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Characterization of "*Candidatus* Nebulobacter yamunensis" from the cytoplasm of *Euplotes aediculatus* (Ciliophora, Spirotrichea) and emended description of the family *Francisellaceae*☆

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ABSTRACT

Our knowledge of ciliate endosymbionts occurrence and diversity greatly expanded in the last decades, due to the development of characterization methods for uncultivable bacteria. Symbionts related to human pathogens such as rickettsiae and francisellae have been detected inside the cytoplasm of different ciliate species. In the present work, we have characterized a novel *Francisella*-related bacterium inside the rich prokaryotic community harbored by a population of *Euplotes aediculatus* (Ciliophora, Spirotrichea). Following the "Full-Cycle rRNA Approach" we obtained the almost full-length 16S rRNA gene sequence of this bacterium, and developed probes for diagnostic fluorescence *in situ* hybridizations. Attempts to culture the endosymbiont outside of its host failed. We classified this novel organism in a new taxon for which we propose the name "*Candidatus* Nebulobacter yamunensis". In order to investigate its evolutionary relationships, we have also performed phylogenetic analyses on the class *Gammaproteobacteria* and the order *Thiotrichales*, which include the monogeneric family *Francisellaceae*. We found highly supported evidences for the establishment of a new monophyletic taxon including *Francisella* species, other organisms currently *incertae sedis*, and "*Candidatus* Nebulobacter yamunensis". These organisms form a clade sharing a signature sequence not present in other *Thiotrichales* bacteria. Moreover, most of them have developed an intracellular life cycle inside eukaryotic organisms. We emended the original description of family *Francisellaceae* in order to encompass all members of the described clade.

Introduction

Bacteria inhabiting the cytoplasm or a different compartment of an eukaryotic cell are usually labeled as "endosymbionts". The biodiversity and frequency of this category of bacteria in ciliates (Ciliophora, Alveolata) have been recently proven to be very high [9,13,15,28,46,48,49,52,55,56,59,60,62; reviewed in 14,20,21,58]. One of the most striking features of these ciliate–bacteria relationships is their variability. The endosymbiont can act as a parasite [11] as well as a mutualist providing advantages for its host in different systems [29,57] or under different conditions [25,26,51]. It can be highly infectious or rely on vertical inheritance [11,61]. In most cases, these intracellular bacteria show some kind of dependence upon their host, and do not grow on standard culture media outside of their natural habitat in the eukaryotic cell [21]. As can be expected from such different behaviors, they belong to different taxonomic groups [28,48,48,52,56,59,62], and their adaptations to the endosymbiotic life-style have evolved many times independently.

Some of the bacterial endosymbionts of ciliates have received more attentions than others [21]. Bacteria belonging to the genera *Holospora* and *Caedibacter*, for example, have been studied for decades because of their peculiar morphological characteristics and the relevant effects that they produce on their hosts [16,47]. They are also harbored by one of the most studied ciliate taxon, the genus *Paramecium*. The genus *Euplotes*, another very well known ciliate, is usually cited by symbiontologists regarding the presence of the obligate endosymbiont *Polynucleobacter necessarius* in one clade of fresh- and brackish-water species [24,60,61]. Strains of these *Euplotes* species cannot divide properly when the bacteria are removed with ampicillin, and eventually die [24,57].

Since the introduction of reliable molecular methods to study uncultivable bacteria [2,3], a vast number of less-known and much more diverse organisms have been described. In most cases their ecological role, if any, is unknown. Most of them belong to the phylum *Proteobacteria*, and specifically to the classes

☆ *Note*: Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession number HE794998.

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Alphaproteobacteria, *Betaproteobacteria* and *Gammaproteobacteria* [3,28,48,52,55,61]. Even those endosymbionts belonging to the same order can be only distantly related to each other [13,56]. Because the number of papers on this subject is growing, it is probable that the taxonomic diversity of bacterial endosymbionts of ciliates will increase.

One of the intriguing aspects of this field of research is that some of the recently described ciliate endosymbionts are phylogenetically related to known pathogenic bacteria. Some are assigned to the group of "*Rickettsia*-like organisms" [13,52], bacteria closely related to the family *Rickettsiaceae* (*Alphaproteobacteria*), which includes the etiologic agents of diseases like epidemic typhus (*Rickettsia prowazekii*), Rocky Mountain spotted fever (*Rickettsia rickettsii*) and scrub typhus (*Orientia tsutsugamushi*) [63]. Until now, only one example is known of an endosymbiont belonging to the genus *Francisella* (*Francisellaceae*, *Gammaproteobacteria*), which also includes *Francisella tularensis*, the etiologic agent of tularemia [50]. "*Candidatus* Francisella noatunensis subsp. endociliophora" was discovered inside the species *Euplotes raikovi* [48]. *Caedibacter taeniospiralis*, an endosymbiont of *Paramecium tetraurelia*, is also related to family *Francisellaceae*, although less closely [4]. To our knowledge there are currently no dedicated studies, but it would be very interesting to test the infective capabilities of those ciliate endosymbionts related to human pathogens. This could even allow to uncover new environmental reservoirs of old diseases.

In this work, we have characterized another *Euplotes* endosymbiont related to francisellae, for which we propose the name "*Candidatus* Nebulobacter yamunensis", in accordance to the current rules of nomenclature for uncultivated bacteria [39,40]. We employed the "Full Cycle rRNA Approach" [2] to obtain its 16S rRNA gene sequence, and we developed a specific fluorescent probe in order to diagnose the presence of this novel endosymbiont in future studies. Moreover, we performed detailed phylogenetic analyses aiming not only to elucidate the phylogenetic relationships of the endosymbiont, but also to revise the taxonomy of family *Francisellaceae*. We have identified a more comprehensive taxon whose monophyly is highly supported by all phylogenetic methods employed. This clade includes the genus *Francisella*, *Caedibacter taeniospiralis*, the free-living bacterium *Fangia hongkongensis* and the newly characterized "*Candidatus* Nebulobacter yamunensis". We thus propose to extend the boundaries of the currently monogeneric family *Francisellaceae* Sjöstedt 2005 to encompass all these related organisms.

Materials and methods

Ciliate sampling, culture and identification

Euplotes aediculatus population In was collected on February 2007 in an eutrophic freshwater pond near the Yamuna River (New Delhi, India). Several cells were isolated from the sample and cultured in the original medium periodically enriched with boiled rice grains and diluted with SMB (synthetic medium for *Blepharisma*; [37]). The polyclonal culture was maintained at 19–20 °C on a 12:12 h irradiance of 200 μmol photons m^{-2} s^{-1}. All attempts to isolate monoclonal strains growing under controlled feeding conditions repeatedly failed (single cells did not divide or divided only a few times when fed with *Dunaliella tertiolecta*, *Phaeodactylum tricornutum* or *Enterobacter aerogenes*). The species identification was performed through living and fixed observations with optical and scanning electron microscopes (Fig. 1), as described in Modeo et al. [38]. The pattern of diagnostic characters, especially the number and position of frontoventral cirri, was in accordance with that of *E. aediculatus* [6]. The characterization of the 18S rRNA gene sequence (accession number: FR873713) of this ciliate is reported in Vannini et al. [55] and confirmed the identification.

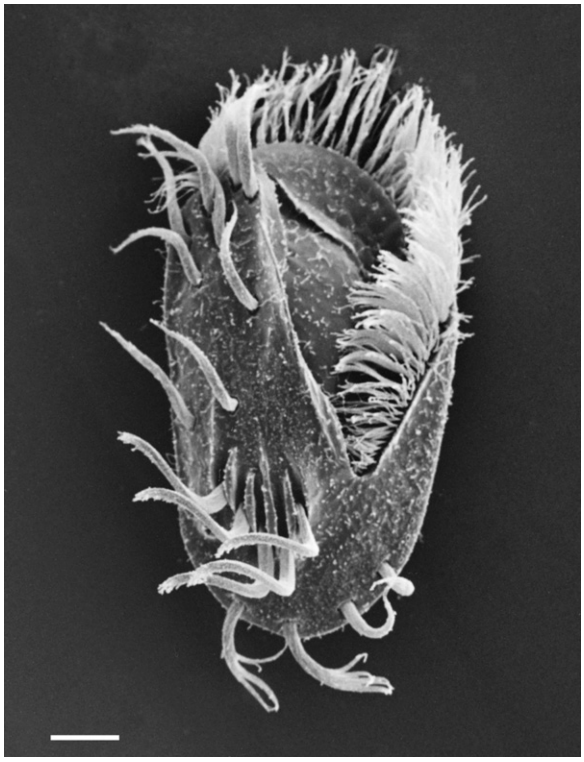

Fig. 1. Ventral view of a *Euplotes aediculatus* cell of the population In at the scanning electron microscope. The bar corresponds to 10 μm.

DNA extraction and 16S rRNA gene sequencing

About 80 cells of the population In were individually collected, washed three times in sterile distilled water and stored in ethanol 70% at −22 °C. Total genomic DNA (tgDNA) was extracted using the NucleoSpin™ Plant II DNA extraction kit (Macherey-Nagel GmbH & Co., Düren NRW, Germany), following the protocol for fungal DNA extraction.

Polymerase chain reactions (PCRs) were performed in a C1000™ Thermal Cycler (BioRad, Hercules, CA) with the TaKaRa Ex *Taq* (TaKaRa Bio Inc., Otsu, Japan). All PCRs started with a preliminary denaturation step at 94 °C for 3 min and a final elongation step at 72 °C for 6 min; denaturation (94 °C), annealing and elongation (72 °C) steps were, respectively, 30 s, 30 s and 90 s long in each cycle. A negative control was included in every reaction. 5 μL of PCR products were evaluated through electrophoresis on 1% agarose gel (*Gelly*Phor LE, EuroClone, Milano, Italy) subsequently stained with ethidium bromide. The remaining products were purified for subsequent uses with the NucleoSpin™ Extract II kit (Macherey-Nagel).

A first attempt to obtain the 16S rRNA gene sequence of the bacterium with primers 16S alfa F19a (5′-CCTGGCTCAGAACGAACG-3′; [62]) and 1492R (5′-GGNWACCTTGTTACGACTT-3′; modified from Lane [30]; annealing temperature: 50 °C, 30 cycles) resulted in a low-quality product. After cloning (kit TOPO TA Cloning®; Invitrogen, Carlsbad, CA) we recovered a putative sequence of the gammaproteobacterial symbiont. We designed two primers of narrower specificity (as described in Petroni et al. [41]) on this sequence. The forward primer Neb_F203 (5′-CTTTAGGGCAGTCGCTATAC-3′) and the reverse primer FNF_R759 (5′-CCACGCTTTCGTCCCTC-3′) were then employed in two new PCR amplifications of the stored tgDNA, the first one with primers