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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

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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 *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)