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Together forever: Inseparable partners of the symbiotic system *Paramecium multimicronucleatum/Ca.* Trichorickettsia mobilis

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Abstract

Ciliates tend to form stable associations with prokaryotes at the cellular level of organization. The ciliate *Paramecium multimicronucleatum* harbors the motile intranuclear endosymbiont *Ca*. Trichorickettsia mobilis (Rickettsiaceae) resistant to antibiotics. We assessed the effect of antimicrobial peptide complex FLIP7 produced by the larvae of the blue blow fly *Calliphora vicina* on the ciliate host viability and ability to maintain the endosymbiont. FLIP7 demonstrated a certain anti-ciliate activity, however, its administration failed to clear the endosymbionts from the host cells, surviving ciliates always keeping Trichorickettsia. Trichorickettsia-carrying ciliates appeared to be more resistant to FLIP7 and its chromatographic fractions, than Trichorickettsia-free strains, suggesting that Trichorickettsia might increase the host fitness. Alongside with the ovoid forms persisting in the host nucleus, another population of bacteria enriched in lipids was observed inside the cytoplasmic vacuoles. These forms were also registered outside the host cell, suggesting a possibility of horizontal transmission under stress. The data obtained support the belief that the symbiotic system between *P. multimicronucleatum* and *Ca.* Trichorickettsia mobilis is a perfect holobiont model to be used in further cytological and genetic studies.

Keywords Holobiont · Ciliate · Rickettsia · Insect immunity · Antimicrobial peptides · Resistance to antibiotics · Host fitness

In 1991 Lynn Margulis proposed the term "holobiont" to emphasize the integrity of the partners in the symbiotic systems (Margulis 1991). According to this concept, a holobiont (a microorganism and all its microbiota) is regarded as an evolutionary single unit undergoing selection. This idea was greeted with fervor by the scientific community, further development of the concept crystallizing into the notion of a hologenome comprising the genomes of all constituents of the symbiotic association (Zilber-Rosenberg and Rosenberg 2008; Bordenstein and Theis 2015; Theis et al. 2016; Bosch and Miller 2016; O'Malley 2017; Simon et al. 2019). Despite the continuous debate on the importance of selection at the holobiont level, at present, the holobiont concept is considered a highly promising approach in evolutionary thinking (Morris 2018; Baedke et al. 2020). Accumulating data on microbial ecology and, in particular, on symbiotic associations formed by unicellular eukaryotic microorganisms and various bacteria could provide a comprehensive range of relatively simple model holobionts to be used in further research.

Ciliates are widely spread unicellular organisms characterized by highly peculiar organization. Their motility is ensured by numerous cilia covering the body, and they possess two types of nuclei: the generative micronucleus and the vegetative (somatic) macronucleus. Due to intensive phagocytosis, ciliates constantly take up various microorganisms, prokaryotic and eukaryotic, many of which are able to escape from the digestive vacuole and survive inside the ciliate, forming stable endocytobiotic systems (Görtz and Fokin 2009; Görtz 2010; Fokin 2012; Schweikert et al. 2013; Vannini et al. 2017; Fokin et al. 2019; Flemming et al. 2020; Sonntag and Sommaruga 2020; Schrallhammer

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and Potekhin 2020; Fokin and Serra 2022). In some cases, bacteria-carrying cell lines isolated from natural populations can be maintained in laboratory for decades, which makes them inspiring models for further elaboration of the holobiont concept (Serra et al. 2019). The ciliate cell offers a number of compartments convenient for the invader, which can reside in the cytoplasm (enclosed in the host-derived vacuole or "naked", i.e. host membrane free), or in either of the nuclei. The relations between the partners in such systems are rather obscure, often making difficult to distinguish between commensalism, mutualism and parasitism. Moreover, the type of the relations seems to be flexible depending on the environmental conditions and the state of the host (Bella et al. 2016).

Among many described associations between the ciliate host and the prokaryotic endosymbiont some have already become generally recognized model symbiotic systems. These are *Paramecium caudatum*/*Holospora obtusa* (Fujishima 2009; Fokin and Görtz 2009; Fokin and Serra 2022), *Paramecium tetraurelia*/*Caedibacter taeniospiralis* (Schrallhammer and Schweikert 2009; Grosser et al. 2018), *Euplotes aediculatus*/*Polynucleobacter necessarius* (Heckmann and Schmidt 1987; Vannini et al. 2005, 2007; Boscaro et al. 2019), to name a few. Besides these relatively well studied symbioses, lately, a great number of novel associations have been discovered, the last decade being especially fruitful in new findings (Szokoli et al. 2016; Serra et al. 2016; Takeshita et al. 2019; Fokin et al. 2019; Beliavskaia et al. 2020; Korotaev et al. 2020; Yakovleva et al. 2020; Castelli et al. 2021a, b; and many others). One of them is a symbiotic association formed by the ciliate *Paramecium multimicronucleatum* and the bacterium *Ca*. Trichorickettsia mobilis, which resides in the host macronucleus (Vannini et al. 2014). The first mention and morphological description of this remarkable endosymbiont made by Vishnyakov and Rodionova (1999) remained unnoticed. Normally, these bacteria are represented by short motile rod-like forms, however, oval non-motile and long septate forms occur as well, especially under antibiotic treatments (Vishnyakov and Rodionova 1999; Mironov and Sabaneyeva 2020). These bacteria often carry virus-like particles and polyhydroxyalcanoate (PHA) inclusions (Vannini et al. 2014; Sabaneyeva et al. 2018). The most peculiar feature of this endosymbiotic bacterium is its high motility in the host nucleus ensured by the presence of numerous flagella on the rod-shaped forms (Vishnyakov and Rodionova 1999; Vannini et al. 2014). Sequencing the SSU rRNA gene demonstrated that this bacterium belongs to *Rickettsiaceae* family, well known for several of its members, causative agents of severe human diseases: *Rickettsia rickettsii* (Rocky Mountains spotted fever), *R. prowazekii* (epidemic typhus), *R. conori* (Mediterranian spotted fever). Since, so far, this endosymbiont could not be maintained outside of the host cell, according to the rules of microbial nomenclature it has been described as a Candidate species, *Ca.* Trichorickettsia mobilis (Vannini et al. 2014). For simplification, hereafter it will be referred to as Trichorickettsia. Although Trichorickettsia inhabits the macronucleus of *P. multimicronucleatum*, it resides in the cytoplasm of several other ciliate species, such as *P. nephridiatum, Euplotes aediculatus* (Vannini et al. 2014) and *P. calkinsi* (Sabaneyeva et al. 2018). Surprisingly, Trichorickettsia has been shown to be ingestion resistant and to persist in the planarian enterocytes for a week after feeding the worms with liver paste enriched in the cell homogenate prepared using Trichorickettsia-bearing ciliates (Modeo et al. 2020).

Our preliminary observations of the living strains carrying intranuclear Trichorickettsia suggested a very stable symbiotic relationship resistant to temperature shifts and starvation; besides, their rate of propagation seemed somewhat higher than that of Trichorickettsia-free *P. multimicronucleatum* strains. However, our attempts to validate/check this observation by comparing the growth rate of the infected strains and the strains cured by antibiotic treatment have failed (Mironov and Sabaneyeva 2020). Surprisingly, neither antibiotics traditionally used for obtaining aposymbiotic cell lines in paramecia, ampicillin and streptomycin (Kusch et al. 2002; Dusi et al. 2014; Bella et al. 2016; Grosser et al. 2018), nor antibiotics generally administered for treatments of rickettsioses in humans, chloramphenicol and tetracycline (Wisseman et al. 1974; Raoult 1989; Rolain 2007; Walker 2009), could clear *P. multimicronucleatum* infected with Trichorickettsia (Mironov and Sabaneyeva 2020). In most of the experiments, both partners of the symbiotic system survived the treatment with these antibiotics. Otherwise, the host cells had died before the complete loss of the bacterial partner was registered (Mironov and Sabaneyeva 2020). These observations suggested a very stable relationship between the partners in this symbiotic system, and made it a good candidate for an "ideal holobiont" model, where the presence of the endosymbiont is a permanent feature. However, it would be too soon to dismiss the possibility that some other experimental approaches might clear Trichorickettsia from the host cells. At present, administration of naturally occurring antimicrobial peptide complexes, for example, those retrieved from insects, can be considered a promising alternative to treatment with antibiotics (Chernysh et al. 2015; Torrent et al. 2012; Rima et al. 2021).

FLIP7 (Fly Larvae Immune Peptides) is an antimicrobial peptide complex produced by bacteria challenged larvae of the blue blowfly *Calliphora vicina* R.-D. (Diptera: Calliphoridae). It is composed of cell membrane disrupting agents, such as defensins, cecropins, diptericins, proline-rich

peptides and several unknown antibacterial substrates (Chernysh et al. 2015, 2018). This cocktail proved to be efficient against a wide range of Gram-positive and Gram-negative bacteria (i.e. *Staphylococcus aureus, Escherichia coli*, *Klebsiella pneumonia* and *Acinetobacter baumanni*) (Chernysh et al. 2015). The present study was undertaken to check whether this complex would affect the symbiotic system and whether it could be used to obtain aposymbiotic (endosymbiont-free) paramecium cell lines.

It should be emphasized that some representatives of *Rickettsiaceae*, such as *Rickettsia prowazekii*, *R. rickettsii*, *R. conorii* and the closely related species *Orientia tsutsugamushi*, are known to cause relapse of the disease by persisting in tissues of the patients recovered after the infections treated with chloramphenicol or doxycycline, sometimes many years after the recovery (Raoult and Roux 1999; Weissmann 2005; Shaked et al. 1989; Kelly et al. 2017; Sekeyová et al. 2019). This ability to form persister cells implies that irrespective of the bacterial load after the treatment even a single bacterium left could serve as a source of recrudescence in future. Thus, in our experiments we evaluated the presence/absence of the bacteria in the host cell disregarding the bacterial load.

1 Materials and methods

1.1 Cell cultures

Two *Paramecium multimicronucleatum* strains infected with the intranuclear motile endosymbiotic bacterium *Ca*. Trichorickettsia mobilis, LSA11-2 and Kr154-4, were used in the experiments. LSA11-2 strain was collected in the channel in Lucca (Italy) by E. Sabaneyeva in 2011; Kr154-4 strain was isolated in Krasnoyarsk (Russia) by A. Potekhin in 2012. Two endosymbiont free strains of the same ciliate species, CyP5-3(Cyprus, Greece) and Esa3-2 (Saarema, Estonia), isolated by N. Lebedeva in 2016 and in 2017, respectively, were used as controls to exclude the possibility of one strain being particularly sensitive. *P. calkinsi* strain CyL8-33 harboring cytoplasmic Trichorickettsia was used only in TEM study. All strains except LSA11-2 were maintained in the RC CCM culture collection at the Core Facility Center for Cultivation of Microorganisms (Saint-Petersburg State University) and were kindly provided by N. Lebedeva.

Ciliates were kept in glass tubes in the lettuce infusion inoculated with bacteria *Klebsiella aerogenes*. 1.5-2 mL of fresh medium were added twice a week.

1.2 FLIP7 complex extraction

FLIP7 extraction was performed as described elsewhere (Chernysh et al. 2015; Gordya et al. 2017). In brief, diapausing *C. vicina* larvae were inoculated with *Escherichia coli* M17 strain (Microgen, Russia) to induce immune response and left at +25 °C. The next day hemolymph was collected through a puncture of the cuticle into the ice-cold tubes and centrifuged to exclude cell fraction and fats (30 min at 10 000 x g at +4 °C). Supernatant (plasma) was acidified with 0.1% trifluoroacetic acid (TFA) to a final concentration of 0.05%, centrifuged to remove insoluble particles (30 min at 10 000 x g at +4 °C) and applied to reversed-phase SepPak C18 cartrige (Waters Corporation, Milford, MA, USA) for fractionation. Highly hydrophilic compounds were removed by cartridge washing with 0.05% TFA. The compounds absorbed in the cartridge were eluted with 50% acetonitrile solution in 0.05% TFA, lyophilized (FreeZone; Labconco, Kansas City, MO, USA) and stored at −70 °C. Prior to use, the lyophilized sample was dissolved in deionized water and sterilized by filtration through a membrane with a pore size of 0.22 µm (Milliex-GS; Merck Millipore, Billerica, MA, USA).

For further fractionation 20 mg of the lyophilized extract was dissolved in deionized water and applied to Shimadzu LC20 Prominence HPLC system (Shimadzu, Japan) equipped with reversed-phase Vydac C18 column (250 x 10 mm, 5 µm, Grace, USA), equilibrated with 0.05% TFA. Substances were eluted with a linear gradient of acetonitrile (ACN) from 2 to 60% for 60 min on the 2.5 ml / min flow rate. Chromatographic fractions were automatically collected with 1 min intervals. The fractions' optical densities were registered by means of a UV detector at wavelength of 214 nm. The fractions were lyophilized, dissolved in deionized water and tested against infected and non-infected ciliates.

1.3 Whole FLIP7 treatments

In preliminary experiments aimed to approximately determine the range of FLIP7 concentrations to be used in further experiments, only one of Trichorickettsia-carrying strains, Kr154-4, and the endosymbiont-free CyP5-3 strain, which served as a control, were used. Ciliates were washed in the sterile lettuce infusion to get rid of food bacteria and were placed in wells of the 24-well plate containing 0.5 mL of the fresh sterile lettuce medium, 50 cells per well. It should be noted that since FLIP7 production is a costly and laborious procedure, we could not obtain it in quantities which would permit experiments employing many replicates and obtaining quantitative data. Only one replicate was used in each experiment, however, the experiments with low

concentrations of total FLIP7 were repeated three times for three strains, each in four concentrations.

The stock solution of FLIP7 was administered to the wells to adjust the final concentrations to 0.2, 2, 10 and 20 mg/mL. The effect was checked immediately after FLIP7 administration and the observations were continued throughout 1 h after the start of the experiment. In the trials with lower FLIP7 concentrations (20 and 100 μg/mL) the cells were checked for viability in 1 h, 2 h and on the 3d day after administration of FLIP. The check for the ciliate viability was performed by observing the cells in a well using a stereomicroscope MSP-1 (LOMO microsystems, Russia). In order to check if immotile cells were alive, the well was slightly rotated to make the ciliates detach from the bottom of the well. All moving ciliates were considered viable, in rare cases when the ciliates did not resume movement, they were registered as dead. Viability of the motile intranuclear endosymbionts was registered in the ciliates immobilized using a special chamber under a Leica B6000 microscope equipped with DIC and a digital camera DFC 500. The ciliates were considered able to maintain the endosymbiont after the antimicrobial treatment in case at least 5 randomly taken cells in a row from the same well demonstrated motile bacteria in the macronucleus.

Additionally, fluorescence in situ hybridization (FISH) with the species-specific oligonucleotide probe was carried out to confirm the presence of bacteria in the host.

In the second set of experiments the FLIP7 concentration range was 20, 100, 200, and 400 μg/mL. Cells of the two Trichorickettsia-carrying strains, Kr154-4 and LSA11-2, and the control Esa3-2 strain were washed and placed in the wells, as described above. The cells were not fed during the experiment, but 2–3 drops of sterile lettuce infusion were added once in 3 days to compensate evaporation. Each experiment (all four concentrations tested for the three strains) was repeated three times at different days. The effect of FLIP7 was assessed in 1 h and on the 7th day after the start of the experiment. Each experiment was repeated three times. Besides living cell observations, fluorescence *in situ* hybridization using species specific probe, RickFla_430, was carried out to reveal the endosymbiont location in the host cell. In order to follow the changes in the distribution and fine structure of both, the host and the endosymbionts, the cells were fixed for TEM in a week after administration of the whole FLIP7 (100 μg/mL).

1.4 Assessment of FLIP7 HPLC fractions' effects

Analysis of the anti-ciliate activity of 47 HPLC fractions (4–50) was performed using the strain Kr154-4. The cells were washed and placed in the wells as in the previous experiments. The final concentration of all fractions used corresponded to 1.2 mg of whole FLIP per mL (hereinafter – "FLIP7 equivalents/mL"). The ciliates were checked for viability in 5, 30, 60 and 180 min after the start of the experiment. Most active and several "sublethal" fractions were selected, and these were tested at lower concentrations (120, 240 and 360 μg of FLIP7 eqvl./mL). The cells were checked in 1 h after the start of the experiment, which we designated as a short-term experiment. In the long-term experiments viability of the infected Kr154-4 ciliates and the control CyP5-3 cells was checked on the 7th day after administration of the most active fractions at the concentration of 120 μg of FLIP7 eqvl./mL. Cell counts were performed by taking the ciliates one by one using a thin capillary for the fractions.

1.5 Fluorescence *in situ* hybridization

Cells were fixed in 4% paraformaldehyde in 0.1 M PBS for 1.5 h in the glass wells at 4°C, and transferred to the Super Frost slides (Menzel-Gläser, Germany). The excess fixative was removed and the cells were washed for 5 min in 0.2M PBS. After washing, the excess buffer was removed and substituted with 70° methanol for further fixation. After postfixation, which lasted for 15–30 min, the ciliates were briefly washed with 0.2M PBS. Hybridization was performed according to Manz (Manz, 1992) using hybridization buffer containing 30% formamide and the species-specific probe RickFla_430 (5'-TCTTCCCTGCTAAAAGAACTTT-3') (Vannini et al. 2014) labeled with Cy3 and the nearly universal eubacterial probe Eub338 (5'-GCTGCCTCCCG-TAGGAGT-3') (Amann et al. 1990) labeled with FAM in a wet chamber placed in a TDB-120 dry block thermostat (SIA BIOSAN, Latvia) at 46 °C for 1.5 h. Then the slides were washed twice in the washing buffer at the same formamide concentration at 48 °C, each time for 30 min. Then the slides were mounted in Mowiol 488 mounting solution containing PPD antifade and DAPI to counterstain the nuclei prepared in accordance with the manufacturer's instructions. The slides were analyzed with a Leica SPE5 confocal laser scanning microscope. Three lasers with the 365, 488, and 555 nm wave lengths were used for scanning. The obtained images were processed and analyzed with a Fiji 64 software.

1.6 Transmission Electron Microscopy

Cells were fixed with 2.5% glutaraldehyde diluted with 0.1 M cacodylate buffer (pH 7.4) for 1 h. Then the cells were washed in the same buffer containing 12.5% sucrose and postfixed in 1.6% OsO_4 (1 h at 4 °C). After postfixation the cells were dehydrated in ethanol gradient followed by ethanol/acetone mixture (1:1), 100% acetone and embedded

Table 1 The effect of whole FLIP7 on the viability of *P. multimicronucleatum* and their ability to maintain Ca. *Trichorickettsia mobilis* in the short term (1 h) and long term (7 days) experiments after administration. Ciliate strains: "*T. m.* – free" – Esa3-2 (control); "*T. m.* – bearing-1" – Kr154-4; "*T. m.* – bearing-2" – LSA11-2

Ciliate status	FLIP7 concentration							
	20 µg /mL		100 µg /mL		200 µg /mL		400 µg /mL	
	1 h	7 days	1 h	7 days	1 h	7 days	1 h	7 days
T. m. - free	alive / T-	alive / T-	alive / T-	dead / T-	dead / T-	dead / T-	dead / T-	dead / T-
T. m. – bearing-1	alive / T+	alive / T+	alive / T+	alive / T+	dead / T-	dead / T-	dead / T-	dead / T-
T. m. – bearing-2	alive / T+	alive / T+	alive / T+	alive / T+	alive / T+	alive / T+	dead / T-	dead / T-

in Epoxy embedding medium (FlukaChemie AG, St. Gallen, Switzerland) according to the manufacturer's protocol. The blocks were sectioned with a Leica EM UC6 Ultracut, and ultrathin sections were stained with aqueous 1% uranyl acetate followed by 1% lead citrate. All samples were examined with a JEM-1400 transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 90 kV.

2 Results

2.1 The effect of the whole FLIP7 on *Paramecium multimicronucleatum/Ca.* Trichorickettsia mobilis symbiotic system

Preliminary experiments using high (0.2, 2, 10 and 20 mg/mL) and low (20 and 100 µg/mL) FLIP7 concentrations were performed only once to estimate the range of concentrations to be tested further. 50 cells of the endosymbiont bearing Kr154-4 strain and 50 cells of the control CyP5-3 strain were subjected to treatment with the high FLIP7 concentrations (50 cells of each strain per each concentration) and checked immediately after the treatment and throughout 1 h after the beginning of the experiment. Administration of FLIP7 at high concentrations caused death of ciliates, both, of the endosymbiont bearing Kr154-4 strain and of the control CyP5-3 strain. The highest concentration of FLIP7 (20 mg/mL) caused death of all Trichorickettsia-carrying ciliates as early as in 1 min after the administration of antimicrobial peptide cocktail, while in the lowest concentration (0.2 mg/mL) all infected cells died in 20 min. The control Trichorickettsia-free cells demonstrated similar timing data for all concentrations. Preliminary experiment with lower FLIP7 concentrations (20 and 100 µg/mL) lasted for 3 days, since ciliate death was not registered in 1 or 2 h after FLIP7 administration in any strain. Trichorickettsia-free ciliates died on the 3^{d} day after the start of the experiment in FLIP7 at 100 µg/mL. Trichorickettsia-carrying cells remained viable.

The range of FLIP7 concentrations used in further experiments was 20, 100, 200, and 400 µg/mL. Viability of ciliates of the two Trichorickettsia-bearing strains, Kr154-4 and LSA11-2, and of the control Trichorickettsia-free strain Esa2-3 was tested in three independent experiments performed at different days. In each experiment 50 cells of each of the three strains were subjected to the treatment with four different concentrations of FLIP7 (20, 100, 200, and 400 µg/mL). The effect of the short-term (1 h) and long-term (7 days) treatments with lower FLIP7 concentrations on the viability of the ciliates and the ability of the infected cells to maintain the endosymbiotic bacteria in their macronucleus is summarized in Table 1. At 20 µg/mL, FLIP7 did not affect ciliate viability in the experimental (Kr154-4 and LSA11-2) and in the control (Esa2-3) strains. The same was true for the short-term treatment with 100 µg/mL FLIP7, however long incubation of ciliates at this concentration of the antimicrobial complex led to cell death in the control strain, while both infected strains remained viable. The control strain and one of the infected strains, Kr154-4, demonstrated susceptibility to FLIP7 used at 200 µg/mL as early as in 1 h after the beginning of the experiment, while the other Trichorickettsia-bearing strain, LSA11-2, appeared to be more resistant. Therefore, in the further experiments using FLIP7 fractions only the more susceptible strain Kr154-4 was used. The highest concentration administered in these experiments (400 µg/mL) proved to be deleterious for both, endosymbiont-bearing and endosymbiont-free strains.

As demonstrated by living cell observations and fluorescence in situ hybridization (FISH) with the species-specific oligonucleotide probe RickFla_430, in all experiments the surviving ciliates of the LSA11-2 and Kr154-4 strains maintained *Ca*. Trichorickettsia mobilis in their macronucleus (Figs. 1 and 2). Interestingly, in 7 d after FLIP7 administration at 100 µg/mL the cytoplasm of living host cells abounded in vacuoles containing spherical particles, presumably modified Trichorickettsia (Fig. 1a). In FISH experiments, most part of Trichorickettsia were revealed in the macronucleus of the host cell, however, single bacteria surrounded by DAPI-positive chromatin were released from

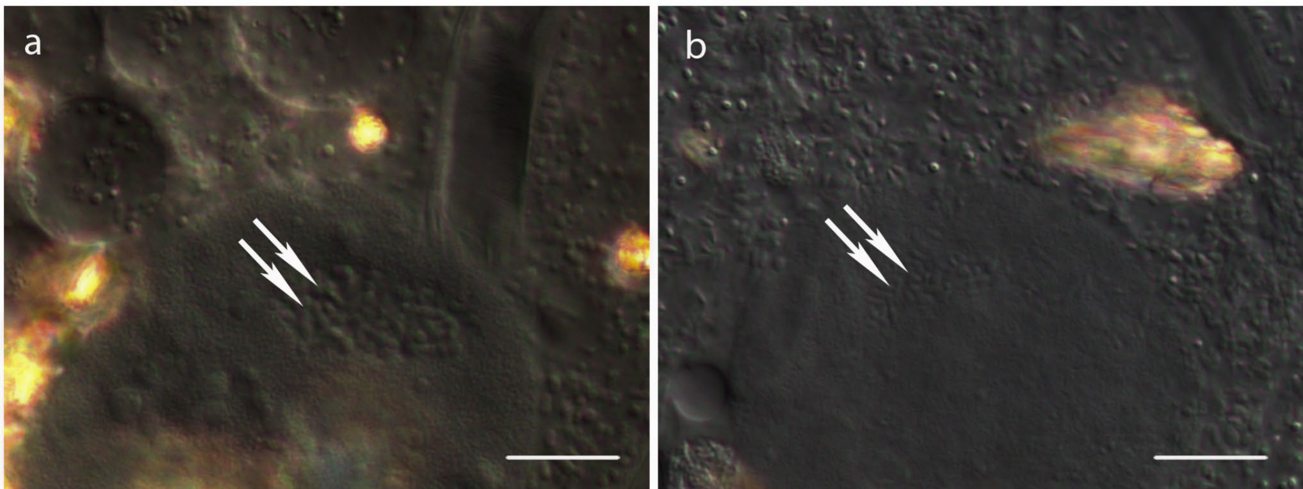

Fig. 1 Living *Paramecium multimicronucleatum* with endosymbiotic Trichorickettsia in the macronucleus, DIC. a – 7 days after FLIP7 administration, b – untreated cell. Arrows point to Trichorickettsia. Scale bar: 10 µm

the host macronucleus into the host cytoplasm (Fig. 2). In TEM sections of the ciliates fixed in a week after administration of the whole FLIP7 at 100 µg/mL, ovoid forms of Trichorickettsia resided in the host nucleus (Fig. 3a, b). Their appearance resembled that of the bacteria found in non-treated paramecia (Fig. 3c): these forms usually contained virus-like electron-dense particles and occasionally had electron-lucid areas, corresponding to PHA inclusions. In the cytoplasm of Trichorickettsia-bearing ciliates subjected to FLIP7 treatment large vacuoles packed with bacterial forms much smaller in diameter than the oval forms and overloaded with PHA inclusions were registered (Fig. 3a, c). No virus-like particles could be discerned in Trichorickettsia located in the vacuoles. Surprisingly, a few smaller forms with PHA inclusions were noted outside the host cell in the vicinity of the ciliate cortex, suggesting that these forms could be released from the host cell (Fig. 3d).

2.2 The effect of FLIP7 HPLC fractions

In order to determine the effective fractions of FLIP7, ciliates of the infected strain Kr154-4 were subjected to the treatment with 47 different fractions of FLIP7 (final concentration 1.2 mg of FLIP7 eqvl./mL). The activity of the fractions against the ciliates is shown in Fig. 4. In 5 min after the treatment all ciliates died in the presence of fractions 12, 16–19, 23, 26–39. In the case of three fractions (7, 15 and 20) ciliates appeared swollen in 20 min after administration of the fraction and died in 30 min; these fractions were considered “sublethal”. In fractions 6, 14, 24 and 25 the ciliates did not demonstrate any changes in their appearance, however, they also died within 30 min, so these fractions were also registered as “sublethal”. In the rest of the fractions the ciliates stayed viable, motile bacteria being present in the macronucleus.

Since the goal of the further study was not only to compare the viability of Trichorickettsia-bearing and Trichorickettsia-free cell lines and the ability of the former to maintain their endosymbionts in various FLIP7 fractions, but also to detect the fraction which would clear Trichorickettsia from the host cells, so that we could obtain an aposymbiotic cell line, 11 fractions (7, 12, 15, 17, 20, 23, 26, 30, 31, 33 and 36) were selected for further experiments. Among the selected fractions were both, the fractions shown to be deleterious for the host cell (12, 17, 23,26, 30, 31, 33 and 36) and the “sublethal fractions” (7, 15 and 20). As seen from Table 2, at lower concentrations (120, 240 and 360 µg of FLIP7 eqvl./mL), these fractions demonstrated different effects after a short-term treatment (1 h). Both, the endosymbiont harboring Kr154-4 cells and the control CyP5-3 cells survived in all fractions used at a concentration of 120 µg of FLIP7 eqvl./mL. Administration of these fractions at a concentration of 240 µg of FLIP7 eqvl. /mL led to the death of the Trichorickettsia-free control ciliates in the fractions 20 and 33, while in all other fractions the ciliates survived. Remarkably, Trichorickettsia-carrying Kr154-4 cells survived in all fractions at this concentration. With a concentration of 360 µg of FLIP7 eqvl. /mL, fractions 20, 26, 33 and 36 caused death of the control non-infected ciliates within 1 h after administration of the fraction, and only fraction 33 proved to be lethal for the Trichorickettsia-carrying Kr154-4 strain. In all concentrations of all FLIP7 fractions tested, motile endosymbiotic bacteria were always registered in the surviving ciliates of the Kr154-4 strain.

In the long-term experiments, Trichorickettsia-bearing strain Kr154-4 and Trichorickettsia-free strain CyP5-3 were treated with the same selected fractions at the low

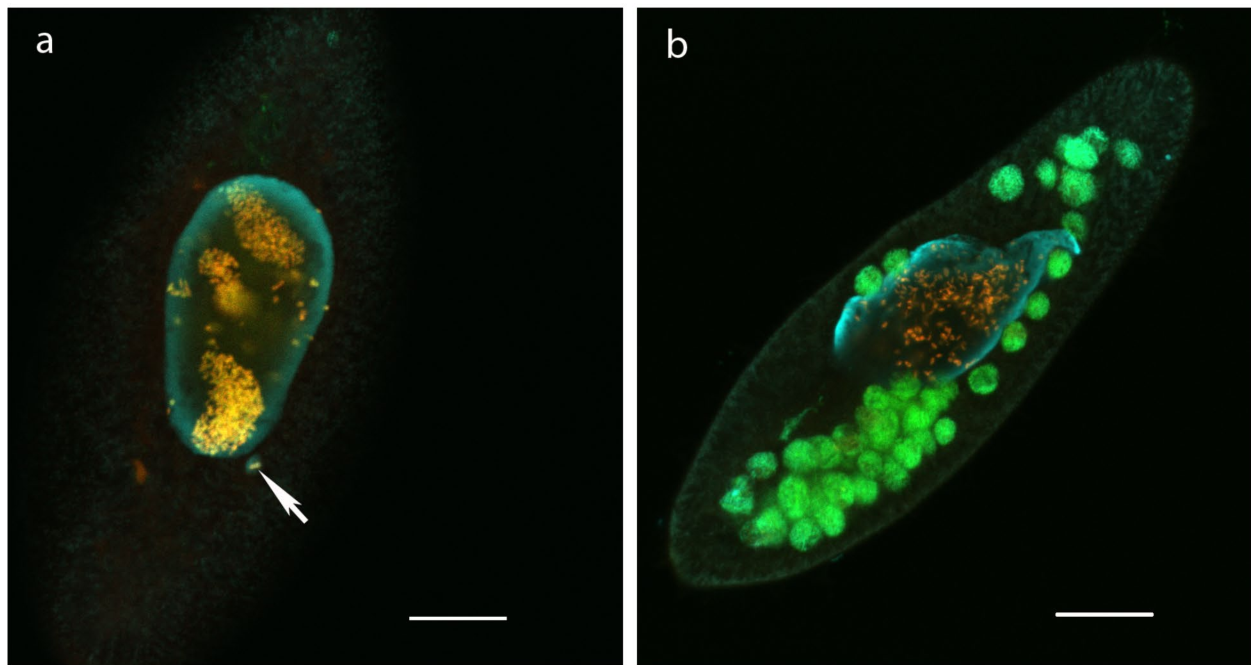

Fig. 2 Fluorescence in situ hybridization of *P. multimicronucleatum* carrying endosymbiotic Trichorickettsia with the species-specific RickFla_430 probe (red signal) and the nearly universal probe for eubacteria Eub338 (green signal), confocal laser scanning microscopy. a – 7 days after FLIP7 administration, b – untreated cell. Trichorickettsia are orange-yellow due to overlay of the two channels. Arrow points to the bacteria released from the host nucleus. Scale bar: 20 µm

concentration (120 µg of FLIP7 eqvl./mL) for a week. Viability of the Trichorickettsia-bearing Kr154-4 cells and the endosymbiont-free strain CyP5-3 differed drastically by the 7th day after administration of most of these fractions. All of the endosymbiont-free cells died in all the fractions administered at 120 µg of FLIP7 eqvl./mL tested, while the ciliates of the endosymbiont harboring strain Kr154-4 died only in the 7th, 17th and the 33[d] fraction. Surprisingly, in the 12th and the 30th fractions the host cell number seemed to increase (68–70 cells instead of 50 taken into the experiment), implying that about at least one third of the cells must have divided at least once and suggesting that these fractions did not affect the host cell cycle. As in the previous experiments, all surviving cells of the Kr154-4 strain continued to maintain motile bacteria in their macronucleus.

3 Discussion

Antimicrobial peptides are suggested to be very promising agents against a wide range of Gram-negative and Gram-positive bacteria including antibiotic-resistant strains (Chernysh et al. 2015) as well as some fungi, protists and viruses (Boulanger et al. 2006; Huan et al. 2020; El-Dirany et al. 2021). Bacteria of the order Rickettsiales are characterized by a wide host range, *Wolbachia*, a representative of the family *Anaplasmataceae*, parasitizing insects (Dumler and Walker 2005). This fact suggests that antimicrobial peptides produced by insects might possess a certain activity against rickettsia, which are known to be resistant to many antibiotics (Rolain et al. 1998; Rolain 2007). Nevertheless, in our experiments, we failed to obtain aposymbiotic (endosymbiont-free) cell lines by administration of the antimicrobial peptide complex FLIP7 or its fractions to the *P. multimicronucleatum* strains bearing Trichorickettsia in the macronucleus. The response of Trichorickettsia-harboring strains to the treatments with FLIP7 and its HPLC fractions conforms to our results obtained in the experiments using antibiotic treatments (Mironov and Sabaneyeva 2020) and, in a way, resembles the "all-or-none" principle of physiology. Either no effect is registered, the host cells looking healthy and keeping proliferating endosymbionts in their macronuclei, or the ciliates die, presumably, together with their endosymbionts. This feature of Trichorickettsia is in

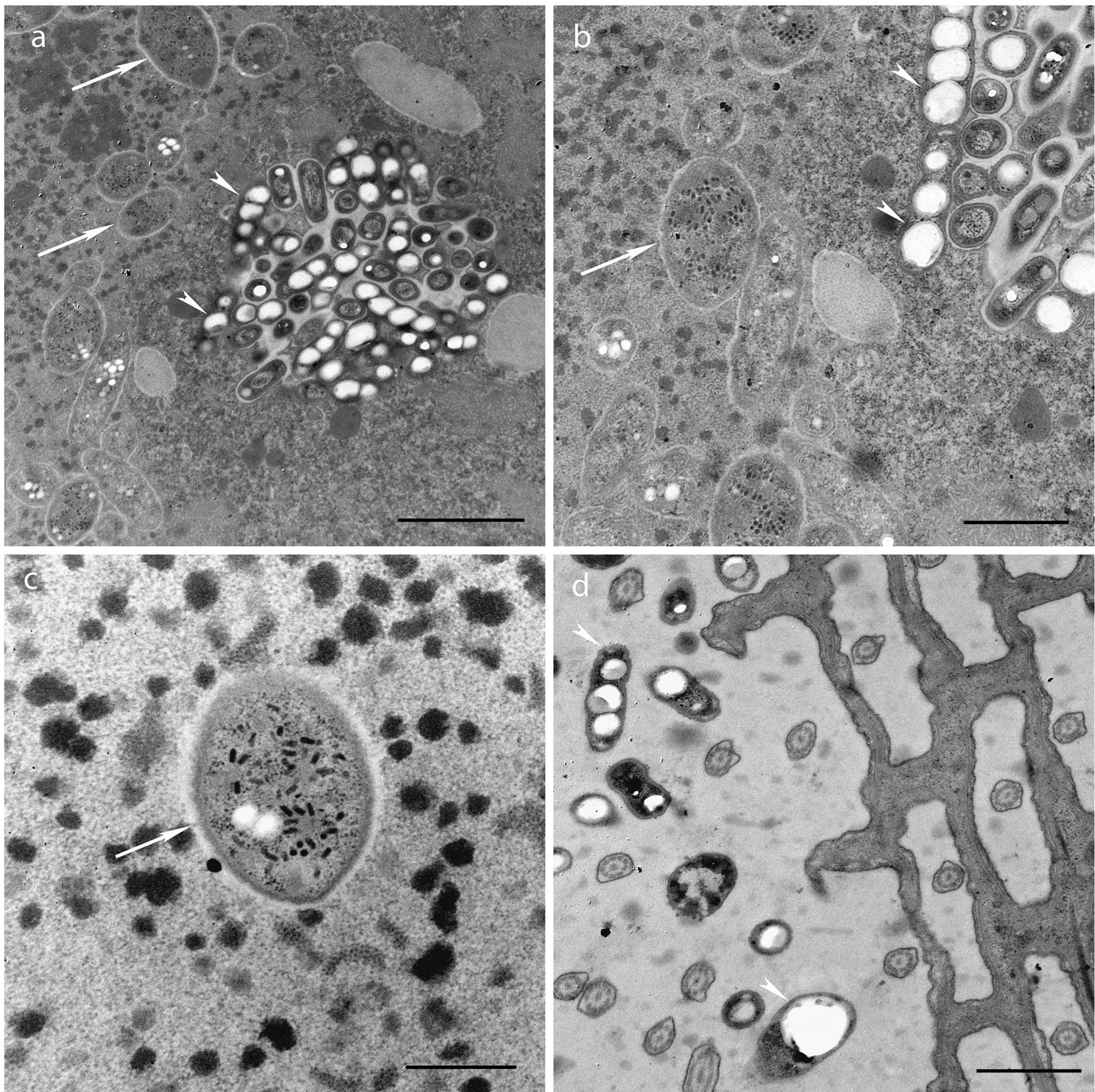

Fig. 3 Fine structure of the two forms of Trichorickettsia in *P. multimicronucleatum*, transmission electron microscopy. a, b, d - after FLIP7 administration, c – control. Arrows point to the forms with virus-like particles, arrowheads – to the smaller forms with lipid granules. Scale bar: a – 2 µm, b, d – 1 µm, c – 0.5 µm

good agreement with the well-known resistance to antibiotic treatments demonstrated by pathogenic bacteria from the family Rickettsiaceae causing severe human diseases (Rolain et al. 1998; Rolain 2007).

High concentrations of FLIP7 proved to be harmful to ciliates, arguing for its anti-protist activity. Surprisingly, the strains harboring Trichorickettsia demonstrated higher viability after administration of FLIP7 or its HPLC fractions than the control strain, which was especially evident in the experiments using fractions 20, 26 and 36. It is indeed remarkable that the control non-infected ciliate strain appeared more sensitive to the antimicrobial peptide complex and its fractions than the Trichorickettsia-bearing strains. Although it would be too early to irrevocably state that it is the presence of the endosymbiotic bacteria in the macronucleus of *P. multimicronucleatum* that makes the

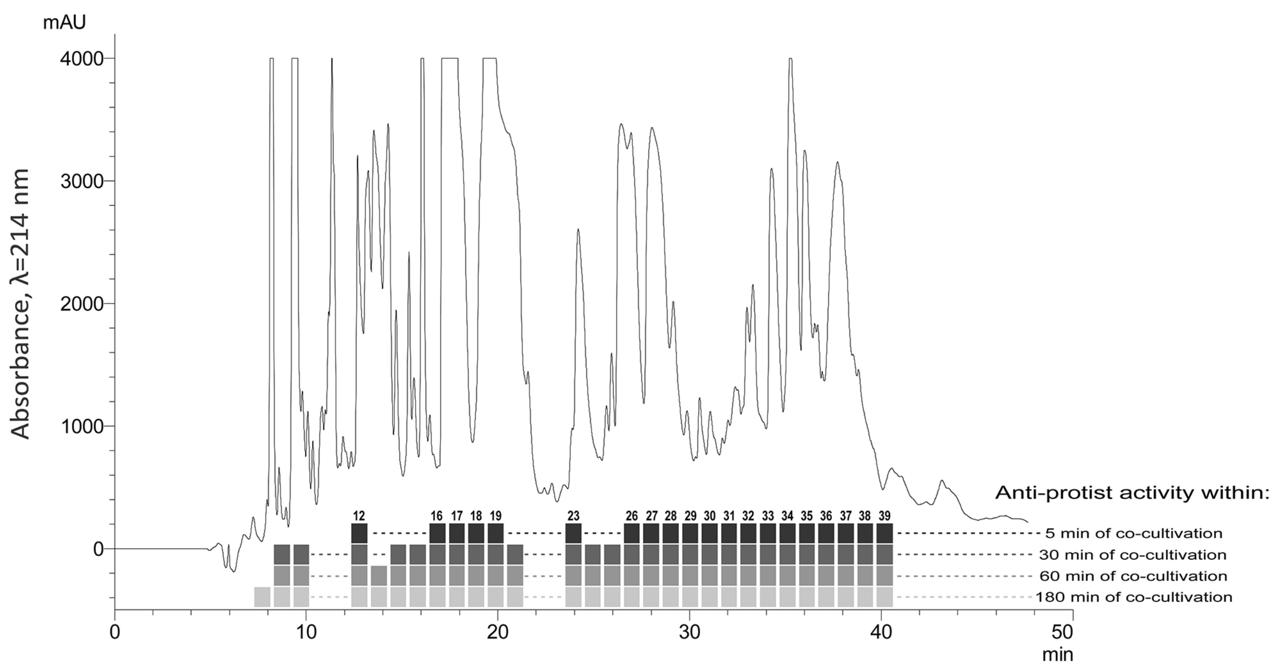

Fig. 4 Anti-ciliate activity of chromatographic fractions of the antimicrobial peptide complex FLIP7 (1.2 mg FLIP7 eqvl./mL) tested on Trichorickettsia-bearing *P. multimicronucleatum*. The anti-ciliate activity of each fraction was mapped on the chromatographic profile (histograms in the areas of the corresponding fractions) with respect to the time of ciliate death

strains resistant against antimicrobial peptides, this possibility should be taken into consideration. Since we have failed to obtain an aposymbiotic cell line, it is not feasible to find an unequivocal answer to the question whether Trichorickettsia confers to its host special features, or resistance to antibiotics and antibacterial peptides is just an intrinsic character of the particular ciliate strains independent on the presence of the endosymbionts.

In our HPLC chromatography experiments the fractions of FLIP7 were eluted basing on their hydrophobic properties. The rate of cytotoxicity of most of the FLIP7 fractions which were found to be effective against ciliates in our study in general corresponded to that of the fractions which have been shown to contain antimicrobial peptides of *Calliphora vicina* (Chernysh et al. 2018; Gordya et al. 2018), implying that FLIP7 activity against ciliates is due to antimicrobial peptides. However, some of the fractions (from 7 to 20) demonstrating anti-ciliate activity do not conform to the known AMP-containing fractions, which might mean that these fractions comprise some compounds with specific anti-protist properties.

In the short-term experiments, only one of the fractions (33), appeared to be effective against Trichorickettsia-carrying ciliate. This fraction has been shown to contain a cecropin with MW 4156 Da (Gordya et al. 2018). Insect cecropins are linear amphipathic alpha-helical peptides particularly effective against Gram-negative bacteria (Chernysh et al. 2018) as well as against some protists (Boulanger et al. 2006; El-Dirany et al. 2021). Possibly, it is the presence of cecropins that account for the anti-ciliate activity of this fraction.

Trichorickettsia-carrying P. multimicronucleatum subjected to FLIP7 treatments harbored two distinct populations of the endosymbiont: the nuclear ovoid form containing virus-like particles and the cytoplasmic smaller form enriched in PHA inclusions. Thus, the stress caused by administration of the antimicrobial peptide complex FLIP7 causes release of the bacteria from the nucleus into the cytoplasm similarly to the effect observed after some antibiotic treatments (Mironov and Sabaneyeva 2020). Although the smaller forms were registered within vacuoles in the host cytoplasm, they resembled “naked” (host membrane-free) Trichorickettsia scattered in the cytoplasm of *P. calkinsi* (Sabaneyeva et al. 2018), the main difference being the presence of flagella in the latter case.

Trichorickettsia localized in the vacuoles in the host cytoplasm and those registered outside of the host cell were rich in polyhydroxyalcanoate granules (PHA), the presence of which has been already recorded in cytoplasmic Trichorickettsia inhabiting *P. calkinsi* (Sabaneyeva et al. 2018). PHA are known to be synthesized by bacteria in unbalanced growth conditions; they serve as a source of energy and carbon supply and facilitate bacterial survival under stressful conditions (Kadouri et al. 2005; Tian et al. 2005; Pötter and Steinbüchel 2006). Interestingly, some lipid modifications have been shown to serve as important mechanisms of

Table 2 Viability of Trichorickettsia-bearing and Trichorickettsia-free *P. multimicronucleatum* strains after administration of chromatographic fractions of the antimicrobial peptide complex FLIP7 in the short-term experiment. «+» - ciliates survive within 1 h after fraction administration, «-» - ciliates die within 1 h

Fr. No.	Ciliate status	FLIP7 chromatographic fraction concentration		
		120 µg FLIP7 eqvl. /mL	240 µg FLIP7 eqvl. /mL	360 µg FLIP7 eqvl. /mL
7	T. - free	+	+	+
	T. - bearing	+	+	+
12	T. - free	+	+	+
	T. - bearing	+	+	+
15	T. - free	+	+	+
	T. - bearing	+	+	+
17	T. - free	+	+	+
	T. - bearing	+	+	+
20	T. - free	+	-	-
	T. - bearing	+	+	+
23	T. - free	+	+	+
	T. - bearing	+	+	+
26	T. - free	+	+	-
	T. - bearing	+	+	+
30	T. - free	+	+	+
	T. - bearing	+	+	+
31	T. - free	+	+	+
	T. - bearing	+	+	+
33	T. - free	+	-	-
	T. - bearing	+	+	-
36	T. - free	+	+	-
	T. - bearing	+	+	+

resistance to antimicrobial peptides in Gram-negative bacteria (Moravej et al. 2018). PHA are mostly found in free-living bacteria, however, they have been demonstrated in the symbiotic species, *Rhizobia* and *Burkholderia*, as well (Lakshman and Shamala 2003; Kim et al. 2013). The ability of *Burkholderia* to produce PHA is believed to play an important role in sustaining its infectious levels and, moreover, to affect the host fitness (Kim et al. 2013). Last, but not least, it has been proposed, that PHA granules are produced in environmentally acquired endosymbionts, and not in vertically transmitted bacteria (Kim et al. 2013). Likewise, it seems that Trichorickettsia exploits the same strategy. On the one hand, synthesis of PHA in these endosymbionts could be concomitant with the development of small forms, which can be later released from the host cell to ensure their survival in the environment and, possibly, their horizontal transmission. It should be noted, that although Trichorickettsia and the closely related genus, *Ca.* Megaira, are not host specific and, thus, theoretically, could possess infectious capacity, the way of their transmission still remains unclear (Modeo et al. 2020). On the other hand, it is the synthesis of PHA by symbiotic Trichorickettsia that might account for the higher fitness of the Trichorickettsia-carrying ciliate strains compared to Trichorickettsia-free ones. The mode of the anti-protist action of antimicrobial peptides is believed to be based firstly on membrane permeabilization (Torrent et al. 2012), and the presence of extra lipid supply could be an important factor ensuring resistance of ciliates to the membrane-tropic chemical compounds.

4 Conclusions

The antimicrobial peptide complex FLIP7, especially some of its HPLC fractions, is active against the ciliate *P. multimicronucleatum.* Trichorickettsia-carrying strains demonstrated higher resistance to FLIP7 and its fractions, than Trichorickettsia-free strains, suggesting that this endosymbiont could contribute to the host fitness. Administration of antimicrobial complex failed to produce any aposymbiotic cell lines, which implies that *P. multimicronucleatum/Ca.* Trichorickettsia mobilis symbiotic system may be regarded as a single entity and may serve as a perfect holobiont model in further cytological and genetic studies.

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