Host and symbiont intraspecific variability: The case of *Paramecium calkinsi* and "*Candidatus* Trichorickettsia mobilis"

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Abstract

Newly isolated strains of the ciliate *Paramecium* calkinsi and their cytoplasmic bacterial endosymbionts were characterized by a multidisciplinary approach, including live observation, ultrastructural investigation, and molecular analysis. Despite morphological resemblance, the characterized *P. calkinsi* strains showed a significant molecular divergence compared to conspecifics, possibly hinting for a cryptic speciation. The endosymbionts were clearly found to be affiliated to the species "*Candidatus* Trichorickettsia mobilis" (*Rickettsiales*, *Rickettsiaceae*), currently encompassing only bacteria retrieved in an obligate intracellular association with other ciliates. However, a relatively high degree of intraspecific divergence was observed as well, thus it was possible to split "*Candidatus* Trichorickettsia" into three subspecies, one of which represented so far only by the newly characterized endosymbionts of *P. calkinsi*. Other features distinguished the members of each different subspecies. In particular, the endosymbionts of *P. calkinsi* resided in the cytoplasm and possessed numerous peritrichous flagella, although no motility was evidenced, whereas their conspecifics in other hosts were either cytoplasmic and devoid of flagella, or macronuclear, displaying flagellar-driven motility. Moreover, contrarily to previously analyzed "*Candidatus* Trichorickettsia" hosts, infected *P. calkinsi* cells frequently became amicronucleate and demonstrated abnormal cell division, eventually leading to decline of the laboratory culture.

Keywords: Atomic force microscopy (AFM); Bacterial endosymbionts; Ciliophora; Fluorescence in situ hydridization (FISH); rRNA gene insertions; Symbiosis

Introduction

Symbiosis between ciliates and bacteria is a well-known phenomenon (Fokin 2004; Görtz 2001; Ossipov 1981; Preer et al. 1974; Preer and Preer 1984; Schweikert et al. 2013). For a long time investigations of such associations have been focused on specific model systems, mainly *Paramecium*/*Holospora* (Fokin and Görtz 2009; Fujishima 2009), *Paramecium*/*Caedibacter* (Schmidt et al., 1987, Schrallhammer and Schweikert, 2009) or *Euplotes*/*Polynucleobacter* (Boscaro et al., 2013a, Heckmann and Schmidt, 1987, Vannini et al., 2012). However, in the course of time, extensive sampling worldwide has demonstrated that phylogenetic diversity of both hosts and endosymbionts is rather high. Considering the continuous advances in our knowledge due to new discoveries, it is likely that so far we have been dealing only with the tip of the iceberg (Castelli et al. 2016; Fokin 2012; Görtz and Fokin 2009). Moreover, it has been argued that ciliates could serve as a natural reservoir for potential pathogens of animals and humans (Görtz and Michel 2003; Schrallhammer et al. 2011; Watanabe et al. 2016), similarly to

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Acanthamoeba, which can host *Legionella*, the causative agent of severe pneumonia (Magnet et al., 2015, Molmeret et al., 2005), or some strains of *Mycobacterium* (Mura et al. 2006). As revealed by direct sequencing of the SSU rRNA gene of the endosymbionts inhabiting ciliates and by phylogenetic analysis of the obtained sequences, a great number of these bacteria belong to the order *Rickettsiales* as defined by Szokoli et al. (2016a). According to present knowledge, this group comprises only obligatory intracellular bacteria of a wide range of eukaryotic hosts (Dumler and Walker 2005, for a recent review see Castelli et al. 2016), which are also considered closely related to the mitochondrial ancestors (Emelyanov, 2001, Gray et al., 1999, Rodríguez-Ezpeleta and Embley, 2012, Wang and Wu, 2015). This makes studying rickettsial endosymbionts of ciliates enticing from the evolutionary viewpoint. More specifically, some of the microorganisms invading ciliates fall into the family *Rickettsiaceae* (e.g. Ferrantini et al., 2009, Schrallhammer et al., 2013, Vannini et al., 2014). This family includes infamous bacteria, which cause severe diseases in humans, such as epidemic typhus (*Rickettsia* prowazekii), Rocky Mountain spotted fever (*Rickettsia rickettsii*), or Mediterranean spotted fever (*Rickettsia conorii*), reviewed in Raoult and Roux (1997), Renvoisé et al. (2011). Representatives of *Rickettsiaceae* in particular, and *Rickettsiales* in general, have been long believed to invade only arthropods and vertebrates as interchanging hosts. More recently, several non-conventional *Rickettsiales* have been found in ciliates, as well as in several other protists (e.g. Dyková et al., 2003, Hess, 2017, Hine et al., 2002, Kawafune et al., 2014, Kuo and Lin, 2013, Schulz et al., 2016, Yang et al., 2016; for a review see Castelli et al. 2016). These data provide grounds for reconsidering the role of protists as ancestral hosts for these bacteria as well as for their possible role in transmission to other host species and in dissemination of non-conventional *Rickettsiales* (or *Rickettsia*-like organisms: RLO) in the ecosystem (Ogata et al., 2006, Perlman et al., 2006, Vannini et al., 2014, Weinert et al., 2009). Studying these protist-associated RLO might also elucidate peculiarities of rickettsial life strategies and various aspects of the host-bacterium "cross-talk".

In particular, two new members of *Rickettsiaceae*, "*Ca.* Trichorickettsia mobilis" and "*Ca*. Gigarickettsia flagellata", have been recently described in ciliates. Interestingly, these two new bacterial species proved to be the closest known relatives of the genus *Rickettsia* within the family (Vannini et al. 2014). The first species shows a broader host range, infecting *Paramecium multimicronucleatum*, *Paramecium nephridiatum* and *Euplotes aediculatus*, while the second one has been retrieved so far only in *Spirostomum minus*. Although ciliate endosymbionts have been traditionally assumed to manifest cell compartment specificity, "*Ca.* Trichorickettsia mobilis" (hereafter referred to as *Trichorickettsia*) can occur either in the macronucleus (*P. multimicronucleatum*), or in the host cytoplasm (*P. nephridiatum* and *E. aediculatus*). The macronuclear *Trichorickettsia* from *P. multimicronucleatum* is highly motile due to the presence of long flagella, while its cytoplasmic counterpart never showed any motility or any traces of flagella, though its general morphology resembles that of the nuclear Trichorickettsia (Vannini et al. 2014). These observations might point to essentially different features present in the same endosymbiont species when infecting different host species and/or when residing in different cell compartments.

Here we describe new isolates of cytoplasmic *Trichorickettsia* found in two populations of a new host, *Paramecium calkinsi*. We will especially focus on peculiar morphology of the endosymbiont, its numerous flagella, and its life strategy. Differences from previously described cytoplasmic *Trichorickettsia* and nuclear *Trichorickettsia* will be discussed and a subdivision of the species in three subspecies is proposed.

Material and Methods

Cell cultures

The *Paramecium* strains CyL 8–32 and CyL 8–33 were isolated from a population inhabiting a stagnant pool of a wastewater stream (salinity ca. 9‰) in Larnaca (Cyprus) close to the Aliki Lake (N 34°53′ E 33°65′) in 2012, while the strains CyL 1–7, CyL 1–22, CyL 1–24 and CyL 1–26 were isolated in the Oroklini Lake area (N 34°59′ E 33°39′) at the same salinity in 2014. Ciliates were maintained in filtered or artificial seawater diluted with boiled lettuce medium inoculated with *Raoultella planticola* and containing β-cytosterol at final salinity 10‰.

For live cell observations, paramecia were immobilized and squashed with a compression device (Skovorodkin 1990) and analyzed using differential interference contrast (DIC) with a Leica 6000 microscope (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with a digital camera DFC 500. Swimming behavior and cell shape were observed with a dissecting microscope.

Molecular characterization

For total DNA extraction of the strain CyL 8–33, approximately 80 *Paramecium* cells were fixed in 70% ethanol. DNA was extracted with NucleoSpin® Plant DNA Extraction Kit (Macherey-Nagel GmbH & Co. KG, Düren NRW, Germany), following the CTAB protocol for mycelium. For the strain CyL 1–24, approximately 10 *Paramecium* cells were subjected to whole-genome amplification (WGA) with the REPLI-g Single Cell Kit (Qiagen, Hilden, Germany), and the WGA product was employed as template for PCR reactions.

For both strains, the nearly full-length eukaryotic SSU rRNA gene was amplified employing the primers 18S F9 5′-CTGGTTGAT CCTGCCAG-3′ (Medlin et al. 1988) and 18S R1513 Hypo 5′-TGATCCTTCYGCAGGTTC-3′ (Petroni et al. 2002), following an amplification protocol involving 40 cycles, annealing temperature of 52 °C, and 2 min of extension time. The internal transcribed spacer (ITS) was amplified with primers 18S F783 5′-GACGATCAGATACCGTC-3′ (Rosati et al. 2004) and RGD2 5′-GGTCCGTGTTTCAAGACGGG-3′ (Boscaro et al. 2012), following an amplification protocol involving 40 cycles, annealing temperature of 52 °C, and 4 min of extension time.

For bacterial SSU rRNA gene characterization of the strain CyL 8–33, the primers of the PCR reaction mix were the *Alphaproteobacteria*-targeting forward primer 16Sα_F19b 5′-CCTGGCTCAGAACGAACG-3′ and the almost universal *Bacteria* reverse primer 16S_R1522a 5′-GGAGGTGATCCAGCCGCA-3′ (Vannini et al. 2004). A touchdown PCR cycle was employed, as described by Szokoli et al. (2016b). For the strain CyL 1–24, the "*Ca*. Trichorickettsia" targeted primer pair RickFla_F87 5′-CTCTAGGTTAATCAGTAGCAA-3′ and R1455_Rick 5′-CCGTGGTTGGCTGCCT-3′ (Vannini et al. 2014) was used for the first amplification, with the same touchdown protocol. Subsequently, two seminested reactions were performed, namely RickFla_F87 with Rick_R1270 (5′-TTTTAGGGATTTGCTCCACG-3′) (Vannini et al. 2014), and 16S F343 ND (5′-TACGGGAGGCAGCAG-3′) (Vannini et al. 2004) with R1455_Rick, employing an amplification protocol involving 40 cycles with annealing temperature 50 °C, and 2 min of extension time.

The obtained PCR products were purified with EuroGold CyclePure Kit (EuroClone S.p.A. Headquarters & Marketing, Pero Milano, Italy). Direct sequencing was then performed at GATC Biotech AG (Köln, Germany) by using the suitable internal primers for eukaryotic SSU rRNA gene (18S R536: 5′-CTGGAATTACCGCGGCTG-3′, 18S F783: 5′-GACGATCAGATACCGTC-3′, 18S R1052: 5′-AACTAAGAACGGCCATGCA-3′; Rosati et al.

2004), ITS (18S F919 5′-ATTGACGGAAGGGCACCA-3′; Rosati et al. 2004) (RGD2: 5′-GGTCCGTGTTTCAAGACGGG-3′; Boscaro et al. 2012), and bacterial SSU rRNA gene (16S F343 ND, 16S R515 ND: 5′-ACCGCGGCTGCTGGCAC-3′, 16S F785 ND: 5′-GGATTAGATACCCTGGTA-3′, Vannini et al. 2004), respectively. The resulting sequences were deposited at the NCBI GenBank (CyL 8–33 eukaryotic partial SSU rRNA gene joint with ITS: KY996811; CyL 1–24 eukaryotic partial SSU rRNA gene joint with ITS: MG018200; CyL 8–33 prokaryotic partial SSU rRNA gene: MF039744).

Phylogenetic analysis

The partial SSU rRNA sequence of the bacterial endosymbiont of *P. calkinsi* CyL 8–33 (the one from CyL 1–24 was omitted since it was identical) was aligned on the SSU Ref NR99 123 database (Quast et al. 2013). Within the ARB software package 5.2, the automatic alignment tool was employed (Ludwig et al. 2004), followed by manual editing to optimize base pairing in the predicted rRNA structure.

For the phylogenetic analyses, 30 other sequences of the family *Rickettsiaceae* were selected, plus seven members of families *Anaplasmataceae* and "*Ca*. Midichloriaceae" as outgroup. The resulting alignment of 38 sequences was trimmed at both ends to the length of the shortest sequence, while all remaining internal positions were kept, with gaps treated as missing data. The final alignment consisted of 1353 nucleotide positions. After selection of GTR + I + G as the best nucleotide substitution model employing ModelTest 2.1 (Darriba et al. 2012), phylogenetic analysis were performed. PhyML 2.4.5 (Guindon and Gascuel 2003) from ARB package was employed for maximum likelihood (ML) (1000 pseudo-replicates), and MrBayes 3.2.6 (Ronquist et al. 2012) for Bayesian inference (BI) (three runs, each with one cold and three heated Markov chains Monte Carlo, 1,000,000 generations, 25% burn-in). Identity values within *Trichorickettsia* were calculated both on the unmodified dataset from phylogeny ("full-sequence"; Table 1), and on the same alignment, excluding the two insertions that are typical in *Trichorickettsia* ("no-insert": 1133 nucleotide positions; Table 2).

Fluorescence in situ hybridization (FISH)

Cells were fixed in a depression slide with 4% paraformaldehyde in PBS for 30 min, transferred onto a glass slide, and washed in PBS. The excess liquid was removed, and the cells were postfixed with ice-cold 70% methanol. The slides were washed in PBS, and a drop of hybridization buffer containing 10 ng/mL of each of oligonucleotide probes and 15% formamide was added. The slides were covered with parafilm and placed in a wet chamber. The hybridization was performed for 1.5 h at 46 °C, followed by two subsequent incubations with a washing buffer at the same formamide concentration, 30 min each, at 48 °C (Manz et al. 1992). Two oligonucleotide probes were simultaneously used for hybridization: *Trichorickettsia* targeted probe Rick_Fla_430 (5′-TCTTCCCTGCTAAAAGAACTTT-3′), 5′-conjugated to Cy3 (Vannini et al. 2014) and the almost universal bacterial probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′), 5′-conjugated to FITC (fluorescein isothiocyanate) (Amann et al. 1990). The slides were mounted in Mowiol (Mowiol 4.88, Calbiochem) diluted in glycerol containing PPD (*p*-phenylenediamine) and DAPI (4′,6-diamidino-2-phenylindole) according to manufacturer protocol. The slides were analyzed with a Leica TCS SPE2 Confocal Laser Scanning Microscope (CLSM). The images were further processed with Fiji-win32 open access software.

Table 1. Percent identity values among the currently characterized SSU rRNA genes of members of *"Ca.* Trichorickettsia" and *"Ca.* Gigarickettsia" on the "full-sequence" matrix (1353 nucleotide positions). The newly characterized sequence of the endosymbiont of *P. calkinsi* CyL 8-33 is highlighted in bold. Values within the currently accepted species threshold (98.65-98.7%) are highlighted in bold.

	1.	2.	3.	4.	5.	6.	7.
1. *"Ca.* Trichorickettsia mobilis subsp. mobilis" endosymbiont of *P. multimicronucleatum* PS23 HG315612	**100**						
2. *"Ca.* Trichorickettsia mobilis subsp. mobilis" endosymbiont of *P. multimicronucleatum* LSA HG315611	**100**	**100**					
3. *"Ca.* Trichorickettsia mobilis subsp. mobilis" endosymbiont of *P. multimicronucleatum* Pm HG315610	**100**	**100**	**100**				
4. *"Ca.* **Trichorickettsia mobilis subsp. hyperinfectiva" endosymbiont of** *P. calkinsi* **CyL 8-33**	97.7	97.7	97.7	**100**			
5. *"Ca.* Trichorickettsia mobilis subsp. extranuclearis" endosymbiont of *P. nephridiatum* PAR HG315614	98.4	98.4	98.4	97.3	**100**		
6. *"Ca.* Trichorickettsia mobilis subsp. extranuclearis" endosymbiont of *E. aediculatus* In HG315609	98.4	98.4	98.4	97.3	**100**	**100**	
7. *"Ca.* Gigarickettsia flagellata" endosymbiont of *Spirostomum minus* SS03 HG315613	93.6	93.6	93.6	93.4	93.7	93.7	**100**

Atomic force microscopy (AFM)

Cells were placed on a cover slip in a tiny drop of the culture medium, punctured or torn with a needle or the edge of a glass capillary, immediately fixed with 2.5% glutaraldehyde and air-dried. Then the cover slip was washed with distilled water to remove the salt crystals and air-dried once again. The material was examined using NTEGRA Aura (NT MDT, Russia) in a semicontact mode.

Transmission electron microscopy (TEM)

Cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer diluted with filtered seawater at 10‰, washed with the same buffer containing 8.5% sucrose and postfixed with 1.5% OsO_4 diluted with the same buffer containing sucrose. The material was dehydrated in a graded series of alcohol and acetone, and finally embedded in Epoxy embedding medium (Fluka, BioChemika) according to the manufacturer protocol. The blocks were sectioned with a Leica EM UC6 ultracut, stained with 1% aqueous uranyl acetate and 1% aqueous lead citrate and examined with a Jeol JM 1400 (Jeol, Ltd., Tokyo, Japan) electron microscope at a voltage of 90 kV. To obtain images of bacterial flagella, the ciliates were squashed in a tiny drop of culture medium, fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and placed on a nickel grid with the formvar supporting film for 30 min to settle down. Then the grids were washed with distilled water and stained with a drop of 1% uranyl acetate. In 1 min the liquid was removed with filter paper, and the grids were air-dried.

Histochemistry

Nile blue A staining was performed according to the protocol proposed by Ostle and Holt (1982) to reveal the nature of inclusions in the bacterial cytoplasm. The host cells (strain CyL 1–24) were briefly washed in distilled water, transferred onto a slide in a small drop of water and punctured or torn with a needle. The bacteria were heat-fixed and stained with 1% aqueous Nile

Blue A (OOO "Reaktiv", Russia) at 55 °C. Then, the slides were washed with tap water and treated with 8% aqueous acetic acid for 1 min. After that, the slides were rinsed in water again and covered with a cover slip. The edges of the cover slip were sealed with nail polish, and the slide was examined with a Leica TCS SPE2 Confocal Laser Scanning Microscope (CLSM) using an excitation wave length of 488 nm, as suggested by Greenspan et al. (1985).

Table 2. Percent identity values among the currently characterized SSU rRNA genes of members of *"Ca.* Trichorickettsia" and *"Ca.* Gigarickettsia" on the "no insert" matrix (i.e. excluding the two adjacent insertions typical in these bacterial endosymbionts: 1133 nucleotide positions). The newly characterized sequence of the endosymbiont of *P. calkinsi* CyL 8-33 is highlighted in bold. Values within the currently accepted species threshold (98.65-98.7%) are highlighted in bold.

	1.	2.	3.	4.	5.	6.	7.
1. "*Ca.* Trichorickettsia mobilis subsp. mobilis" endosymbiont of *P. multimicronucleatum* PS23 HG315612	**100**						
2. "*Ca.* Trichorickettsia mobilis subsp. mobilis" endosymbiont of *R multimicronucleatum* LSA HG315611	**100**	**100**					
3. *"Ca.* Trichorickettsia mobilis subsp. Mobilis" endosymbiont of *P. multimicronucleatum* Pm HG315610	**100**	**100**	**100**				
4. *"Ca.* **Trichorickettsia mobilis subsp. hyperinfectiva" endosymbiont of** *P. calkinsi* **CyL 8-33**	**99.6**	**99.6**	**99.6**	**100**			
5. *"Ca.* Trichorickettsia mobilis subsp. extranuclearis" endosymbiont of *P. nephridiatum* PAR HG315614	**99.7**	**99.7**	**99.7**	**99.5**	**100**		
6. *"Ca.* Trichorickettsia mobilis subsp. extranuclearis" endosymbiont of *E. aediculatus* In HG315609	**99.7**	**99.7**	**99.7**	**99.5**	**100**	**100**	
7. *"Ca.* Gigarickettsia flagellata" endosymbiont of *Spirostomum minus* SS03 HG315613	95.0	95.0	95.0	94.7	95.0	95.0	**100**

Results

Live host cell observations

When observed with a dissecting microscope, the cells of CyL 8–32 and CyL 8–33 strains manifested a slightly distorted body shape and peculiar wriggling movement, atypical for paramecia, while other features (clockwise rotation and the contractile vacuole with a single pore and short channels seen in immobilized cells with DIC) were characteristic of *P. calkinsi* (data not shown) (Fokin 2010/11). Though normally euryhaline paramecia are easily acclimated to the standard medium (lettuce infusion inoculated with *R. planticola* at 0‰ (Smurov and Fokin 2001)), this was not the case with these strains, and laboratory culture could be maintained only at 10‰ salinity. The cell pellicle in these strains appeared rather fragile, as the cells were rapidly squashed with the immobilization device, making acquisition of live cell images impossible. The strains CyL 1–7, CyL 1–22, CyL 1–24 and CyL 1–26 had normal body shape and the way of movement typical for *P. calkinsi* (data not shown), but also could not be acclimated to the standard medium at 0‰ salinity.

In all strains, when visible, the micronucleus was of the "endosomal" type; however, the number of micronuclei (Mi) varied in different strains. A typical set of 2 Mi was present in CyL 1–7 and CyL 1–22; CyL 1–24 and CyL 1–26 had 1 or 2 Mi, while in the strains CyL 8–32 and CyL 8–33, only one Mi was present in the cells soon after their isolation from nature and was completely lost in the laboratory culture, producing amicronucleate cell lines. The cells of the strains CyL 8–32 and CyL 8–33 often could not complete cell division and generated boomerang-looking "monsters" (data not shown), which finally led to the extinction of these cell lines after 2 years of cultivation. Though the strains CyL 1–7, CyL 1–22 and CyL 1–26 never demonstrated abnormal

body shape or peculiar movement, they also became extinct after two years of cultivation, and the only surviving monoclonal culture at present is CyL 1–24.

Live bacterial cell observations

When living immobilized paramecia were observed with DIC, their cytoplasm appeared hyperinfected with bacteria, which sometimes could be hardly distinguished from trichocysts (Fig. 1a). In the intact cells, endosymbionts were always packed so densely, that they seemed nearly motionless. At least, it was not possible to tell whether their motility was intrinsic, or caused by the cytoplasm cyclosis. Interestingly, the infected cells often demonstrated few attached trichocysts, most of which were freely circulating in the cytoplasm.

Upon squashing the host cell, numerous bacteria were released, demonstrating morphological heterogeneity: some bacteria had a long twisted bundle of flagella and were motile, while others lacked this bundle and were immobile (Fig. 1b–d). Bacteria bearing a bundle of flagella could attach to one another by coiling their bundles of flagella, sometimes formed chains, or remained anchored to the paramecium cortex at one spot, producing jerky movements and looking like a "bunch of flowers" (Fig. 1b). The length of the bundle of flagella was approximately 4–5 times longer than that of the bacterial cell and reached 10–12 μm (Fig. 1c, d).

Molecular characterization of the host

Eukaryotic SSU rRNA gene sequence of the host species strains CyL 8–33 and CyL 1–24 of 1702 bp was obtained through direct sequencing. The two sequences were identical, and showed ~98.8% identity in NCBI Blast to sequences of *Paramecium calkinsi* (accession numbers: AF100301 and AF100310; Strüder-Kypke et al. 2000).

The direct sequencing of the PCR product of ITS (including partial 18S rRNA gene – ITS1 – 5.8S rRNA gene – ITS2 – partial 28S rRNA gene) from the host strain CyL 8–33 produced a 1126 bp long sequence, in which the positions of rRNA genes (18S, 5.8S and 28S), ITS1 and ITS2 were inferred from the NCBI Blast alignment with *P. calkinsi* from NCBI nucleotide (accession number JF304160; data not shown). The ITS was similarly obtained for strain CyL 1–24, producing a 1140 bp long sequence, which was 100% identical to the other strain (difference in length is only due to the number of sequenced bases at the 3′ end, within the partial 28S rRNA gene). For each strain, the product was joined in the corresponding part with the respective previously obtained partial SSU rRNA gene, producing final sequences (CyL 8–33: 2745 bp, accession number: KY996811; CyL 1–24: 2759 bp, accession number: MG018200). The predicted length of both spacer sequences, in particular ITS1, resulted shorter respect to the reference, namely 94 bp (positions 1725–1818) vs 119 bp for ITS1, and 167 bp (positions 1965–2131) vs 174 bp for ITS2. Moreover, while NCBI Blast for the ITS2 produced consistent results with respect to the SSU rRNA gene, namely 84.7% identity with other *P. calkinsi* strains (accession numbers JF304160, JF304161, JF304162) (Przyboś et al. 2012), no significant hits were obtained for the ITS1. Further attempts to align manually the ITS1 with relatives within the ARB package failed to produce any putatively acceptable result (data not shown).

Molecular characterization and phylogenetic analysis of the endosymbiont

NCBI Blast results of the nearly full-length bacterial SSU rRNA gene sequence of the endosymbiont of *P. calkinsi* CyL 8–33 (1646 bp, accession number: MF039744) showed 97.3–97.8% sequence identity to different strains of *Trichorickettsia* in *P. nephridiatum*, *P. multimicronucleatum* and *E. aediculatus* (Vannini et al. 2014). The corresponding sequence of

the endosymbiont of *P. calkinsi* CyL 1–24 was 100% identical to the one of CyL 8–33, thus it was not included in the further analyses, avoiding redundancy.

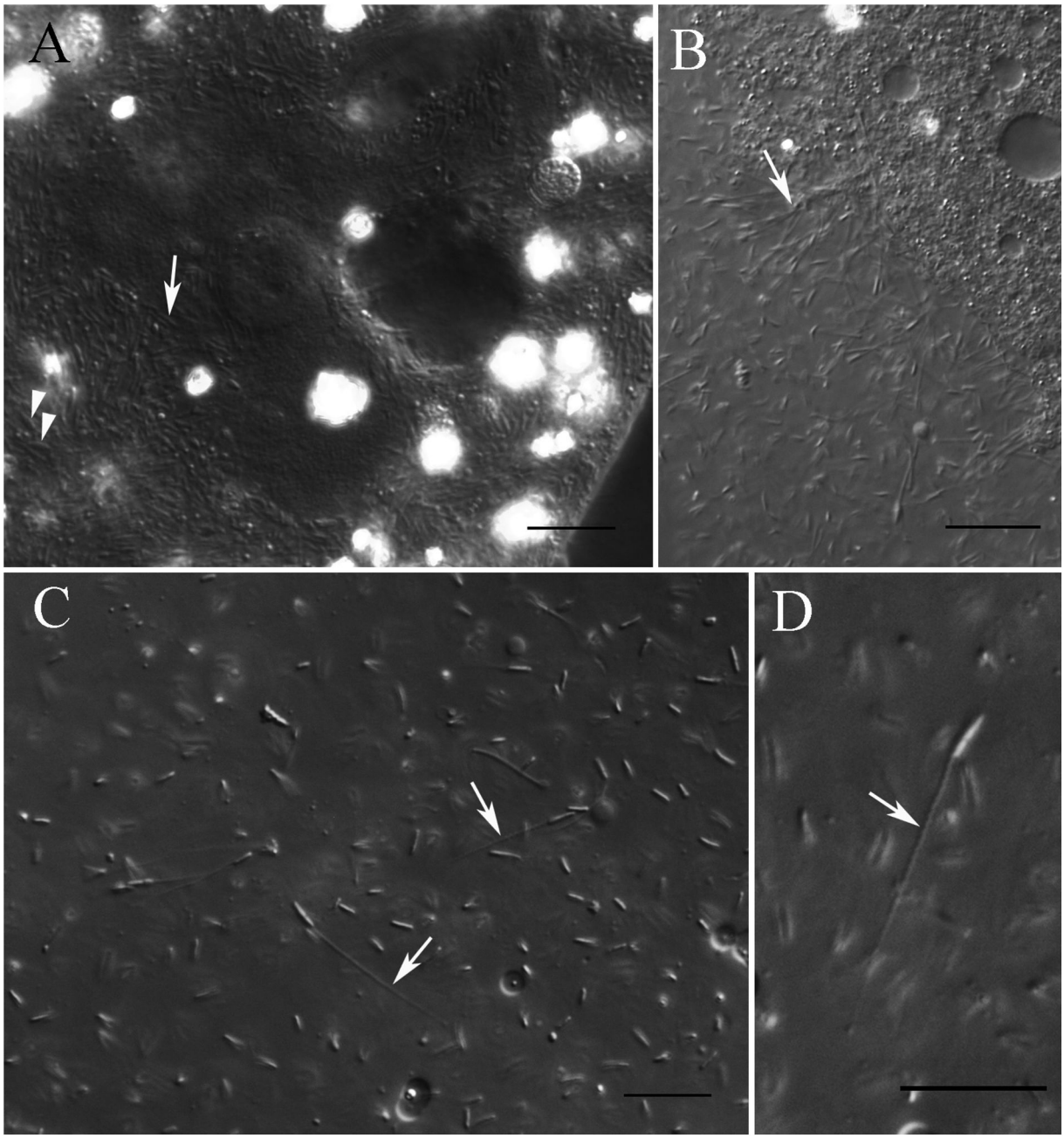

Fig. 1. *Trichorickettsia* in *Paramecium calkinsi* strain CyL 8-33, DIC; a—a fragment of the intact cell, white arrow points to a bacterium in the cytoplasm, trichocysts are shown with arrowheads; b—bacteria released from a squashed cell form a bouquet at the cell cortex (arrow); c—note different appearance of bacteria in the medium; d—a tail-bearing bacterium at a larger magnification, note the twisted appearance of the tail; in c and d arrows indicate the tail. Scale bar 10 μm.

The phylogenetic analysis on representatives of family *Rickettsiaceae* resulted fully consistent with previous analyses on these obligate intracellular bacteria (e.g. Schrallhammer et al., 2013, Vannini et al., 2014). Within the family, well-recognized major clades were retrieved with good support, such as the genera *Rickettsia* and "*Ca*. Megaira" and their main subclades (Fig. 2). As expected, the newly characterized endosymbiont of *P. calkinsi* CyL 8–33 clustered with high support (94 ML; 0.95 BI) within the *Trichorickettsia* clade, together with previously characterized endosymbionts of *Paramecium* and *E. aediculatus*. This clade is closely related to "*Ca*. Gigarickettsia flagellata", endosymbiont of the ciliate *S. minus*, and with genus *Rickettsia*. Within the *Trichorickettsia* clade, the identity values are in the range 97.3–100% (Table 1, "full sequence"). Three subclades appear evident in the new analysis (Fig. 2). The first clade

(100 ML; 100 BI) is represented by the endosymbionts of *P. multimicronucleatum* (three different strains: PS23, LSA, Pm; sequence identity values: 100%). The newly characterized endosymbiont of *P. calkinsi* CyL 8–33 constitutes the second clade, which is more closely associated to the endosymbionts of *P. multimicronucleatum* (96 ML; 0.95 BI), and appears fast-evolving, considering the relatively longer length of its branch in the tree (Fig. 2). The third and last clade is formed by the endosymbionts of *P. nephridiatum* PAR and *E. aediculatus* In, with quite strong support as well (100 ML; 0.75 BI; sequence identity value: 100%). Considering that most of the SSU rRNA gene sequence divergence within *Trichorickettsia* was previously observed in two adjacent inserted regions (Vannini et al. 2014), sequence identity values were calculated excluding those regions (Table 2; "no insert"). As expected, in the "no insert" nucleotide matrix the similarity values among all *Trichorickettsia*, including the newly characterized endosymbiont of *P. calkinsi* CyL 8–33, were considerably higher than in "full-sequence", resulting in the range 99.5–100%.

Fig. 2. BI phylogenetic tree of 31 representatives of the family *Rickettsiaceae* inferred on 1353 SSU rRNA gene nucleotide positions with the GTR + I + G substitution model. The outgroup is represented collapsed as a trapezoidal shape, labeled as "other *Rickettsiales*" (a total of 7 representatives of closely related *Anaplasmataceae* and "Ca. Midichloriaceae" were employed). Posterior probabilities (after 1,000,000 generations) and ML bootstrap values (1000 pseudoreplicates) for each node are reported in the corresponding branch (values below 0.85170 were omitted, respectively). Three genus-level clades are evidenced by the boxes on the right-hand side, namely *Rickettsia,* "*Ca.* Trichorickettsia", and "Ca. Megaira". The newly characterized endosymbiont of *P. calkinsi* CyL 8-33 is evidenced in bold. Next to each representative of "Ca. Trichorickettsia" the presence/absence of observed flagella (F+/F—) and the host subcellular localization in the macronucleus or cytoplasm (M/C) is schematized. The included representatives of each subspecies can be distinguished by a combination of these two features. The scale bar stands for 10% estimated sequence divergence.

FISH visualization of the endosymbiont

FISH using Cy3-labeled *Trichorickettsia*-targeted probe RickFla_430 (Vannini et al. 2014) produced positive signal in *P. calkinsi* CyL 8–33 and CyL 1–24, proving their identity to *Trichorickettsia.* The full overlap with the signal of the FITC-labeled bacterial probe EUB338, resulting in yellow fluorescence of endosymbionts, indicated that, despite the observed morphological heterogeneity, a single species of endosymbiont was present (Fig. 3). The endosymbionts revealed striking density, and, as seen with CLSM, were evenly distributed throughout the host cytoplasm, without any evident preference to any particular region (e.g., cortex or perinuclear area). No endosymbionts have ever been detected in the nuclei. Consistently with live observations, Mi was not detected by DAPI staining in the strains CyL 8–32 and CyL 8–33.

Fine structure of the endosymbiont revealed with AFM and TEM

The size of the endosymbionts, estimated both with AFM and TEM, was ca. 1.5–3 μm, length, and 0.25–0.35 μm, width. Scanning the surface of the endobionts with AFM as well as negative contrast TEM analysis of the bacteria released from the host cytoplasm and stained with uranyl acetate produced a somewhat unexpected result: contrary to DIC images, all examined bacteria demonstrated numerous fine flagella on their surface (Figs. 4a, b, 5). In many bacterial cells the flagella appeared to twist around each other forming a bundle, originating not at the bacterial tip, but always slightly at a side. Interestingly, the origin of the bundle of flagella was usually at the same position in different bacteria. The length of the bundle could not be estimated with these techniques due to tangling of flagella of closely located bacteria. The length of a single flagellum, estimated in TEM images of bacteria, released from the host cytoplasm, was ca. 19–20 μm, while its diameter was ca. 17–18 nm (Fig. 5).

In fine sections of infected paramecia, numerous fine flagella were seen to surround each endosymbiont, occupying a substantial area in the host cytoplasm (Fig. 6a–d). The flagella were evenly distributed all over the bacterial surface, implying that the bacteria are peritrichs (Fig. 6b). The flagella appeared thin (14–15 nm), with a somehow ragged appearance, similarly to those of *Trichorickettsia* endosymbiont of *P. multimicronucleatum* (Vannini et al. 2014).

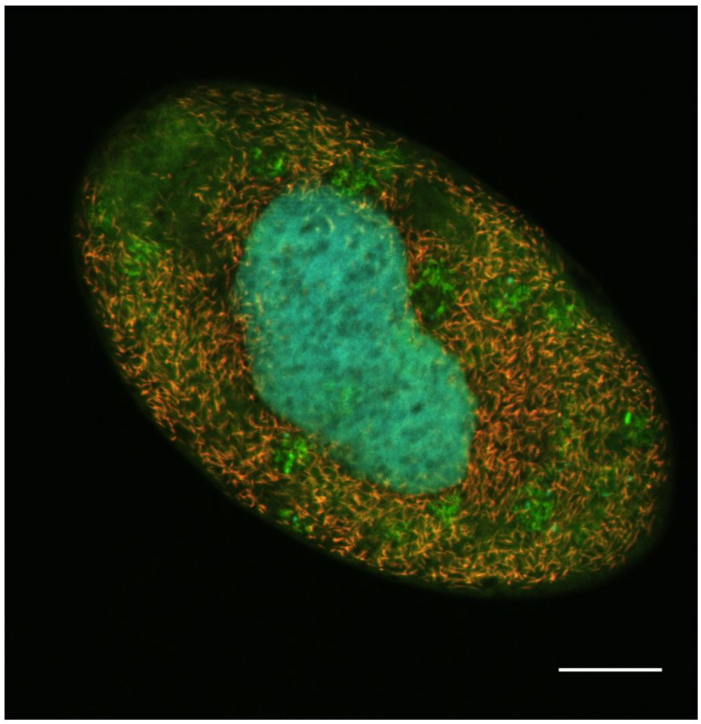

Fig. 3. *Trichorickettsia* in *Paramecium calkinsi* strain CyL 8-33, CLSM. FISH with *Trichorickettsia* probe RickFla_430 (red) and the universal bacterial probe EUB338 (green), macronucleus (Ma) counterstained with DAPI (cyan). A merged image of a Z-stack in the mid-plane of the ciliate obtained in 3 channels. Note the absence of bacteria in Ma and the hyperinfection of the cytoplasm, food bacteria (green) are revealed with EUB338 probe only. Scale bar: 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

In a random transverse section through the *Paramecium* cell, the number of endosymbionts per 100 μm^2 could reach 23–25 (data not shown), supporting our living cell observations and FISH data concerning high bacterial density in the host cytoplasm. The area occupied by the bacterial flagella was devoid of host cell ribosomes or other organelles, however, no host membranes separating the bacterium from the host cytoplasm were observed (Fig. 6a–d). The endosymbionts revealed a two-membrane cell wall typical for Gram-negative bacteria; ribosomes and nucleoid could be detected in their cytoplasm. Practically all bacteria demonstrated large electron lucid

"holes", usually up to 0.3 µm in diameter, but sometimes reaching 0.5 µm (Fig. 6a–d). These "holes" resembled biopolyester droplets (most often, polyhydroxyalkanoate granules), common to many bacteria and washed out in the course of processing for TEM (Pötter and Steinbüchel, 2006, Tian et al., 2005). At the sites of the largest droplets' location bacteria appeared swollen. Contrary to *Trichorickettsia* infecting the cytoplasm *of P. nephridiatum* and the macronucleus of *P. multimicronucleatum* (Vannini et al. 2014), no viral capsid-like structures were detected in the bacteria invading the cytoplasm of *P. calkinsi*.

Histochemistry

Nile blue A staining of *Trichorickettsia* released from the host cell revealed brightly fluorescent roundish granules (0.3–0.4 µm, diameter) in all bacteria present in the field of view (Fig. 7). The number of granules per bacterial cell varied from 2 to 7. The cell wall of bacteria did not demonstrate any fluorescence.

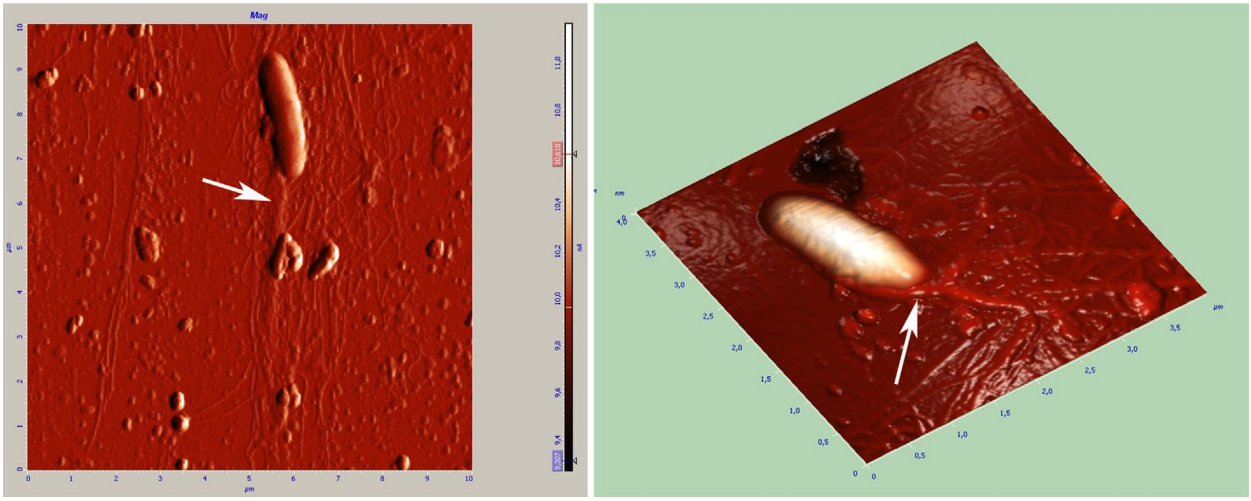

Fig. 4. AFM images of *Trichorickettsia* released from the host cell *(Paramecium calkinsi* strain CyL 1-24); a—Mag, arrow points to the tail formed by numerous fine flagella; b—3D reconstruction showing twisted flagella, arrow. Frame 5x5 µm.

Discussion

Identification of the partners in the symbiotic association

Molecular characterization of the symbiotic partners revealed that the host strains CyL 8–33 and CyL 1–24 are identical to each other, and closely related with previously characterized *P. calkinsi*. This finding is in good agreement with the observed morphological features. However, the sequence divergence was found to be relatively high in SSU rRNA gene (98.8% identity) and ITS2 (84.7% identity). In the case of ITS1, the sequence was significantly shorter in length with respect to relatives due to indels, and the observed divergence was much higher, even preventing to produce an alignment with relatives. Therefore, molecular data show that the intraspecific variability of the morphospecies *P. calkinsi* is rather high, and may even suggest that the characterized strain could represent a new cryptic species, similarly to the cases already evidenced for the genus *Paramecium* (e.g. Catania et al., 2009, Przyboś and Tarcz, 2016, Tarcz et al., 2014). Further analyses, including sequences of additional molecular markers and deeper morphological investigations for potential finer discriminatory characters, will be necessary to fully clarify this point. Such analyses were beyond the aim of the present study.

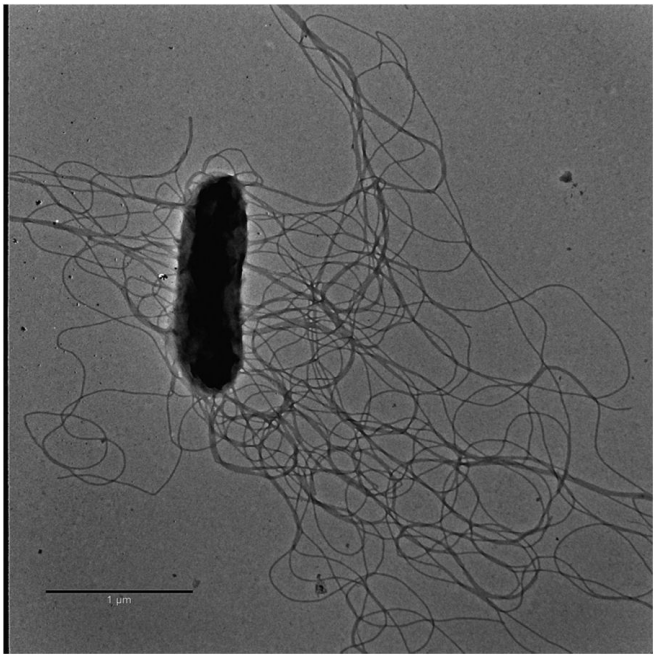

Fig. 5. TEM images of *Trichorickettsia* released from the host cell *(Paramecium calkinsi* strain CyL 1-24), negative staining; note numerous flagella. Scale bar: 1 µm.

Trichorickettsia subspecific division

The endosymbionts of *P. calkinsi* CyL 8–33 and CyL 1–24 are identical in their SSU rRNA genes as well. According to phylogeny, the newly characterized endosymbionts are part of the clade *Trichorickettsia*, which encompasses other previously characterized endosymbionts of ciliates (Fig. 2). In accordance with the "full-sequence" matrix (Table 1), several identity values within *Trichorickettsia* (range 97.3–100%) fall below the commonly accepted species threshold for bacterial species of 98.65–98.7% (Kim et al., 2014, Stackebrandt and Ebers, 2006). Therefore, following the taxonomic threshold rule, the three subclades evidenced in phylogeny (Fig. 2) could be elevated at the rank of species. However, it should be taken in account that *Trichorickettsia* (and "*Ca*. Gigarickettsia") SSU rRNA genes contain two inserted elements that are hypervariable, possibly non-functional and subjected to weak purifying selection (Vannini et al. 2014). It can even be hypothesized that they are removed from the mature 16S rRNA, as it occurs in similar cases for other symbionts such as *Caedibacter caryophilus* (Springer et al., 1993, Brown et al., 2015). As a matter of fact, if those insertions are not considered, the subclades can still be discriminated only by the divergence in the remaining part of the gene sequence, but at a significantly lower extent, with identity values (99.5–100%) above the species threshold (Table 2; "no insert"). Therefore, in the case of "*Ca*. Trichorickettsia", it seems more appropriate to follow a less strictly splitting taxonomic approach. Accordingly, we propose to keep all "*Ca*. Trichorickettsia" under a single species, and to elevate the three subclades only at the subspecies level, which may be amended in the future in case a conclusive answer on the nature of the inserted elements is found. We consequently named "*Candidatus* Trichorickettsia mobilis subspecies mobilis" the type subspecies retrieved in *P. multimicronucleatum*; "*Candidatus* Trichorickettsia mobilis subspecies hyperinfectiva" the newly described one; and "*Candidatus* Trichorickettsia mobilis subspecies extranuclearis" the one retrieved in the cytoplasm of *E. aediculatus* and *P. nephridiatum.*

The nature of numerous flagella of *Trichorickettsia*

Considering that, in general, each intracellular bacterium appears to have preferred host subcellular location(s), the ability to move inside the host cell can be regarded as one of the most

important characteristics of such endosymbionts. In this respect, according to the traditional view reported in the Bergey's Manual of Systematic Bacteriology (Dumler and Walker 2005), all *Rickettsiales* have been thought to be non-flagellated bacteria. At the same time, several *Rickettsia* species have long been known to recruit and use host cell actin as an alternative strategy for their movement (Heinzen et al., 1999, Heinzen, 2003), though in a way seemingly different from that of *Listeria* or *Shigella* (Gouin et al. 2005). However, more recently, it has become clear that several *Rickettsiales* do possess flagella, or at least flagellar genes, including representatives of each of the three families (i.e. *Rickettsiaceae*; *Anaplasmataceae*; "*Ca*. Midichloriaceae") (e.g. Boscaro et al., 2013b, Kwan and Schmidt, 2013, Martijn et al., 2015, Sassera et al., 2011, Schulz et al., 2016, Vannini et al., 2014). These findings suggest the presence of flagella in the ancestors of *Rickettsiales*, a feature that must have been lost in several current lineages in the course of evolution.

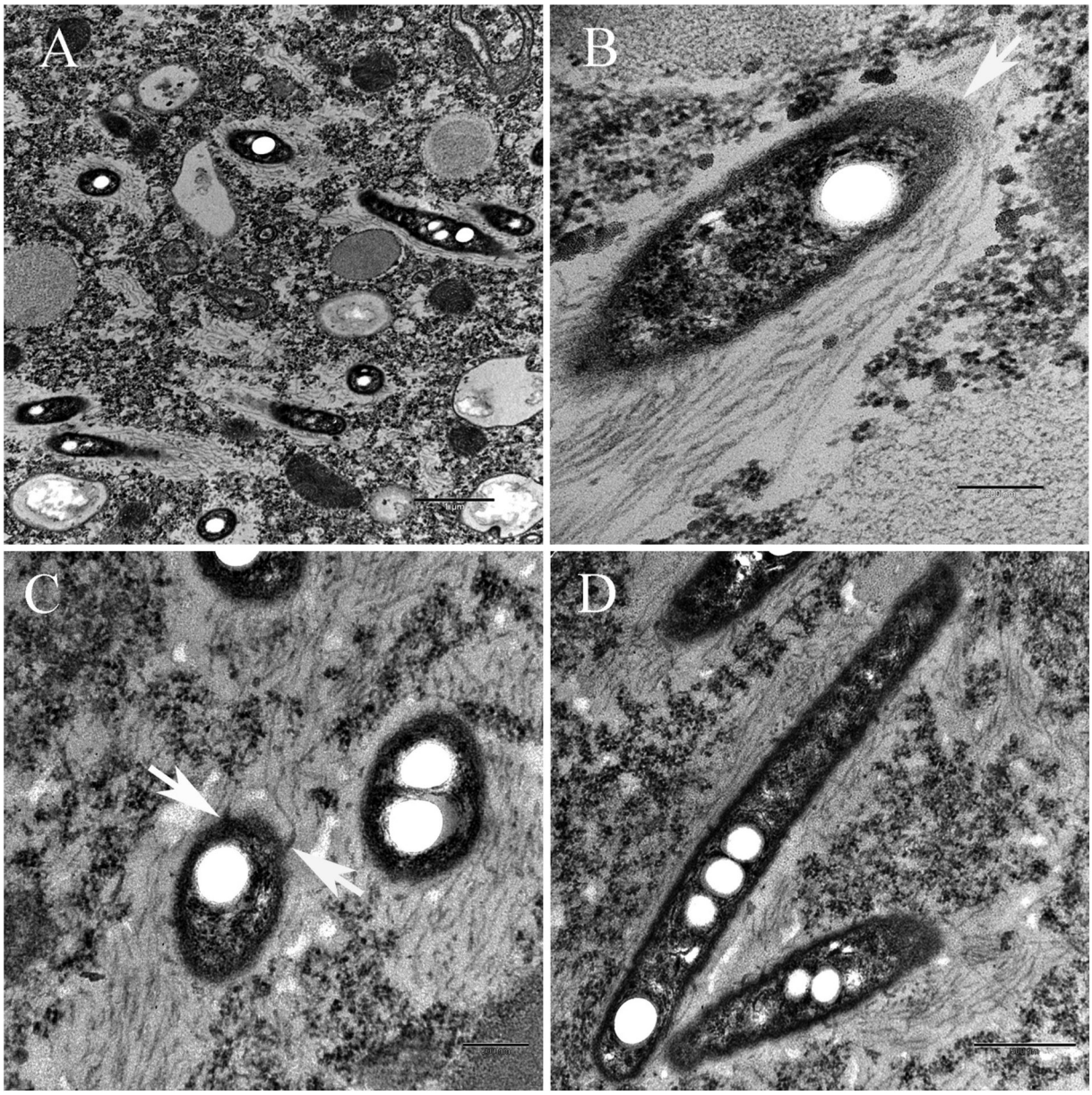

Fig. 6. *Trichorickettsia* in the cytoplasm of *Paramecium calkinsi* CyL 8-33, TEM, fine sections; a—a fragment of the host cytoplasm, note the area occupied by bacterial flagella; b—flagella seen at the front tip of the cell, arrow; c—a hook-like structure anchoring the bacterial flagellum, arrow; d—biopolyester granules located in one part of the cell. Scale bar: a—1 µm, b, c—200 nm, d—500 nm.

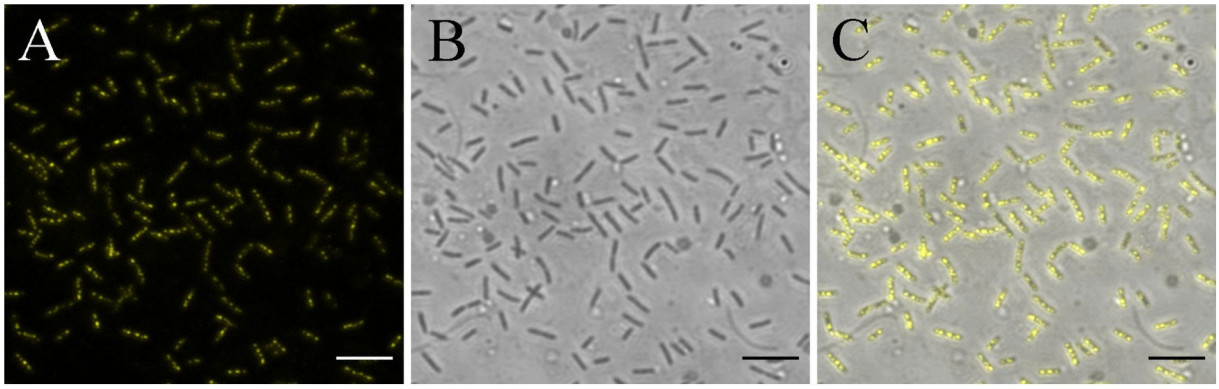

Fig. 7. Nile blue A staining of inclusions in *Trichorickettsia* released from the host cell *(Paramecium calkinsi* strain CyL 1-24), CLSM. a—Fluorescent mode, b—transmission mode, c—overlay. Scale bar: 5 µm.

The feature, distinguishing cytoplasmic *Trichorickettsia* in *P. calkinsi* from its counterpart sharing the same subcellular compartment in *P. nephridiatum* and *E. aediculatus* and bringing it closer to the nuclear *Trichorickettsia* in *P. multimicronucleatum* (Vannini et al. 2014), is the presence of numerous long flagella on all examined bacteria, as shown both with AFM and TEM (Fig. 4, Fig. 5, Fig. 6). Interestingly, this is fully consistent with phylogeny, in which both flagellated and (seemingly) non-flagellated *Trichorickettsia* form monophyletic groups (Fig. 2). Considering also the presence of flagella in "*Ca*. Gigarickettsia flagellata", which forms the sister group of all *Trichorickettsia* (Fig. 2), it is likely that flagella were present in the ancestor of this clade of endosymbionts of ciliates. The lack of flagella in a *Trichorickettsia* subspecies could be explained by a lineage-specific loss of flagellar genes (or by alteration(s) of genes required for flagellar gene expression), although at present it is not possible to exclude that the flagellar gene expression in *Trichorickettsia* is conditional, and dependent on not yet identified external factors, possibly related to a combination of host species and subcellular compartment. Obviously, the fine dimensions of spread flagella did not permit to register them with DIC.

The ragged appearance of the bacterial flagella in TEM suggests that they may be unsheathed. Indeed, the thickness of the appendages estimated with TEM (14–15 nm) is in good agreement with the measurements given by Kirov et al. (2002) for an unsheathed bacterial flagellum (12–20 nm). The ability of flagella to twist around each other forming a bundle when the bacteria are released from the host cytoplasm is a common feature in free-living peritrichs.

The possibility that observed appendages in *Trichorickettsia* could in reality be built of the host cell actin like in a number of *Rickettsia* spp. (Heinzen et al. 1999) rather than true flagella seems very unlikely, not only for the consistence with the presumed presence of flagella in *Rickettsiales* ancestor (Sassera et al. 2011), but also for several morphological features. First the thickness of the appendages observed by TEM (14–15 nm in cross sections, 17–18 nm in bacteria released from the host cytoplasm and stained with uranyl acetate) is similar to bacterial unsheathed flagella, and is not compatible with an actin filament (7 nm). Second, if we consider appendages on the surface of *Trichorickettsia* to be host actin microfilaments and not flagella, it would be difficult to explain the presence of such structures on the bacterial front pole (Fig. 6b) from the biomechanical point of view. Actin-based propulsion of bacteria would more likely require unipolar location of actin microfilaments to produce directional movement, as observed in the case *of Listeria or Shigella* (Gouin et al. 2005). Besides, the appearance of the appendages on *Trichorickettsia* in TEM images differs from the aspect of the tail trailing after *Holospora* using host cell actin to escape from the host food vacuole (Sabaneyeva et al. 2009).

Life strategy in the cytoplasmic *Trichorickettsia*

It is noteworthy, that the longevity of ciliate strains infected with different *Trichorickettsia* subspecies is strikingly different: *P. multimicronucleatum* strains bearing nuclear "*Ca*. Trichorickettsia mobilis subsp. mobilis" never showed hyperinfection, and some of them, in particular Pm (Vannini et al. 2014), have been maintained in the laboratory for about 20 years. On the other side, *P. calkinsi* strains with "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva" in the cytoplasm were always hyperinfected, were difficult to keep in the laboratory conditions, and most of them were lost after two years of cultivation. Moreover, nearly all populations of *P. multimicronucleatum* isolated in Italy bear *Trichorickettsia* in their macronucleus (our observations, unpublished), suggesting that they could display a competitive advantage with respect to *Trichorickettsia* free *P. multimicronucleatum*.

Histochemical analysis of the inclusions present in the cytoplasmic *Trichorickettsia* which are visualized as electron-lucid "holes" in electron micrographs strongly argues for their lipid nature. Commercial Nile blue A, spontaneously oxidized to Nile Red, has been reported to selectively stain polyhydroxybutyrate granules in bacteria (Ostle and Holt 1982). Polyhydroxyalkanoate (PHA) granules are synthesized in bacteria under unbalanced growth conditions with the excess amount of carbon source and serve as a source of energy and carbon depot (Pötter and Steinbüchel, 2006, Tian et al., 2005). Moreover, PHA granules are known to enhance the bacterial survival under stress conditions (Kadouri et al. 2005). Interestingly, PHA granules have been reported not only in free living bacteria, but also in symbiotic bacteria, such as *Rhizobium* (Lakshman and Shamala 2003) and *Burkholderia* (Kim et al. 2013). In the latter species, symbiont's ability to synthesize PHA granules has been shown to be important for sustaining its infectious levels and to affect the host fitness. It has been proposed that the synthesis of PHA granules occurs only in environmentally acquired symbionts, but not in vertically transmitted ones (Kim et al. 2013). Abundance of PHA granules in cytoplasmic *Trichorickettsia* provides further supports to our hypothesis concerning the different life style in "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva", leading to the host cell death. Storage of nutrients and energy supply would be necessary for the bacterium to survive after the host cell lysis until it encounters a new host.

It is worth mentioning that, interestingly, also within the closely related genus *Rickettsia* the representatives of two different subclades are known to demonstrate quite different life styles: spotted fever group (SFG) *Rickettsia* are maintained in the cell in low numbers and are quickly spread from cell to cell, while typhus group (TG) *Rickettsia* accumulate in the host cell until its lysis (Sahni and Rydkina 2009). It is tempting to speculate that nuclear *Trichorickettsia mobilis* subsp. *mobilis* could behave somehow similarly to the SFG *Rickettsia*, whereas cytoplasmic "*Ca*. Trichorickettsia mobilis subsp. hyperinfectiva", causing hyperinfection could be considered analogous of TG *Rickettsia* life style.

The notion that protists, including ciliates, were the probable ancestral hosts of *Rickettsiales* is currently widely accepted (e.g. Castelli et al., 2016, Perlman et al., 2006, Vannini et al., 2014). Additionally, investigations on *Rickettsiales* and other intracellular bacteria in protists proposed further features on these associations. In the study on *Legionella* infection in *Acanthamoeba*, the latter has been figuratively, but very precisely, referred to as "a training ground for intracellular bacterial pathogens" (Molmeret et al. 2005). Moreover, it has been also proposed that "amoeba-like phagocytic ancestral protozoa could have served a genetic melting pot", providing for gene exchange between different invading bacteria and promoting their adaptation to intracellular life style (Ogata et al. 2006).

Along the same lines of reasoning, ciliates in general and the genus *Paramecium* in particular can be considered as a true evolutionary gym for *Rickettsia*-related bacteria, as supported by our findings of *Trichorickettsia* in different *Paramecium* species, and its ability to invade various compartments of the ciliate cell, showing different life strategies and motility in different compartments.

Trichorickettsia subspecies description

Description of "*Candidatus* Trichorickettsia mobilis subspecies mobilis" (Tric.ho.ric.ket'tsi.a mo'bi.lis; Gr. masc. n. *thrix*, hair, N.L. fem. n. *Rickettsia*, from the name of a related genus, N.L. fem. n. *Trichorickettsia*, hairy *Rickettsia*; L. adj. *mobilis*, motile). Rod-shaped bacterium, macronuclear symbiont of the protist ciliate *P. multimicronucleatum*, displaying flagella and swimming behavior inside the host cell. Electrondense cytoplasm, frequently hosting cylindrical particles arranged in a regular fashion and resembling viral capsids. Basis of assignment: SSU rRNA gene sequence (accession number: HG315612). Uncultured thus far.

Description of "*Candidatus* Trichorickettsia mobilis subspecies hyperinfectiva" (hy.per.in.fec.ti'va, Gr. prep. *hyper*, highly, extremely, L. adj, *infectiva*, infectious, N.L. fem. adj. *hyperinfectiva*, highly infectious) Rod-shaped bacterium, displaying peritrichous flagella. Cytoplasmic symbiont of the protist ciliate *P. calkinsi*. Electrondense cytoplasm, no viral particles observed. Basis of assignment: SSU rRNA gene sequence (accession number: MF039744). Uncultured thus far.

Description of "*Candidatus* Trichorickettsia mobilis subspecies extranuclearis" (ex.tra.nu.cle.a'ris; L. prep, *extra*, outside, L. n. *nucleus*, nucleus, kernel, N.L. fem. adj. *extranuclearis*, occurring outside the host nucleus). Rod-shaped bacterium. Cytoplasmic symbiont of the protist ciliates *P. nephridiatum* and *E. aediculatus*. Electrondense cytoplasm, in *P. nephridiatum* hosting icosahedral particles arranged in a regular fashion and resembling viral capsids. Basis of assignment: SSU rRNA gene sequence (accession number: HG315614) Uncultured thus far.

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