Rediscovering the genus Lyticum, multiflagellated symbionts of the order Rickettsiales

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Among the bacterial symbionts harbored by the model organism Paramecium, many still lack a recent investigation that includes a molecular characterization. The genus Lyticum consists of two species of large-sized bacteria displaying numerous flagella, despite their inability to move inside their hosts’ cytoplasm. We present a multidisciplinary redescription of both species, using the deposited type strains as well as newly collected material. On the basis of 16S rRNA gene sequences, we assigned Lyticum to the order Rickettsiales, that is intensely studied because of its pathogenic representatives and its position as the extant group most closely related to the mitochondrial ancestor. We provide conclusive proofs that at least some Rickettsiales possess actual flagella, a feature that has been recently predicted from genomic data but never confirmed. We give support to the hypothesis that the mitochondrial ancestor could have been flagellated, and provide the basis for further studies on these ciliate endosymbionts.

In the mid-twentieth century, T. Sonneborn revealed two features of Paramecium that had a lasting impact beyond the field of protozoology. First, he identified many morphologically identical strains of “Paramecium aurelia” that were not sexually compatible1. This observation provided one of the first and most extreme cases of a “sibling species” complex – fifteen different species have been described within the P. aurelia species complex until now2-3. Another important discovery was that of “cytoplasmic particles” of various kinds, found many times in several Paramecium species and often able to confer non-genetically inherited traits4. Years later, all these particles were identified as bacterial endosymbionts5.

Many of these symbionts have peculiar biological properties, and sometimes remarkably distinctive morphologies. Examples include the infectious Holospora with its specialized nucleus-invading form6, and the “killer” symbionts, that confer to infected paramecia the ability to kill uninfected “sensitive” strains present in the same culture medium5,7. Much interest was directed to the unusual bacteria belonging to genus Caedibacter (formerly “kappa particles”) and their complex cytoplasmic inclusions, the “R-bodies”8-13. Other equally intriguing killer symbionts were characterized, and among them were those belonging to the genus Lyticum5-7.

Lyticum bacteria appear as large rods (2.0–10.0 μm long) harbored in the hundreds in the cytoplasm of three different species of the P. aurelia complex1,5,14,15. They are non-motile, despite being covered by numerous flagella16. The two species were formally described as Lyticum flagellatum (formerly ”lambda particle”, type species of the genus) and Lyticum sinuosum (“sigma particle”)17. They differ in shape (respectively, straight vs. curved rods) and host specificity (respectively, Paramecium tetraurelia or Paramecium octaurelia vs. Paramecium biaurelia)5,18.

The original descriptions of Lyticum and many other symbionts detected in the last century left many questions unanswered. One of the most important issues from an evolutionary point of view concerns the phylogenetic relationships of these bacteria.

The study of prokaryotic symbionts of protozoa is currently attracting a renewed interest, and is performed with the aid of molecular tools complementing ultrastructural methods like electron microscopy [e.g.19-23]. In recent years, the focus has shifted to the remarkable biodiversity of these organisms and their close relationships with human pathogens, e.g. Rickettsia[26-29] and Francisella[30,31].

In this work, we have characterized the symbionts of P. octaurelia strain 299 and P. biaurelia strain 114 following a multidisciplinary approach. They represent the type strains of L. flagellatum and L. sinuosum,
respectively. We also reported a recently sampled environmental isolate (P. biaurelia USBL-3611) infected by L. sinuosum, for which only one host strain was known so far. Morphology, ultrastructure and killer capabilities of the two bacterial species were investigated and molecular tools for their identification developed and tested. Moreover, we established their phylogenetic relationships, placing them inside the order Rickettsiales (Alphaproteobacteria) together with other obligate intracellular symbionts. This discovery not only clarifies the Lyticum affiliation, but also provides evidence supporting the hypothesis that Rickettsiales, the extant bacteria most closely related to the mitochondrial ancestor, were ancestrally flagellated. This finding provides a relevant contribution in inferring the features of the free-living ancestor of both Rickettsiales and mitochondria, supporting the view that it was motile.

**Results**

**Morphology and ultrastructure.** The cytoplasmic symbionts of P. octaurelia 299 (L. flagellatum) are straight rod-shaped bacteria 0.6–0.9 × 2.0–4.0 μm in size (Fig. 1a–c), while those harbored by P. biaurelia USBL-3611 are bigger – up to 1.1 × 7.8 μm – and curved (Fig. 1d–f), perfectly fitting the description of L. sinuosum. Both are covered by numerous thick, peritrichous flagella about 4 μm long, clearly visible in TEM sections and negative staining. Nevertheless, in vivo observations did not show any sign of motility. The cytoplasm of both kinds of bacteria is homogeneous, with no visible inclusion. They both feature a Gram-negative type cell organization, with two membranes, and the symbionts are enclosed in a membrane-bound vesicle, often with several bacteria inside the same vesicle (Fig. 1b, f). These results are in good accordance with previous descriptions.

**Molecular characterization.** The 16S rRNA gene sequences of the symbionts harbored by strains 114 and USBL-3611 are identical. They differ by 6 out of 1331 (0.5%) sites from the homologous sequence of the 299 symbiont. The most similar sequences available according to NCBI blastn are those of the Acanthamoeba spp. UWC8 and UWC36 symbionts (87.1–88.0% similarity), which belong to the “Candidatus Midichloriaceae” family within Rickettsiales.

Hybridizations with the genus-specific oligonucleotide probe LytiProb_433 (that provides no match on RDP) gave clear signals deriving from bacteria localized in both 299 and USBL-3611 cells at LytiProb_433 (that provides no match on RDP) gave clear signals the features of the free-living ancestor of both Rickettsiales and mitochondria, supporting the view that it was motile.

**Discussion**

The infected Paramecium strains 299 and 114 were sampled almost a century ago. Nevertheless, cultures of these ciliates still retain their original symbionts, although those of strain 114 are almost instantly lost after adaptation to standard cultivation conditions. On the other hand, the stability of the L. flagellatum-P. octaurelia 299 relationship supports the hypothesis that the symbiosis is obligate for the host, which possibly depends on metabolites provided by the bacteria. L. sinuosum has been reported so far only in P. biaurelia 114. We obtained a new environmental isolate of P. biaurelia which is infected by the same bacterial species, as can be inferred by morphology and the identity of 16S rRNA gene sequences. Interestingly, the monoclonal strain P. biaurelia USBL-3611 was established from a water sample collected in the surroundings of the Indiana University, where T. Sonneborn was working at the time of his Lyticum description.

The morphological difference between the two Lyticum species corresponds to a difference in their 16S rRNA gene sequences, albeit small. Due to the diagnostic characters separating the two bacteria and the species-specific probes herein developed we recommend maintaining their status of separate but closely related species.

Although the identification of the described symbionts is sound, we could not repeat previous results concerning the killer trait. This was not entirely unexpected: the original literature describes the death of non-infected paramecia induced by Lyticum as extremely rapid (10–40 minutes), but triggered only in some Paramecium strains belonging to P. triaurelia, P. pentaurelia and P. novaurelia. Those strains were not available for the killer tests performed in this study. Therefore, our results suggest that those sensitive strains were the exception, and not the rule. The common adaptive explanation of the killer trait as a competitive advantage for the hosts would not apply to Lyticum, which apparently has no effect on most strains of the P. aurelia complex, including those belonging to the same species as their hosts (P. biaurelia, P. tetraurelia and P. octaurelia). It is also possible, of course, that the Lyticum killer effect requires specific physiological conditions in the sensitive, the killer and/or its symbiotic bacteria, and that those requirements were not met in our experiments. However, also the recently sampled strain USBL-3611 did not act as a killer. This result makes it highly unlikely that an “ageing” effect of the cultures is responsible for the loss of killer activity.

**Lyticum** clearly belongs to the recently established candidate family “Ca. Midichloriaceae” within Rickettsiales, like several other symbionts of ciliates, amoebas and metazoa [e.g. 1, 4]; a member of this group was also associated to fish disease. The present study enables, for the first time, the assignment of a valid genus to this clade. Like other cytoplasmic bacteria belonging to “Ca. Midichloriaceae” and Anaplasmataceae, but in contrast to members of Rickettsiaceae, Lyticum symbionts are enclosed with an additional membrane, likely of host origin.

On the basis of genome annotations and phylogenomic analyses recently performed on “Candidatus Midichloria mitochondrii”, a hypothesis concerning the presence of flagella and motility in the Rickettsiales mitochondria ancestor was proposed, even though none of the so far characterized Rickettsiales bacteria actually possesses flagellar structures. Additionally to genome-derived evidences, further support is provided by the expression of flagellar genes on
RNA and in one case also on protein level by “Ca. Midichloria mitochondrii”. This hypothesis would confer an important role to motility in the establishment of the ancient symbiotic relationship that turned free-living bacteria into organelles. Our results support this view, revealing for the first time that heavily flagellated bacteria can be found among members of the order, and suggesting that the last common ancestor of Rickettsiales, or at least of “Ca. Midichloriaceae”, possessed flagella. The next step required for corroborating this scenario would be obtaining the sequence of Lyticum flagellar genes, and comparing them with those found in the “Ca. Midichloria mitochondrii” genome to test the alternative hypothesis that they were acquired independently.

“Ca. Midichloria mitochondrii” displays no flagella and is non-motile. Curiously enough, the Lyticum species do not use their flagella for movement. The question arises whether flagella or single flagellar proteins can also serve other than locomotion related functions. In a syntrophic symbiosis between a fermentative bacterium and a methanogenic archaean, the significant role of the flagellar cap protein FliD to synchronize their metabolism was described. One might speculate about an involvement of the numerous Lyticum flagella in establishment or maintenance of the symbiosis with Paramecium, hence this question awaits future analyses.

**Methods**

**Hosts identification and culture.** The P. octaurelia strain 299 and the P. biurelia strain 114 were kindly provided by T. G. Doak and M. Lynch (Indiana University). The P. biurelia strain USBL-3611 was collected in 2011 from a small pond near Spencer (IN, USA, 39°17'45"N, 86°48'10"W). In order to confirm the identity of the host strains, morphological diagnostic features were checked and the mitochondrial cytochrome c oxidase subunit 1 gene (cox1) was sequenced according to Barth and colleagues; sequences are available at EMBL database with the accession numbers HF969031-3. The cultures were maintained at 19°C on a 12:12 h light/dark cycle and fed with Raoultella planticola inoculated in modified Cerophyl medium according to Boscaro and colleagues or, alternatively, with Enterobacter aerogenes inoculated in lettuce medium at room temperature. Strain 114 was obtained several times, but the symbionts were always lost shortly after the paramecia started to propagate. Thus, Transmission Electron Microscopy (TEM), fluorescence in situ hybridizations (FISH) and killer tests could not be performed on this strain.

Transmission electron microscopy. Ciliate cells were harvested by gentle centrifugation and fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at room temperature. After washing in buffer, cells were post-fixed in 1% OsO4 in 0.1 M cacodylate buffer (1 hour at room temperature). Three washing steps in this buffer were performed prior to dehydration in an acetone series and consecutive infiltration into Spurrs resin. Images were taken with a Zeiss EM 10 electron microscope at 60 kV. Alternatively, the cells were fixed in a mixture containing 2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hours at room temperature followed by a wash in the same buffer containing 12.5% sucrose and post-fixation in 1.6% OsO4 (1 hour at 4°C).

Figure 1 | Morphology and ultrastructure of Lyticum species. Negative staining (a) and ultrathin sections (b, c) of L. flagellatum harbored by P. octaurelia strain 299. Negative staining (d) and ultrathin sections (e, f) of L. sinuosum harbored by P. biurelia strain USBL-3611. Bars stand for 1 μm. Arrowheads highlight some of the flagella, arrows point at symbiosomal membranes. M, mitochondria.
Figure 2 | Genus- and species-specific in situ detection of Lyticum flagellatum and Lyticum sinuosum. Merge of the signals from probes EUB338 marked with fluorescein (green) and LytiProb_433 marked with Cy3 (red) on P. octaurelia strain 299 (a) and P. biaurelia strain USBL-3611 (b). The signals coincide, and Lyticum bacteria appear yellowish. Merge of the signals from probes Lflag_268 marked with Cy3 (red) and Lsinu_268 marked with fluorescein (green) on P. octaurelia strain 299 (c) and P. biaurelia strain USBL-3611 (d). At 20% formamide concentration, the probes used in competition are able to discriminate between the species. Bars stand for 20 μm.

Figure 3 | Phylogenetic position of Lyticum species. Bayesian tree built on the unmodified character matrix (60 sequences, 1331 characters) employing the GTR + I + G model (with the continuous gamma distribution approximated by 4 discrete categories). Numbers associated to each node correspond to ML bootstrap values and posterior probability values (values below 70% are omitted); numbers inside trapezoids show the number of sequences used to represent that clade. The bar stands for an estimated sequence divergence of 10%. Ca., Candidatus; unc. bac., uncultured bacterium.
The cells were dehydrated through a graded series of alcohol and acetone and embedded in Epoxy embedding medium (Fluka, BioChemika). Polymerization was carried out according to the manufacturer’s protocol. Ultrathin sections were cut using a Reichert-Jung Ultracut E or Leica UC6, and stained with aqueous 1% uranyl acetate and 1% lead citrate. The samples were visualized using a Jeol JEM-1400 at 89 kV.

For negative staining of bacteria, several Paramaecium cells were briefly washed in distilled water, squashed with a thin glass capillary in a drop of water, and a drop of the resulting suspension was placed on a Pioloform coated grid. Bacteria were allowed to precipitate for 2–3 min, then a drop of 1% uranyl acetate in distilled water was added for no longer than 1 min. The liquid was then absorbed with filter paper and the grid was air-dried.

16S rRNA gene sequencing. The almost complete 16S rRNA gene sequences were obtained through several PCR amplifications of overlapping regions and direct sequencing of the products (299 symbiont), or by cloning of PCR products, RFLP analyses and sequencing of 5 clones showing the most representative pattern to produce a consensus (for details of primers and PCR reactions, see Supplementary Methods Online). The sequences are available at EMBL with the accession numbers HP969034-44.

FISH. Hybridizations were performed according to the protocol of Manz and colleagues on individually collected Paramaecium cells fixed with 2% paraformaldehyde (w/v). Preliminary FISH experiments were performed with the esubacterial probe EUB338 and the alphaproteobacterial probe ALFb1. Oligonucleotide probes specific for the obtained 16S rRNA gene sequences were developed [LytiProb 433 5’-TTCTTCTTCACACAAAGAC-3’], genus Lyticus specific [Lifag, 268 5’-GCTAAAGTGAAGGCTTGTA-3’], L. filagellatum specific [Lanii, 268 5’-GCTAAAGTGAAGGCTTGTA-3’], L. sino assim specific. These novel probes were tested with a wide range (0–50%) of formamide concentrations in the hybridization buffer. Paramaecium strains containing different alphaproteobacterial symbionts were employed as negative controls. Probe specificities were checked also in silico with the ProbeMatch tool of the Ribosomal Database Project (RDP) website and probe data were deposited at probebase.

Phylogenetic analyses. Non-identical 16S rRNA gene sequences obtained were aligned with 42 homologous sequences of Rickettsiales alphaproteobacteria (as outgroup) using the ARB software package. The almost complete 16S rRNA gene sequences were in silico hybridization detection of archaeal and bacterial endosymbionts in the anaerobic ciliate Paramecium. J. Gen. Microbiol. 154, 497–504 (2008).

Killers tests. 5 cells of the putative killer strains (299 or USRL-361I) and 5 cells of putative sensitive Paramaecium strains (see Supplementary Table S1 online) were put together in a depression slide containing 50 µL of sterile Cerephil or lettuce medium. Numbers of motile cells were checked after 30 and 60 minutes. 10 cells of putative sensitive were employed as controls in each experiment, which was independently repeated three times. Attempts with sterile water instead of medium and/or extended observation periods were also performed.

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Author contributions

V.B., M.S., K.A.B., S.K., F.S. and E.V.S. participated in experimental procedures and data analysis. V.B. wrote the main manuscript text. T.U.B., M.S., F.V., E.V.S. and G.P. coordinated the work. All authors reviewed the manuscript.

Additional information

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