaped under light microscope (Fig. 11); I did not observe any visible difference between the two bands of putative plastids (sonication breaking) under light microscope. When the sonication step was performed for 8 times per sample and 4 times per sample, and then the samples were separated on sucrose gradient, there were in both cases two bands in the same position (1.8/1.3M sucrose and 1.3/0.8M sucrose).

The percoll gradient with cells broken by sonication (8 times) showed two bands at 80%/60% and 40%/30% and some clumps in between. In both bands were putative plastids, as they say in protocols around 40% percoll there should accumulate broken plastids while intact plastids should form band at 60% (diatoms protocol), in the upper band there was only a little of subjects while in lower brown band there were many putative plastids.

The putative plastids from band at 80%/60% percoll (sonication breaking) were a little bit more round in shape than putative plastids from sucrose gradient (sonication breaking) that were more half moon shaped. This could be caused by different qualities of percoll and of sucrose.

TRANSMISSION ELECTRON AND FLUORESCENCE MICROSCOPY

The transmission electron microscopy of four bands from sucrose gradient (Multiflex breaking) proved that in all four samples there were whole cells, then also "cells" that were smaller in size, their plastid was extended throughout the inside compartment and the cell wall was also present, and finally the "spins" which were more curl. The objects I called putative plastids (in all the text above), are most probably the whole cells as well. The preservation of *C. velia* is virtually very bad. Thanks to its thick cell wall the resin is not able to penetrate into the cell properly and consequently the sample drops out the ultra-section (Fig 14.).

The samples from sucrose gradient separation (sonication) were during the preparation steps microwaved, this treatment should help penetration and therefore the samples should be better for TEM. The result of transmission electron microscopy of bands from sucrose gradient separation (sonication breaking) were evident, the objects I considered to be plastids were the whole cells with intact cell wall. The only visible differences when compared to results from samples (Multiflex breaking) were: the cells from sonication were more damaged inside and lower amount of the pieces of the cell wall in sample treated with sonication – this is consistent with the fact that less cells were broken up by sonication. The intact cells were in all samples (sonication breaking) from both bands (upper and lower band - 1.8/1.3M sucrose and 1.3/0.8M sucrose). The subjects from sample that was treated with sonication for 4 times had more preserved the inner compartment than those from sample that was treated with sonication for 8 times.

The results from TEM demonstrated that both Multiflex breaking and sonication breaking were not able to break *C. velia*. The objects I considered to be plastids were finally intact cells. When I observed these "putative plastids" under light microscope they were half-moon shaped and different from the round cell of *C. velia*. I observed the autofluorescence for the putative plastids in most cases all over the objects that was caused probably by the fact that the inner structures of the cell were damaged and so the thylakoid membranes were released and showed up the autofluorescence throughout the cell (Fig. 13).

The same problem as for TEM is the cell wall for fluorescence dyes; they are not able to penetrate through. The results of DAPI and SYBR Green staining were practically zero. The fixating of material was also not helpful, even the cultivation with SYBR Green for more than 48 hours did not show up any positive result. The Propidium Iodide seemed to dye some structures but because the autofluorescence of plastid is red as well as is the emission of Propidium Iodide it is difficult to say whether the signal is from the fluorescence dye or from the plastid which is stretched through a large amount of the cell. The protocol for staining of

C. velia needs to improve the step of preparation of the cells. It would be useful to try treatment with ethanol plus acetic acid, and let the samples stay more hours in fixation solution (Algal culturing techniques, Anderson 2005).

LIGHT INTENSITY, SALINITY AND FLAGELLATES

We used to culture *C. velia* in f/2 medium of salinity 35g/L. It was issued that at salinity of 20g/L the highest percentage of motile forms occurred (Guo et al. 2010) and that if the light intensity grew up (from 3.15 $W.m^2$ to 35.8 $W.m^2$) the number of flagellates also grew up (Oborník et al. 2010). In the combinations I used: cultures at 20g/L salinity and cultures at 35g/L salinity and under two different light conditions, the highest number of flagellates, up to 80%, I observed for culture conditions: f/2 medium with salinity 20g/L under higher light intensity. The light seems to me triggers the increase in number of motile forms more than the salinity. I would have to make more repeats of experiments (this was done only twice) and quantify the number of flagellates under more stable method to be able present more reliable statement. It is very difficult to somehow quantify the flagellates, because of their very fast movement. The fixation in methanol or formaldehyde is not possible, because they lose their elongated shape, throw away the flagella and become round-shaped. Only Lugol solution preserves the flagellates, so it would be possible to fix the culture in Lugol solution and then count the number of flagellates vs. coccoid cells in Burker chamber.

RIFAMPICIN ACTION

Rifampicin is referred to block the RNA synthesis in organelles (http://www.drugbank.ca/), it can act on molecular level at very low concentrations, for inhibitory studies of heme oxygenase activity in *Cyanidium caldarium* it is 5μg/mL (= 0.6μM) (Rhie and Beale 1994). In *Chlamydomonas reinhardtii* studies the full inhibition of chloroplast transcription can be obtained by treating cells for 1 hour with 350μg/mL (425.3μM), and treatment at this concentration for up to 20 hours is lethal for the cells. However the IC50 after 48 hours in *P. falciparum* is about 2.19μM (Strath et al. 1993), primary we wanted to see if there would be any effect in *C. velia* when using the same concentration as used for *P. falciparum*.

I used treatment with rifampicin at concentrations 2μM, 6μM, 20μM and 40μM, none of these doses stopped growing the cultures. But the growth of cultures with added rifampicin was inhibited depending on the particular dose (2μM, 6μM, 20μM and 40μM). It seems that rifampicin has some limiting effect on growing curve of *C. velia*, but the decrease of growing

to 50% (regarding to control) is at doses of 20μM rifampicin – ten times higher than in *P. falciparum* (Strath et al. 1993).

I also observed the flagellate rhythm for 6μM, 20μM and 40μM rifampicin, I omitted the 2μM, because there seemed to be no difference in behaviour of cultures with 2 and 6μM rifampicin. When the cultures were grown at 40 moL/m^2s the highest amount of flagellates was shifted 1-2 day later for cultures with 20μM and 40μM when compared to culture with 6μM rifampicin and control. I thought that there might be any reaction to presence of rifampicin so that the flagellates in cultures with rifampicin would appear earlier and in higher number than in control to escape the environment with added drug. This was not confirmed. The quantity of motile cells at the peak-day seemed to be the same for cultures with 6μM and 20μM rifampicin as well as for control, only for culture with 40μM rifampicin the number was always little lower. There did not seem to be any large difference in biorhythm of flagellates in cultures with and without rifampicin. To confirm if there is any pattern or not in appearance and amount of flagellates with respect to presence of rifampicin in medium it is necessary to do more repeats and work out the counting of the motile cells.

Last thing to mention is the fact that I observed flagellates in cultures (at the day when there was the highest percentage of flagellates, in cultures with 6μM, 20μM and 40μM rifampicin and also for control) one hour and also two hours after the light period. It was reported that motile forms disappear when there is dark period (Oborník et al. 2011), so it indicate that more observation of the rhythm of flagellates is needed.

CONCLUSION

Isolation of intact organelles from secondary alga *Chromera velia* is not an easy objective. Methods I have used so far (enzymatic treatment, Multiflex, sonication) did not produce expected results. Multiflex and enzymatic treatment (mix of enzymes; see methods for details) were not sufficient to break the cells on an appropriate level. In the case of sonication, the cells also remained intact; however, plastids inside the cells were broken and pigments were released to cytoplasm. Thus even whole cells being brownish-green in color remained isolated chloroplasts under the light microscope and were incorrectly classified as putative plastids. Since the cells wall of *C. velia* is extremely thick, particularly sonication destroy the plastid before breaking down the cell wall. I would also highlight the necessity to confirm the status of isolated organelles by TEM.

To see whether *C. velia* might be suitable for testing antimalarial drugs, I have tested rifampicin, active substance usually used to fight *Plasmodium,* and its influence of the growth of *C. velia.* I demonstrate here that concentration 10 times higher than those working in *Plasmodium*, shows comparable inhibition effect (to 50%) to the growth of *C. velia*. I propose that this phenomenon is due to extremely low permeability of thick cell wall for active substance. Similarly, it is very difficult do mark any *C. velia* cellular structures using DNA staining dyes, again due to low permeability of cell wall.

According to the mentioned phenomenon, I conclude that *C. velia* is probably not suitable for testing drugs against apicomplexan parasites, due to necessity to use incomparably higher doses when compared to *Plasmodium*. I also conclude that methods I have used so far are not sufficient to isolate intact organelles from this secondary alga.

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COLOUR SUPPLEMENT

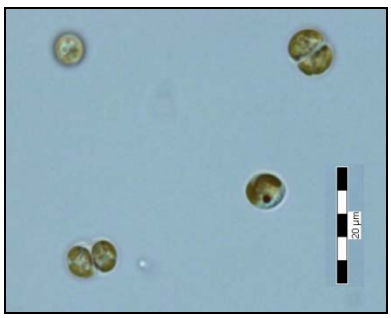
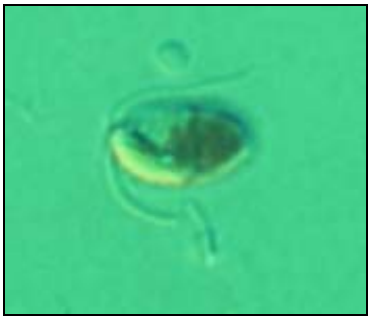


Fig. 3.



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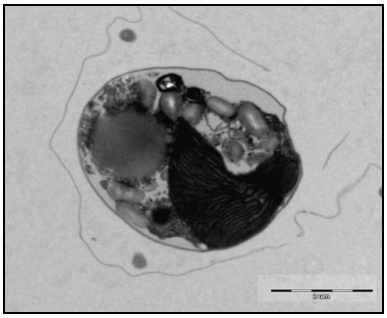


Fig. 5.

The light microscopy of coccoid (**Fig. 3.)** and flagellate (**Fig. 4.** – fixed in Lugol solution) forms of *C. velia*, TEM of *C. velia* (**Fig. 5.),** the plastid is clearly visible.

The gradient separation:

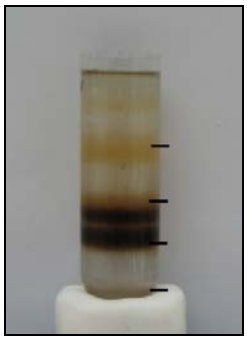


Fig. 6.

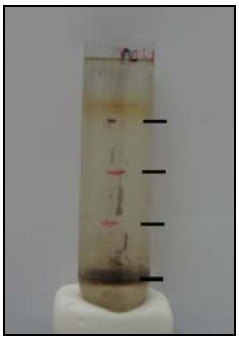


Fig. 7.

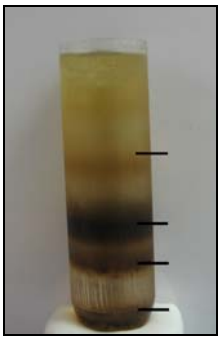


Fig. 8.

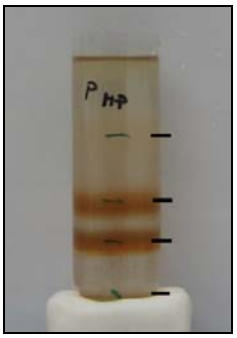


Fig. 9.

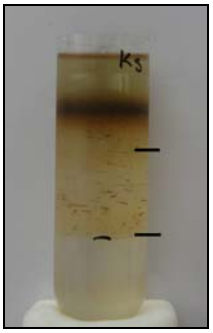


Fig. 10.

Sucrose gradient of cells treated with sonication, stratification from the bottom: 2M, 1.8M, 1.3M, 0.8M, and 0.5M sucrose **(Fig. 6.**). Percoll gradient of cells treated by sonication, stratification: 80%, 60%, 50%, 40%, and 30% percoll (**Fig. 7.).** Sucrose gradient of cells treated with Multiflex, stratification: 2M, 1.8M, 1.5M, 0.8M, and 0.5M sucrose (**Fig. 8.**) and 2M, 1.8M, 1.3M, 0.8M, and 0.5M sucrose (**Fig. 9.**). Percoll gradient of treated with Multiflex, stratification: 80%, 60%, and 40% percoll (**Fig. 10.**).

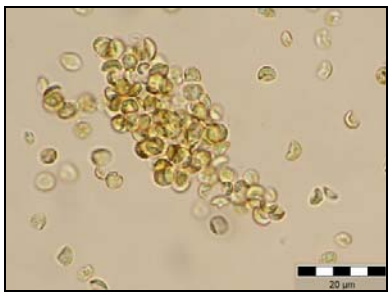


Fig. 11.

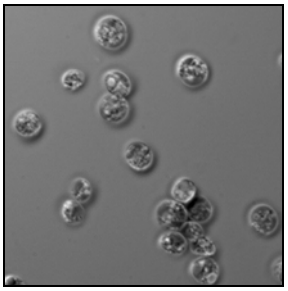


Fig. 12.

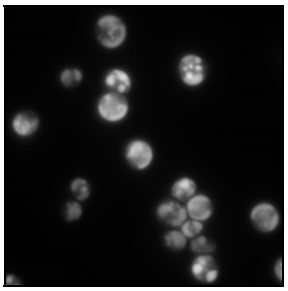
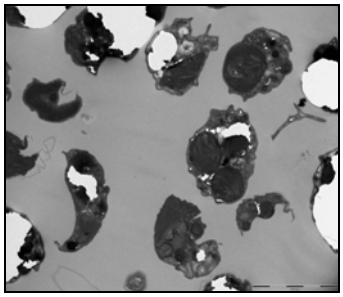


Fig. 13.

Light microscopy (**Fig. 11.**) and Fluorescence microscopy magnification 63x, in normal light (**Fig. 12.**) and under filter cy4 (**Fig. 13.**) of the band (1.8M/1.3M) from sucrose gradient with cells treated by sonication.

Transmission Electron Microscopy:



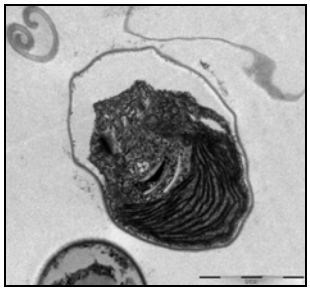


Fig. 14. **Fig. 15.**

TEM of bands from sucrose gradient, Multiflex breaking.

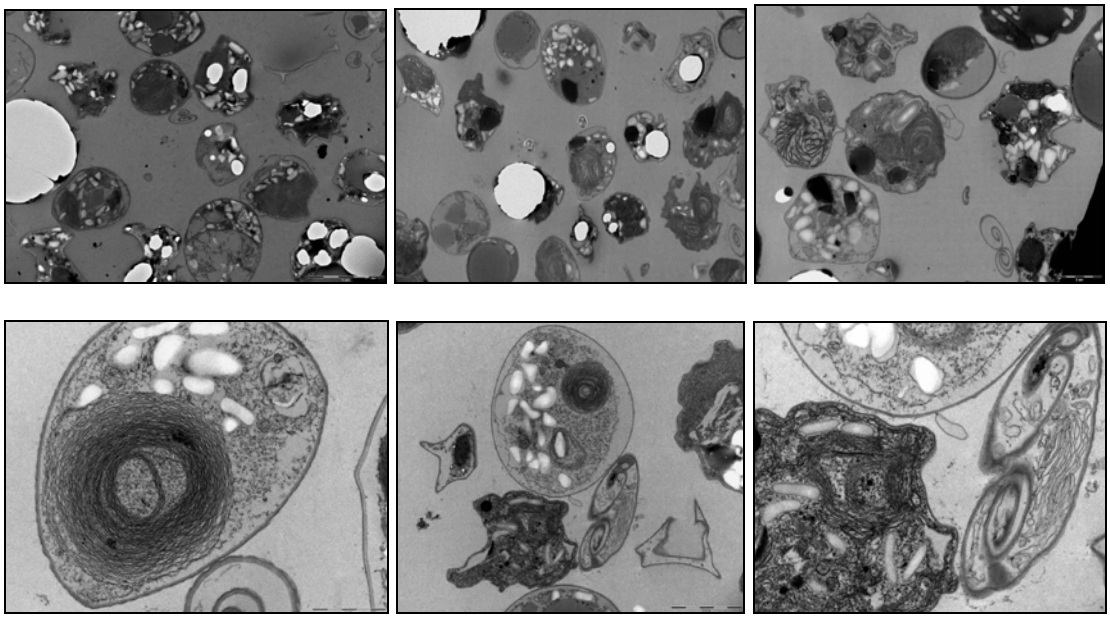
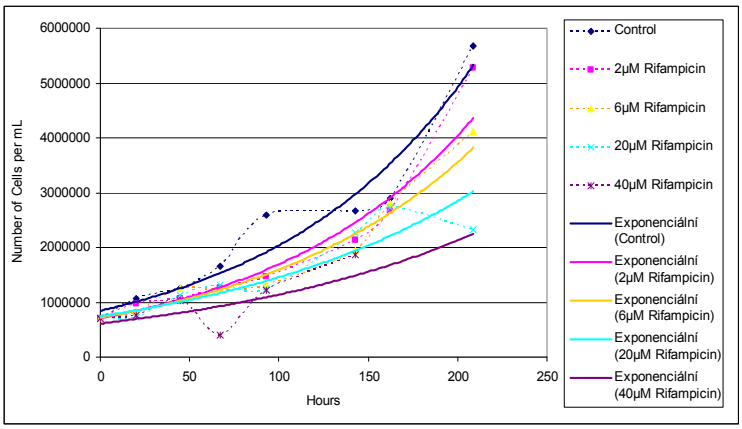


Fig. 16. – 21.

TEM of bands from sucrose gradient, Sonication breaking



Graph 1. of Inhibitory study of rifampicin