Protistology 15 (1), 24–33 (2021)

Protistology

Symphony of minimalism: peculiar endosymbiosis of mycoplasmas and protists

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| Submitted February 28, 2021 | Accepted March 26, 2021 |

Summary

The interaction of minimal biosystems, such as the smallest prokaryotic cells and the most simply arranged unicellular eukaryotic organisms, can become a very attractive model from both fundamental and applied points of view. This mini-review considers so far the only one known phenomenon of true endosymbiosis between some of the simplest prokaryotic cells capable of autonomous replication (*Mycoplasma hominis* and Candidatus *Mycoplasma girerdii*), and a minimalistic unicellular obligate human parasite (*Trichomonas vaginalis*). The description of the *T. vaginalis – M. hominis* "Trojan horse" supports the speculation about the potential of protists as the probable underestimated natural reservoir for mycoplasmas – pathogens of plants and animals. Fantasizing about the creation of artificial endosymbionts on the platform of natural minimal cell models and the perspectives of their use hopefully will be a tempting bonus to the reader.

Key words: endosymbiosis, minimal biosystems, minimal cells, mycoplasma, natural reservoir, protists

Allegro. Mycoplasmas, protists and the endosymbiotic wardrobe

The interspecies interactions, including those among unicellular organisms, characterize the high organization level of living matter. These interactions can be manifold – from obligate parasitism to the closest symbiosis and almost cell-organelle relationship.

It is a well-known phenomenon that in some cases bacteria are able to resist intracellular killing after being harvested, establishing endosymbiosis with protists (Nowack and Melkonian, 2010). Recently, an even more intriguing evidence has begun to emerge, namely the possibility of lengthening the chain of symbiotic interactions up to three organisms or more. As an example, protozoa in the *Listeria–Tetrahymena–Amoeba* food chain can act like a "Trojan horse" in a way of pathogen delivery to consumers of a higher order (Pushkareva et al., 2019). In those experiments, saprophytic and pathogenic bacteria, *Listeria innocua* and *Listeria monocytogenes*, were able not only to survive after entering the cell of an intermediate host (*Tetrahymena pyriformis*). There was a successful following transmission of these microorganisms further along the food chain to *Amoeba proteus*, where *L. monocytogenes* continued to multiply, increasing the possibility of getting mixed infections for a probable future host.

The phenomenon of symbiosis between bacteria and protists is attractive from both fundamental

doi:10.21685/1680-0826-2021-15-1-3

and applied points of view because protists may represent a still insufficiently estimated biological niche for pathogenic microorganisms. In this context, it is curious to know whether extremely simple biosystems, such as minimal prokaryotic cells (mycoplasmas) and minimal eukaryotic organisms (some unicellular protists) can interact with each other. After all, the metabolism of the simplest cells implies a high degree of minimalism, their genomes are notably reduced (Fraser et al., 1995; Derelle et al., 2006), and their abilities to interact, in theory, should be minimalized likewise. The interaction of the minimal prokaryotic cell with the minimal eukaryotic organism could serve as a good model for studying the basic reciprocal processes occurring in the intracellular parasite-host system.

Mycoplasmas (class Mollicutes) are the smallest known prokaryotic microorganisms capable of autonomous self-replication (Pettersson et al., 1996). The average size of mycoplasma cells is 0.3–0.5 μm in diameter (Fig. 1), which is close to the theoretically calculated equivalent of the minimal volume for a living cell, at which all its life support systems work stably (National Research Council (US) Steering Group for the Workshop on Size Limits of Very Small Microorganisms, 1999). Mycoplasmas do not have a cell wall; their genomes are highly reduced and in some species are characterized by the alternative genetic code (where the TGA stop codon encodes the amino acid tryptophan); moreover, mycoplasma cells have a very limited set of metabolic pathways (Browning and Citti, 2014). It is conceived that the pathways preserved during the process of reductive evolution of mycoplasmas are of fundamental importance for the life support of any cell (Wong and Houry, 2004). It is well known that mycoplasmas often demonstrate obligate parasitism, including intracellular parasitism in plant, animal or human cells (Borchsenius et al., 2016). However, what about their possible interactions with extremely simple eukaryotic organisms, apart from their parasitic lifestyle? Is that cooperation tight enough and beneficial for both actors?

It would be truly wrong to classify protists as a group of the simplest organisms in general, basing only on the fact that most protozoa are unicellular. However, among unicellular protists, there is indeed the smallest free-living photosynthetic eukaryotic organism, *Ostreococcus tauri* (Courties et al., 1994), each cell of which contains a nucleus, a chloroplast (with four or five thylakoids and one starch grain), one mitochondrion, one Golgi body and a very much reduced cytoplasmic compartment (Chrétiennot-Dinet et al., 1995). As far as it is known from the literature published to date, the presence of intracellular bacterial symbionts was not shown for *O. tauri*. Perhaps there is simply no room for them in such a tiny volume? However, the benefits of co-cultivation *O. tauri* with a heterotrophic bacterial partner *Dinoroseobacter shibae* were successfully demonstrated (Cooper et al., 2019). This member of the Rhodobacteraceae family of alpha-proteobacteria, genera of which are frequently found associated with marine algae, can help to alleviate the B12 and B1 auxotrophy of *O. tauri*. In turn, *D. shibae* itself lacks the complete pathway to synthesize niacin (B3), biotin (B7), and p-aminobenzoic acid (a precursor for B9), and the alga is able to satisfy the reciprocal vitamin requirements of its bacterial partner in a stable long-term co-culture.

In true endosymbiosis, the benefits should be even more pronounced for both participants, and minimal biosystems are no exception. Examples of relatively simple protists are known, which, however, successfully contain intracellular bacterial symbionts in their cytoplasm. One such example is an obligate human parasite, *Trichomonas vaginalis*, which is unable to survive in the environment (Henriquez et al., 2021). It was *Trichomonas* who became the first object where a successful endosymbiosis of mycoplasmas and protists was discovered: *T. vaginalis* and *Mycoplasma hominis* / Candidatus *Mycoplasma girerdii* (Rappelli et al., 1998; Fettweis et al., 2014). The described close interaction of the simple biological systems, like a "symphony of minimalism", deserves a special discussion. However, before talking in more detail about this phenomenon, let us have a closer look at the participants, their life style and consequences of independent existence for the host organism.

Rondo. Fantastic pathogens and where to find them

According to the data reported by the World Health Organization (Tien et al., 2020), *T. vaginalis* is one of the most common pathogens causing sexually transmitted infections (together with *Chlamydia trachomatis, Neisseria gonorrhoeae* and *Treponema pallidum* they represent 376 million new infections annually). Trichomoniasis itself is linked with serious health concerns such as pregnancy

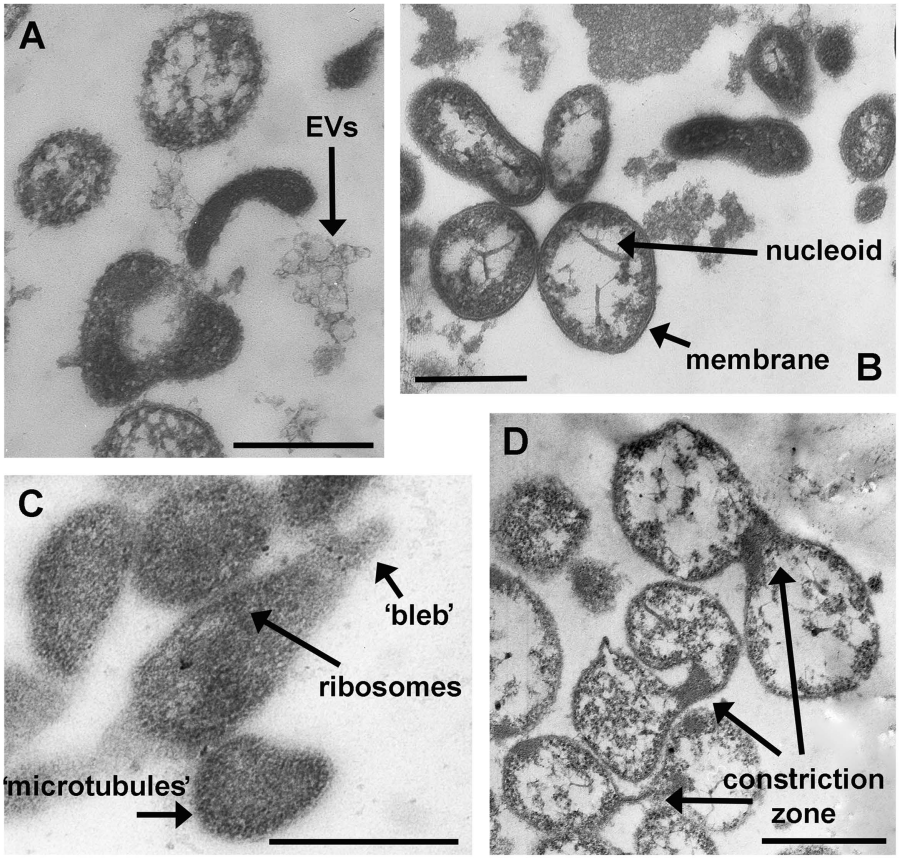

Fig. 1. Transmission electron microscopy images of the minimal prokaryotic cells (*Mollicutes*, mycoplasmas). A – *Mycoplasma hominis* H34, an opportunistic / pathogenic bacterium of humans; B – *Ureaplasma parvum* ser. 3, a human pathogen; C – *Mycoplasma gallisepticum* A5969, a pathogen of poultry; D – *Acholeplasma laidlawii* PG8a, a phytopathogen. *Mycoplasma* spp. samples for transmission electron microscopy were prepared using standard techniques, including post fixation with osmium-potassium ferrocyanide (in D) and embedding into the LR-white resin. Ultrathin sections were stained with uranyl acetate and lead citrate. Micrographs of mycoplasmas demonstrate certain cellular structures (pointed by *arrows*). EVs – extracellular vesicles, 'bleb'- the terminal organelle of *M. gallisepticum*. Scale bars: 500 nm.

outcomes, infertility, predisposition to cervical and prostate cancer, and increased transmission and acquisition of HIV (Hashemi et al., 2021).

T. vaginalis is an anaerobic / microaerophilic unicellular protist, its mode of existence can be defined as obligate parasitism (Leitsch, 2021). It has motile and symptom-causing trophozoites of average size 10 μm × 7 μm, characterized by the absence of mitochondria and the presence of anaerobic hydrogenosomes (Henriquez et al., 2021). Trophozoites can convert to pseudocysts during chemical and temperature perturbations, or spherical, immotile cyst-like structures, resistant to osmotic lysis and detergent treatments, during long cultivation (Beri et al., 2020). Of particular note, these cyst-like structures remained viable even in chlorinated swimming pool water, implicating the possibility of their role as environmentally resistant structures involved in non-sexual mode of parasite transmission.

M. hominis is a sexually transmitted opportunistic human mycoplasma, which resides within the same natural niche as the *T. vaginalis* protozoan, the urogenital tract (Margarita et al., 2020). This microorganism is potentially pathogenic and can cause both genitourinary and extragenital infections, including urinary tract infections, bacterial vaginosis, pelvic inflammatory disease, cervicitis and pyelonephritis, septic arthritis, prosthetic joint infection, brain abscess and meningitis, infective endocarditis following prosthetic valve replacement, abscess formation following post-transplant and

surgical manipulation. The infection of the host organism with *M. hominis* can lead to autoimmune infertility and increased risk of preterm birth, chorioamnionitis, and postpartum fever. *M. hominis* has also been suggested as a possible cause of bacteremia, respiratory distress and pneumonia in neonates, neonatal systemic inflammatory response syndrome, meningitis, meningoencephalitis and brain abscess, pneumonia and bronchopulmonary dysplasia (Ahmed et al., 2021).

M. hominis has the second smallest genome among self-replicating organisms: 665,445 bp, 537 coding sequences, and a significantly reduced metabolic capacity (Pereyre et al., 2009). The mycoplasma shares notable cell polymorphism common for most species of the class Mollicutes. Small sphere-, rod-, and dumbbell-like cells, as well as cells connected with thin tubules, prevail in *M. hominis* culture at the exponential growth phase (Vishnyakov et al., 2009). The size in diameter of the smallest cells is about 80 nm; large cells may have diameters of up to one μm. Electron dense regions of the cytoplasm contain ribosome-like structures, and the central transparent zone has numerous DNA fibrils (Anderson and Barile, 1965). *M. hominis* hydrolyses arginine as its major energy source (Razin et al., 1998), and is capable of invasion and replication within human cells (Hopfe et al., 2013).

Thus, we have two relatively simple biosystems – the prokaryotic cell, which properties are close to the estimated model of a minimal cell, and the eukaryotic unicellular organism, also characterized by minimalistic features. Both microorganisms inhabit the human urogenital tract and can be transmitted from person to person mainly through sexual contacts. Perhaps it was the common ecological niche that contributed to the establish-ment of close endosymbiosis between these two species.

Minuet. A pair dance of the little ones

In 1998, it was shown for the first time that *M. hominis* is able not only to infect *T. vaginalis* cells, but also to replicate in association with them. Moreover, it was demonstrated *in vitro* that *T. vaginalis* infected with *M. hominis* is able to transmit the infection to human epithelial cells, and the infected cells are able to transmit *M. hominis* to *T. vaginalis* backwards (Rappelli et al., 1998). The authors then proposed that the ability of *M. hominis* to parasitize and to replicate in association with *T. vaginalis* could play a key role in this association. The intracytoplasmic location of *M. hominis* in *T. vaginalis* cells was demonstrated afterwards (Dessì et al., 2005).

As it was shown later, this kind of relationship is mutually beneficial. *T. vaginalis*, symbiotically associated with *M. hominis*, turned out to be equipped with two arginine dihydrolase (ADH) pathways of distinct evolutionary origins (mycoplasmal and protozoal). The overall increase of ATP in protozoan–bacterium consortium was most likely due to the mycoplasma arginine dihydrolase (ADH) pathway (Morada et al., 2010): the presence of *M. hominis* resulted in an approximately 16-fold increase in intracellular ornithine and a threefold increase in putrescine, compared with control *T. vaginalis* cultures. Consequently, *M. hominis* might enhance the growth rate of *T. vaginalis* by increasing intracellular ATP (Dessì et al., 2019). In turn, *M. hominis* could benefit from a constant supply of putrescine, which this mycoplasma is not capable of synthesizing. This become possible due to an increased putrescine production by *T. vaginalis* ornithine decarboxylase when associated with *M. hominis* (Shah and Swiatlo, 2008). Additionally, the intracellular bacteria are "invisible" to the host immune system and thus protected from the action of many antibiotics. Moreover, it has been clearly established that, after co-culturing with *T. vaginalis,* there was an increase in *M. hominis* resistance to clindamycin, moxifloxacin, ciprofloxacin and gentamicin (Fürnkranz et al., 2018). In this case, *T. vaginalis*, like the protozoa in the *Listeria–Tetrahymena–Amoeba* food chain, can play a role of a "Trojan horse" for the host organism, with direct benefits for its bacterial symbiont (Dessì et al., 2019).

The symbiotic association between *T. vaginalis* and *M. hominis* is potentially involved in the modulation of host cell cytotoxicity and immune-mediated pathogenesis (Dessì et al., 2019). *T. vaginalis* with intracellular *M. hominis* showed an increased amoeboid transformation rate, enhanced ability to phagocytize yeast cells, and an increased haemolytic activity *in vitro* (Margarita et al., 2016). Thereby, *M. hominis* symbiotically associated with *T. vaginalis* might account for the enhanced parasite virulence. The presence of *M. hominis* in the *T. vaginalis* cytoplasm significantly upregulates the host inflammatory response to the protozoan, and a marked chronic inflammatory state is a condition that predisposes the genital microenvironment to tumor transformation and maintenance, strengthening the oncogenic potential of the individual symbionts (Henriquez et al., 2021). Selective

Fig. 2. *Trichomonas vaginalis – Mycoplasma hominis* "Trojan horse". Symbiotic relationship between *T. vaginalis* and *M. hominis* can lead to severe diseases of the host (human) organism. ADH – arginine dihydrolase.

treatment of trichomoniasis with metronidazole could induce a massive release of *M. hominis* from dead parasites leading to bacterial invasion of placental membranes and amniotic fluid (Thi Trung Thu et al., 2018). Thus, these low virulent microorganisms together are able to produce severe diseases, helping each other to colonize successfully the host organism (Fig. 2).

Until 2014, the symbiosis between *T. vaginalis* and *M. hominis* had represented the unique case of an endosymbiotic association between two obligated human parasites, able to produce infections in the same anatomical site and causing independent diseases in humans (Dessì et al., 2019). Then, the second example of endosymbiosis between mycoplasmas and protists was described through metagenome sequencing and assembly of DNA from four discrete mid-vaginal samples, one of which was obtained from a pregnant woman with trichomoniasis (Fettweis et al., 2014).

Genomes of four strains of the microorganism named Candidatus *Mycoplasma girerdii* were reconstructed. It was shown that metabolic circuitry of Ca. *M. girerdii* is distinct from most true Mollicutes, possibly reflecting a unique history of genome reduction in close association with the anaerobic, hydrogenosome-bearing eukaryote *T. vaginalis*. In particular, Ca. *M. girerdii* and *T. vaginalis* may share the capacity for anaerobic pyruvate metabolism via pyruvate-ferredoxin oxidoreductase (Costello et al., 2017). It should be especially noted that Ca. *M. girerdii* is an uncultivable mycoplasma. This suggests that such kind of symbiosis must be very close. *M. girerdii* DNA could be found only in *T. vaginalis*-positive vaginal samples (Fettweis et al., 2014). Apparently, outside of the host organism or intermediate transmitter Ca. *M. girerdii* is not viable.

These examples of endosymbiosis are the only ones of this kind of interactions known for mycoplasmas and protists to date (Henriquez et al., 2021).

A case, potentially similar to mycoplasma-protist interaction has been observed, for example, for the *Giardia* spp. protozoan cells, where intracytoplasmic mycoplasma-like structures were described (Feely et al., 1988). However, no bacterial species have been isolated from that protist yet (Henriquez et al., 2021). Based on the described facts, one can speculate about the potential of interactions between mycoplasmas and protists in general, paying particular attention to the mutual influence and enhancement of the pathogenic properties of parasitic microorganisms.

Rondo sonata. Polyphony of endosymbioses

There are numerous bacterial endosymbioses with protists (Vouga et al., 2015; Gomaa et al., 2018; Henriquez et al., 2021), including such simply arranged microorganisms as Chlamydiales (Corsaro and Venditti, 2006). How can the existence of mycoplasmas inside protists be useful for both of them, even for a short time? It is well known that *Legionella pneumophila* can be protected from extreme temperatures when hosted in protozoa like *Hartmanella* sp. or *Acanthamoeba castellanii*: cysts of these protists are able to resist severe stresses and help *L. pneumophila* to survive inside them (Jules and Buchrieser, 2007). Moreover, free-living *Acanthamoeba* represent the main environmental reservoir for *L. pneumophila* (Scheikl et al., 2016).

For mycoplasmas, this kind of endosymbiosis would be even more beneficial comparing to other bacteria, since they are lacking a cell wall and, therefore, are more susceptible to fluctuations in environmental parameters and various external stresses. Only one mycoplasma, *A. laidlawii*, which is able of free living, is currently known (Laidlaw and Elford, 1936); the rest of Mollicutes are obligate parasites of plants, insects, animals or humans (Razin et al., 1998). One can assume that mycoplasmas could "weather the storm" inside the protozoan cysts when, for any reason, they are eliminated from the host organism and enter the environment without being adapted to survive therein. Such a possibility of forced temporary endosymbiosis has to be examined closely in the future.

Mycoplasmas do not form classical resting stages (spores), but during unfavorable conditions, they have been noticed to produce the so-called mini-bodies, a kind of viable but non-culturable forms (Chernov et al., 2007; Vishnyakov et al., 2015). These mini-bodies can survive for a long time and reverse into the normal cells after transferring to the rich medium. Facts are known for some bacteria that their viable but non-culturable forms can be resuscitated upon being internalized by the amoebae (Henriquez et al., 2021). In particular, extracellular pyruvate and glutamate can drive the shift of viable but non-culturable microorganisms to the replicative form, perhaps acting as antioxidants and facilitating cell recovery, as it has been shown for *L. pheumophila* in *Acanthamoeba* spp. (Ducret et al., 2014). This could be possible and beneficial for mycoplasmas as well. In addition, as in the case of *T. vaginalis* and *M. hominis*, we cannot exclude that true endosymbiosis is also possible, when both participants have their advantages.

These findings are important not only from a basic-science point of view. Many intracellular protist parasites can cause serious diseases in humans (Vouga et al., 2015). At the same time, there are examples of diseases caused by intracellular parasitic bacteria, usually transmitted to humans from infected animals, without their participation in the transmission process. As an example, a case of community-acquired pneumonia due to *Chlamydia caviae*, a minimalistic intracellular parasite (Read et al., 2003) with zoonotic potential, in a patient with no direct animal exposure was described, raising questions about the zoonotic reservoirs and putative transmission routes (van Grootveld et al., 2018). Could it be similar to what was described by Pushkareva et al. (2019) for *Listeria* or by Jules and Buchrieser (2007) for *Legionella*? Could protists be considered as a potent vector or mediator in the transmission of pathogenic mycoplasmas from one animal to another, or from person to person in the case of humans, excluding their direct contact with each other? Looking for such examples is worth spending the effort.

Among protozoans, it would be both interesting and important to search for potential natural niches for mycoplasmas – poultry pathogens (*M. gallisepticum, M. synoviae, M. meleagridis, M. iowae*) or cattle pathogens (*M. bovis, M. mycoides*, etc). The possibility of indirect transmission of these pathogens between animals, their long-term preservation in the environment as protozoan endosymbionts, the ability to survive unfavorable environmental conditions by passing through the cyst stage in protists can rise the risks of serious economic harm to agriculture that so far has neither been calculated nor foreseen in advance.

As for the interaction of minimal biosystems, we can see the effect of the mutual benefit of such

coexistence using the example of *T. vaginalis* and *M. hominis*. Thus, evidently, minimal biosystems can "fall in love" with each other in the form of true symbiosis, especially occupying a similar niche, and feel stronger together. Moreover, the symbiosis of the simplest organisms is more beneficial for them than the independent existence, since in symbiosis they can share/gain the missing components of the metabolic pathways.

Additionally, mycoplasmas and protists can be considered as a promising model system for studying tight symbiosis, which could theoretically turn into an organelle-host cell relationship. On this basis, one can try to build artificial endosymbiotic systems for theoretical or utilitarian purposes. Such attempts to create synthetic endosymbionts using different microbial platforms already exist. For example, one system has been built recently on the platform of the *Saccharomyces cerevisiae* yeast and *Escherichia coli* (Mehta et al., 2019). This study examined the fundamental changes that could have occurred during the transformation of ancient endosymbionts into organelles.

It would be also fascinating to create an artificial symbiont using plant mycoplasmas (*A. laidlawii* or Candidatus *Phytoplasma* spp.) and the simplest photosynthetic eukaryote, *O. tauri*. Such synthetically obtained organism could become the effective model system for studying the basics of interactions between the photosynthetic eukaryotic cell and phytopathogenic mycoplasmas. Mycoplasma plant infections are widespread throughout the world. Evidence of phytopathogenicity and association with specific diseases is available for all phytoplasma species (Candidatus *Phytoplasma*), as well as for a number of representatives of *Spiroplasma* and *Acholeplasma*. Currently, more than 40 phytoplasmas are known to successfully infect about 1000 species of both wild and agricultural plants (Namba, 2019). Despite the fact that phytoplasmas are obligate parasites with highly reduced genomes and weakened metabolic potential, they cause significant damage to agriculture and crop production. The creation of a relevant model system for studying their properties is one of the priority tasks.

Another option is an attempt to hook up a poultry pathogen *M. gallisepticum* to the cell of some free-living cyst-forming amoeboid protist and try to get the mycoplasma through the cyst phase. Such artificial endosymbiosis could shed light on a potential ecological niche and the possibility of indirect infection by pathogenic mycoplasmas from animal to animal. *M. gallisepticum* is known as a particularly dangerous pathogen (Lierz et al., 2008). This mycoplasma causes chronic respiratory diseases in poultry, characterized by conjunctivitis and sinusitis, which lead to a considerable decrease in their weight and egg production and can significantly degrade the quality of agricultural products (Ley, 2003). Respiratory mycoplasmosis causes serious damage to poultry farms and tangible economic losses in all regions of the world (Gharaibeh and Hailat, 2011; Shil et al., 2011; Stipkovits and Szathmary, 2012). These findings allow concluding that the risk of contamination of poultry with pathogenic protists, which contain endosymbiotic mycoplasmas inside their cells, and the possible intensification of infection may be largely underestimated.

Acknowledgements

This research was funded by the Budgetary Program # FMFU-2021-0006 at the Institute of Cytology RAS. The author is personally grateful to Dr. Andrew Goodkov and Prof. Sergey Borchsenius for the exciting philosophical discussions on the topic and general support.

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