Novel Trypanosomatid-Bacterium Association: Evolution of Endosymbiosis in Action

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ABSTRACT  We describe a novel symbiotic association between a kinetoplastid protist, Novymonas esmeraldas gen. nov., sp. nov., and an intracytoplasmic bacterium, “Candidatus Pandoraea novymonadis” sp. nov., discovered as a result of a broad-scale survey of insect trypanosomatid biodiversity in Ecuador. We characterize this association by describing the morphology of both organisms, as well as their interactions, and by establishing their phylogenetic affinities. Importantly, neither partner is closely related to other known organisms previously implicated in eukaryote-bacterial symbioses. This symbiotic association seems to be relatively recent, as the host does not exert a stringent control over the number of bacteria harbored in its cytoplasm. We argue that this unique relationship may represent a suitable model for studying the initial stages of establishment of endosymbiosis between a single-cellular eukaryote and a prokaryote. Based on phylogenetic analyses, Novymonas could be considered a proxy for the insect-only ancestor of the dixenous genus Leishmania and shed light on the origin of the two-host life cycle within the subfamily Leishmaniinae.

IMPORTANCE  The parasitic trypanosomatid protist Novymonas esmeraldas gen. nov., sp. nov. entered into endosymbiosis with the bacterium “Ca. Pandoraea novymonadis” sp. nov. This novel and rather unstable interaction shows several signs of relatively recent establishment, qualifying it as a potentially unique transient stage in the increasingly complex range of eukaryote-prokaryotic relationships.
While the most intricate relationships seem to have evolved between different bacteria and sap-feeding insects (8), unicellular eukaryotes have also engaged in such associations, leading to profound changes in their lifestyle and ensuing important evolutionary and ecological implications. The best known examples include cyanobacteria in the cercozoan Paulinella chromatophora, nitrogen-fixing bacteria in parabasalids of termites, and methanogenic archaea in anaerobic ciliates and pelobiontids (9, 10). However, the findings of endosymbiotic bacteria in parasitic protists are frequently confined to mere descriptions, as is the case for cytoplasmic bacteria in the apicomplexan Gregaria garnhami (11), the dinoflagellate Hematodinium sp. (12), the heterokont Blastocystis sp. (13), or the ciliate Balantidium joculatorum (14). More attention has been given to the recently discovered facultative symbiosis between two sexually transmitted agents, the flagellate Trichomonas vaginalis and the bacterium Mycoplasma hominis (15). This association is of medical importance, as it likely leads to a more severe disease manifestation (16).

The most extensively studied endosymbiont-containing protists belong to the family Trypanosomatidae ( Euglenozoa, Kinetoplastea), a group of obligatory parasites found in a wide range of arthropods, vertebrates, and plants (17). The best-known representatives are dixenous species (i.e., with two alternating hosts in the life cycle) of the genera Trypanosoma and Leishmania that cause severe diseases in humans and domestic animals, whereas the widest segment of this group’s diversity is represented by monoxenous insect parasites (18, 19). Among those, members of the subfamily Strigomonadinae (genera Strigomonas, Angomonas, and Kentomonas) harbor obligatory symbiotic bacteria of the genus “Ca. Kinetoplastibacterium” (20–22). A common ancestor of this group acquired a betaproteobacterium of the family Alcaligenaceae (22, 23). The ensuing long-term coevolution led to significant changes in the morphology, metabolism, and physiology of both partners of the association. Thus, each trypanosomatid cell bears a single bacterial cell in its cytoplasm, which undergoes a synchronous division with the host cell and is vertically transmitted (24, 25). The endosymbionts lack the cell wall, presumably to ensure intense metabolic exchange with the host cell (26). In endosymbiont-containing trypanosomatids, the corset of subpellicular microtubules gets reorganized in comparison to those of other groups, possibly as a consequence of the extensive branching that is evident in the mitochondrion of those cells. The enlarged mitochondrion might be a consequence of an increased energy consumption by these flagellates compared to the energy consumption of their asymbiotic kin (20, 27). Another characteristic feature is a reduction of the paraflagellar rod (28). It was proposed that the close association of the bacterium with glycosomes in the host cell cytoplasm ensures provision with ATP from the trypanosomatid host (29), which also supplies its partner with phosphatidylinoline required for the endosymbiont’s envelope (30). In return, the bacterium provides enzymes for completing the metabolic pathways for biosynthesis of heme, vitamins, coenzymes, lipids, and essential amino acids within the host cell (31–34). Moreover, the endosymbiont also supplies its host with purines and boosts the production of polyamines, leading to accelerated host cell division (35–37). Trypanosomatis artificialy deprived of bacteria can survive in culture, and yet, they are unable to colonize insect hosts (38), likely due to the altered expression of surface glycoconjugates and gp63-like protease (39, 40).

The endosymbiotic association described above was so far considered a singular event in the evolutionary history of trypanosomatids. However, in the course of a broad-scale survey of biodiversity in Ecuador (41), we have isolated and cultured a new species of trypanosomatid possessing intracytoplasmic bacteria. Neither the eukaryotic host nor the bacterial endosymbiont has close relatives involved in similar endosymbiotic consortia, thus confirming an independent origin of this novel association. Furthermore, the phylogenetic positions of both the trypanosomatid and the bacterial partner of this newly discovered endosymbiotic system suggest that their relationship has been established relatively recently. Here, we characterize this association by describing the morphology and phylogenetic affinities of both organisms, as well as details of their interactions and phylogenetic affinities. We argue that this symbiotic consortium represents a very good model for studying the initial stages of endosymbiosis between a bacterium and a protist.

RESULTS

Isolation, light microscopy, and cultivation. A specimen of Niesthrea vincentii ( Hemiptera: Rhopalidae) collected in July 2008 in the vicinity of Atacames (Esmeraldas Province, Ecuador) was found to be positive for trypanosomatids. The primary culture, labeled E262AT, was established and passaged in brain heart infusion (BHI) medium supplemented with hemin and antibiotics. Next, the trypanosomatids in the primary culture were compared to the corresponding original environmental isolate 262AT (41) by sequencing the spliced leader (SL) RNA gene from both sources. Their sequences exhibited 99% similarity (GenBank accession number KP717858), confirming the identity of the cultured isolate. The clonal axenic culture E262AT.01 was obtained using the limiting dilution method and shown to carry an 18S rRNA sequence (GenBank accession number KT944309) identical to that of the primary culture. Both primary and clonal cultures could be propagated in hemin-free BHI or M199 medium, with the cell division rates being similar regardless of the presence of hemin. Cultured cells could not withstand an elevated temperature (37°C) but, similar to Leishmania, grew faster in the medium with an acidic pH of 5.5 (data not shown).

Light microscopic examination of E262AT.01 revealed the presence of three distinct morphotypes: promastigotes, choanomastigotes with various flagellum lengths, and rarely occurring The amastigotelike cells. Cell measurements are presented in Table S1 in the supplemental material. The proportions of individual morphotypes varied throughout cultivation. Promastigotes prevailed in the early- and mid-log-phase stages, while choanomastigotes dominated in the stationary phase. Cells were also observed forming multicellular rosettes firmly attached to the plastic surface of the cultivation flask (Fig. 1). Occasionally, those rosettes reached a few millimeters in size and contained thousands of cells arranged in multiple layers. Promastigotes divided significantly faster than choanomastigotes. When a stationary-phase culture, composed predominantly of choanomastigotes, was diluted to the same density as a promastigote-dominated culture, it took 14 days to reach the mid-log phase, whereas promastigotes achieved that level in just 8 days (Fig. 1). Alternatively, this lag can be explained by morphotype switching: only promastigotes can divide, and some time is needed for the choanomastigote-promastigote transformation. The addition of antibiotics into the culture medium showed that even at the highest concentrations tested (see Mate-
rial and Methods), elimination of the intracellular bacteria from trypanosomatids did not occur. However, under these conditions, the cells divided considerably more slowly and amassed conspicuously more bacteria than in the absence of antibiotics (data not shown). Importantly, no bacterium-free cells were observed under such growth conditions.

Detection of bacterial endosymbionts by FISH. Giemsa and 4′,6-diamidino-2-phenylindole (DAPI) staining allowed the detection of rod-shaped structures in most E262AT.01 cells. Similar bodies were previously observed in members of the subfamily Strigomonadinae and identified as bacterial endosymbionts (20). In order to confirm the nature of the Giemsa- and DAPI-positive structures, we employed fluorescent in situ hybridization (FISH) using probe Eub338, which recognizes bacterial 16S rRNA (42). An absolute majority of trypanosomatid cells was positive, pointing to their identification as bacterial endosymbionts (Fig. 2A to C). Strikingly, and in contrast to the representatives of the Strigomonadinae flagellates studied so far (24), in the E262AT.01 culture, the number of endosymbionts per host cell varied drastically. It ranged from 0 to 15, with about 6% of the cells lacking an endosymbiont (Fig. 2D). In about 70% of the flagellates, two to six bacteria were usually randomly distributed throughout the cytoplasm (Fig. 2A to C). In some cases, especially in cells with low numbers of bacteria, the latter tended to be located in the vicinity of the nucleus (Fig. 2C). With increasing numbers of bacteria per cell, the proportions of such hosts declined steeply (Fig. 2D).

Isolation of axenic bacterial culture. Upon lysis of the host cells, the bacterial endosymbionts released could be cultured on Trypticase soy agar and propagated in liquid BHI without supplements. The identity of the isolated bacterial culture was confirmed by 16S rRNA gene sequencing (see below). The growth of the bacterial culture was halted by ampicillin and kanamycin at 100 μg/ml and by chloramphenicol at 64 μg/ml (data not shown).

Subcloning of E262AT.01. In order to determine whether it is possible to obtain an endosymbiont-free culture of the E262AT.01, we performed several experiments with cloning by limiting dilution in different media independently in the laboratories in Ostrava, Prague, and České Budějovice (see Table S2 in the supplemental material). None of the 41 subclones obtained,
Phylogenetic analyses. Since individual phylogenetic trees for small subunit (18S) and large subunit (28S) rRNAs and heat shock protein 83 (Hsp83) genes were highly congruent, a concatenated alignment was made to infer the phylogenetic position of the new trypanosomatid. Both maximum-likelihood and Bayesian analyses showed the same topology, positioning it within the subfamily Leishmaniinae (Fig. 3). In fact, the isolate E262AT.01 (hereinafter called *Novymonas esmeraldas* gen. nov., sp. nov.; GenBank accession numbers KT944293, KT944303, and KT944309 for Hsp83, 28S rRNA, and 18S rRNA, respectively) proved to be the closest known relative of the genus *Leishmania*. It is traditionally used for the corresponding culture. The species under study is highlighted.

Bayesian and maximum-likelihood trees of the bacterial sequences were mostly consistent, with just minor differences in the branching order of clades with low bootstrap support (Fig. 4). The trypanosomatid endosymbiont analyzed herein (referred to as “*Candidatus Pandoraea novymonadis*”) is located at the very crown of the genus *Pandoraea* Coenye et al. 2000, being part of the family *Burkholderiaceae* (order *Burkholderiales*, class *Betaproteobacteria*). The affiliation to this genus was supported by high posterior probability and notably high bootstrap values. The exact position of the bacterium within this taxon could not be determined, as the phylogenetic relationships were poorly resolved. Its 16S rRNA gene sequence (GenBank accession number KT944310) differed by 3.3 to 4.8% from those of other *Pandoraea* spp. The branch that it formed on the phylogenetic tree proved to be much longer than those of the previously described members of this genus. Meanwhile, other known endosymbiotic bacteria of trypanosomatids ("*Candidatus Kinetoplastibacterium*" spp., family *Alcaligenaceae*) were only distantly related (Fig. 4).

Ultrastructural characterization of the trypanosomatid-bacteria association. The *Novymonas* cells were further analyzed by scanning electron microscopy (SEM) and high-pressure freeze-etching followed by transmission electron microscopy (HPF-TEM) (Fig. 5 and 6). SEM analysis confirmed the presence of all the main morphotypes (promastigotes, chonamastigotes, and amastigote-like cells) identified by light microscopy (Fig. 5A and B; also data not shown). Upon prolonged cultivation, the prevailing chonamastigotes were found firmly attached to the plastic surface (Fig. 5B). This attachment was mediated by a modified flagellum, which was shortened and widened, forming an attachment pad, along with a gluelike substance cementing cells onto the plastic (Fig. 5B). On the occasional detached cells, we observed that this modified flagellum had a multilobe structure (Fig. 5C). Interestingly, some promastigotes were also found to be attached (Fig. 5B); although in this case, the flagellum was not modified, the gluelike substance was present (data not shown). Examination of the axenic culture of endosymbiotic “*Candidatus Pandoraea novymonadis*” by SEM documented uniformly sausage-shaped bacilli (Fig. 5D), which measured 0.4 to 0.7 μm in diameter and 1.5 to 3.0 μm in length (N = 50).

HPF-TEM revealed typical morphological features of trypanosomatids: an oval nucleus located in the posterior half of the cell, an elongated kinetoplast disk positioned perpendicularly to the basal body of the flagellum, and an extensively branched single mitochondrion. In addition, some distinctive traits were observed, such as the hypertrophied mitochondrion and multiple electron-dense bacteria within the cytoplasm (Fig. 6A to D), which were enclosed in the symbiontophorous vacuoles either individually (Fig. 6B and C) or in pairs, occasionally dividing (data not shown). Quite frequently, vacuoles containing bacteria were accompanied by lysosomes of the host cell (Fig. 6B). Several phases of interaction between the lysosomes, symbiontophorous vacuoles, and bacteria were observed. They ranged from early membrane contacts (Fig. 6E) to complete fusion of organelles and subsequent degradation of the bacterium (Fig. 6B). Intact endosymbionts had an envelope typical for the Gram-negative bacteria: an inner cytoplasmic membrane and a relatively thin cell wall with periplasmic space in between (Fig. 6C). The same structure was found in the free bacteria obtained from the axenic culture, although their periplasmic space was somewhat wider (Fig. 6F).
**Taxonomic summary**

**Trypanosomatid host**

*Class* Kinetoplastea (Honigberg 1963) Vickerman 1976  
*Subclass* Metakinetoplastina Vickerman 2004  
*Order* Trypanosomatida Kent 1880  
*Family* Trypanosomatidae (Doflein 1901) Grobben 1905  
*Genus* Novymonas gen. nov. Kostygov and Yurchenko 2015

Diagnosis: The genus is defined by a unique position on the 18S rRNA-28S rRNA-Hsp83-based phylogenetic tree(s) within the clade Leishmaninae. It does not cluster within either the *Leishmania* clade or the *Leptomonas-Lotmaria-Crithidia* group. The main morphotypes are promastigotes and choanomastigotes.

Etymology: The generic name honors Frederick George Novy, an American bacteriologist and parasitologist who pioneered studies of insect trypanosomatids. He was the first to document structures ("diplosomes") (44) that were later proved to be bacterial endosymbionts in *Strigomonas culicis*. The name also relates to the word *nový* ("new" in many Slavic languages), reflecting the novelty of the discovered trypanosomatid-bacterium association.

**Novymonas esmeraldas** sp. nov. Votýpka, Kostygov, Maslov, and Lukeš (Fig. 2 and 5)
RNA gene sequences. It forms promastigotes and choanomastigotes in culture, with free-swimming promastigotes and attached choanomastigotes in rosettes dominating in log and stationary phases, respectively. Cells in the culture range from 10.9 to 18 \mu m in length and from 1.3 to 4.8 \mu m in width. The length of the flagellum varies from 7.8 to 19.5 \mu m for elongated promastigotes. Spherical choanomastigotes are 4.5 to 9.7 \mu m long and 2.8 to 6.4 \mu m wide, with the flagellum ranging between 8.6 and 20.4 \mu m. The kinetoplast disk is compactly packed and varies between 553 and 938 nm in diameter and 114 to 213 nm in cross section (measured in HPF-TEM pictures). Cells can propagate at low pH but cannot withstand elevated temperature.

Type host: *Niesthrea vincentii* (Hemiptera: Rhopalidae).

Site: Intestine: hindgut. Only short choanomastigotelike cells have been observed in situ.

Type locality: Vicinity of Atacames (Esmeraldas Province, Ecuador, 00°52’31”S; 79°50’32”W).

Type material: The name-bearing type, a hapantotype, is a Giemsa-stained slide of the clonal isolate E262AT.01, deposited in the research collection of the Life Science Research Centre, Ostrava, Czech Republic (accession code 2015/E262AT.01/S). Axenic cultures of the primary (E262AT) and clonal (E262AT.01) isolates are deposited in the research collections of the Life Science Research Centre of the University of Ostrava, Department of Parasitology at Charles University, Prague, and Institute of Parasitology, České Budějovice, Czech Republic, and the Department of Biology, University of California at Riverside, United States.

Etymology: The species name (*esmeraldas*) is derived from the name of the province in Ecuador where the host of this parasite was collected.

Gene sequences: GenBank accession numbers KT944309 (18S rRNA), KT944303 (28S rRNA), KT944298, KT944299 (SL RNA), KT944300 (glycosomal glyceraldehyde-3-phosphate dehydrogenase [gGAPDH]), and KT944293 (Hsp83).

Remarks: Two environmental DNA isolates from biting midges—CAR-B7, collected in September 2012 from *Culicoides cf. fulvithorax* in Dzanga-Sangha Protected Areas, Central African Republic (2°13’N, 16°11’E), and GAB3, collected in June 2014 from *Culicoides cf. distintipennis* in Loango National Park, Gabon (02°20’S, 09°35’E)—as well as the DNA isolate 104SI, sampled in March 2005 from the reduviid *Zelus* sp. in Casanga, Ecuador (00°35’S, 77°33’W), belong to the same species according to 18S rRNA and SL RNA gene sequences.

**Bacterial endosymbiont**

**Class** Betaproteobacteria Garrity et al. 2006

**Order** Burkholderiales Garrity et al. 2006

**Family** Burkholderiaceae Garrity et al. 2006

**Genus** Pandoraea Coenye et al. 2000

“*Candidatus* Pandoraea novymonadis” sp. nov. Kostygov, Grychuk-Ieremenko, and Yurchenko 2015

Species diagnosis and description: Cells are Gram-negative, nonsporulating, rodlike in shape, measuring between 0.4 and 0.7 in length by 1.5 to 3.0 \mu m in width, fitting the genus description (45). They are cultivable axenically and motile. The species is identified by its
Transmission electron microscopy (TEM) images of *Novymonas esmeraldas* sp. nov. and “*Candidatus Pandoraea novymonadis*” sp. nov. (A) General view of *Novymonas* cell showing typical features of trypanosomatids such as the nucleus (n), kinetoplast (k), mitochondrion (m), and flagellar pocket (fp), as well as the bacterial symbionts (b). (B) Interaction between the bacteria and the trypanosomatid cell demonstrating fusion of lysosomes (ly) with bacterium-containing vacuoles in the cytoplasm of the host (ch). Intact and degrading bacteria are labeled ib and db, respectively. (C) Magnification of boxed part of panel B showing the membrane (arrowhead) of the symbiontophorous vacuole (sv), bacterial cell wall (white asterisk), periplasmic space (black asterisk), and internal membrane.

(Continued)
unique position on the 16S rRNA-based phylogenetic tree.

Type host: *Novymonas esmeraldas* (Trypanosomatidae).

Type material: The name-bearing type, a hapantotype, is a Giemsa-stained slide of the axenic culture of “Ca. Pandoraea novymonadis,” deposited in the research collection of the Leishmania Research Centre in Ostrava and the Institute of Parasitology, České Budějovice, Czech Republic (access code 2015/E262AT.01/Pandoraea).

Etymology: The species name (*novymonadis*) refers to the specific trypanosomatid host.

Gene sequences: GenBank accession number KT944310 (16S rRNA).

**DISCUSSION**

In this work, we have characterized a new endosymbiont-bearing species of the family Trypanosomatidae. In contrast to the previously known bacterium-harboring flagellates of the subfamily Strigomonadinae, which constitute a separate clade (20), this monoxenous species is the closest known relative of the monoxenous genus *Leishmania* and qualifies as a representative of the newly established genus *Novymonas*. Similarly to its relatives, it was isolated in the Neotropics, a region from which all the leishmanias might have radiated (46, 47). Therefore, *Novymonas* may share some preadaptations to dixeny with its sister group, although it is clearly incapable of withstanding elevated temperature, thus proving its monoxenous status. *Novymonas* could be considered a proxy for the monoxenous ancestor of *Leishmania*, and hence, scrutiny of its genetics and biochemistry might shed light on the origin of the two-host life cycle within the *Leishmania*.

However, the new species is even more interesting since it harbors a bacterial endosymbiont in what appears to be an unstable relationship. The endosymbiotic bacterium of *Novymonas* belongs to the genus *Pandoraea* within the family Burkholderiaceae and, therefore, is only distantly related to the other known bacterial endosymbionts of trypanosomatids ("Ca. Kinetoplastibacterium" spp.) that belong to the family Polynucleobacteriaceae (22). Consequently, it represents a separate lineage of intracellular symbionts and may have quite different adaptations to such a lifestyle. Indeed, it appears that the *Novymonas-Pandoraea* endosymbiosis was established relatively recently. The following features favor a late origin of this relationship: (i) the endosymbiont preserves its cell wall; (ii) in contrast to Strigomonadinae (24, 25), the division of "Ca. Pandoraea novymonadis" is not coordinated with the division of the host, resulting in various numbers of bacteria per cell and, thus, an overall instability of this association; (iii) the host employs lysosomes to exercise control over the bacteria; (iv) unlike "Ca. Kinetoplastibacterium" spp. (5, 48), the symbiont of *Novymonas* can be axenically cultivated; and finally (v), other known *Pandoraea* spp. are either emerging opportunistic human pathogens or free-living organisms.

Despite the eventual loss of the bacterial symbionts from a fraction of cells in culture, the endosymbiosis is likely to be obligatory for the trypanosomatid, which cannot be cultivated without *Pandoraea*. Moreover, cultivation under high concentrations of antibiotics leads to the elimination of the endosymbiont-free cells, demonstrating that the bacteria are indispensable under these conditions. It is reasonable to assume that *Novymonas* farms bacteria inside its cytoplasm, using them as a source of some essential nutrients. Hence, the apysymbiotic cells are ultimately doomed. Nevertheless, they emerge at a relatively high frequency in the culture, since mechanisms ensuring synchronization between the cohabitants and a proper segregation of bacteria during host cell division seem to be missing. Under high concentrations of antibiotics, cells divide more slowly and accumulate more endosymbionts. In clonal cultures originating from single cells with different numbers of endosymbionts, the whole spectrum of bacterial load can be observed. All our efforts to obtain an endosymbiont-free clonal culture of *Novymonas* failed, apparently due to the reduced viability of such cells. Counterintuitively, the addition of antibiotics at concentrations affecting trypanosomatids did not trigger the loss of bacteria, implying that the host cells actively protect their endosymbionts.

In the relatively closely related Strigomonadinae—"Ca. Kinetoplastibacterium" system, the division of the endosymbiont was proposed to depend on host factors, as the gene implicated in cell division has been lost from the "Ca. Kinetoplastibacterium" genome (37). Moreover, inhibition of protein synthesis in the protist host blocks the symbiont’s cytokinesis (49). Importantly, all other members of the genus *Pandoraea* are free living, and yet, many of them were isolated as opportunistic agents from cystic fibrosis patients (45, 50, 51). This suggests that representatives of this genus actively explore new evolutionary niches and adapt to host-associated lifestyles.

A similar situation was observed in the ciliate *Euplotes aediculatus*, harboring the bacterium *Polynucleobacter necessarius*, which is from the same family as *Pandoraea* (52). This symbiosis was demonstrated to be obligatory for both the ciliate and the bacterium (53). Nevertheless, free-living strains of presumably the same species, as judged by their 16S rRNA gene sequences, have also been discovered (54). However, the *Euplotes-Polynucleobacter* system is quite different from the *Novymonas-Pandoraea* association, since no lysosome-mediated digestion has been detected in the former partnership. Moreover, another interesting aspect of the association described herein is the fact that the eukaryotic partner is a parasite. The impact of endosymbiosis on a host-parasitic lifestyle is largely unknown. Parasites are usually supplied with essential nutrients by their hosts (55), and it is therefore counterintuitive that some of them may need an additional source. To understand why the *Novymonas* trypanosomatid entered into a lasting, although still unstable and rather unique part-

Figure Legend Continued

(D) Cross section of *Novymonas* cell showing mitochondrial hypertrophy. (E) The early stage of the fusion between a bacterium and a lysosome. (F) Endosymbiotic bacillus in the axenic culture of "Ca. Pandoraea novymonadis" with the same structure of cell covering as is seen in panel C. Scale bars are 1 μm (A, D), 500 nm (B), 100 nm (C), and 200 nm (E, F).
nerness with Pandoraea, the whole genomes of both partners will have to be sequenced, and ideally, Novymonas should be modified into a genetically tractable organism. Both aims are now among our priorities, as we are convinced that this symbiotic relationship may serve as a model to study the evolution of early endosymbiosis in general and in parasitic protists in particular.

MATERIALS AND METHODS

Field work, establishment of primary cultures, cloning, and cultivation. *Niesthrea vincentii* Westwood 1842 (*Hemiptera: Rhopalidae*) was collected in the vicinity of Atacames (Esmeraldas Province, Ecuador, 00°52′31″S; 79°50′32″W) in July 2008. The insects were dissected and examined under a light microscope as described previously (56). The primary isolate E262AT was cultivated in brain heart infusion (BHI) medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 μg/ml hemin (Jena Biosciences, Jena, Germany), pH 7.6, and antibiotics as reported previously (20, 57). The clonal isolate E262AT.01 was obtained using the limiting dilution method as described previously (58). The identity of the clonal line was confirmed by sequencing its 18S rRNA gene. The primary culture and clonal line thus obtained were deposited in the collections of the Department of Parasitology, Charles University, Prague, in the Life Science Research Centre of the University of Ostrava, and in the Institute of Parasitology, České Budějovice, Czech Republic. Of note, cells also grew well in BHI medium without hemin or in M199 medium supplemented with 10% fetal bovine serum (FBS) (both from Life Technologies, Grand Island, NY) and antibiotics as described above.

For growth curves, cells were seeded at a density of 1 × 10^5 cells per ml in BHI medium, pH 7.6, and incubated at 23°C or 37°C for 20 days with counting every other day.

In order to eliminate symbionts from cultured trypanosomatid cells, we tested a number of antibiotics at different concentrations. Culture were grown in the presence of either ampicillin or kanamycin at 100, 200, 400, and 800 μg/ml or chloramphenicol at 64, 128, and 256 μg/ml.

For the same reason, we also performed a large-scale experiment with subcloning by limiting dilution. The work was done independently in three laboratories (Ostrava, Prague, and České Budějovice). The following media were employed: (i) M199 with antibiotics as described above, pH 7.4; (ii) M199 medium with antibiotics as described above, pH 5.5; (iii) preconditioned supplemented M199 medium, pH 7.4; (iv) BHI-RPMI (1:1) medium with 10% FBS and 200 μg/ml amikacin; and (v) RPMI medium with 10% FBS. In total, we analyzed 11 96-well plates and obtained 41 subclones. Fifty to eighty cells of each subclone were examined for the presence of endosymbions (see Table S2 in the supplemental material).

Isolation of axenic endosymbiont. An amount of 5 × 10^7 cells was spun down and then lysed in 1 ml of distilled water for 3 days until no moving trypanosomatid cells could be observed under the light microscope. The suspension was divided into 100-μl aliquots, which were plated on Trypticase soy agar, LB agar (both from Sigma-Aldrich), or blood agar plates and incubated at 37°C. Colonies grown on Trypticase soy agar were propagated in liquid BHI without supplements, and experiments with antibiotics were performed as described above.

Light and electron microscopy. Light microscopy of Giemsa or 4′,6-diamidino-2-phenylindole (DAPI)-stained smears on poly-l-lysine-coated slides was done as described elsewhere (46, 59) using an Olympus BX51 microscope equipped with a DP70 charge-coupled device (CCD) camera (Olympus, Tokyo, Japan). Standard measurements were performed for 50 cells of each morphotype on Giemsa-stained smears and expressed in micrometers (19). For scanning electron microscopy (SEM), cultured cells were fixed in 2.5% (vol/vol) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and processed as described previously (58, 60).

Samples were observed using a JEOL JSM-7401-F microscope (JEOL, Tokyo, Japan) with an accelerating voltage of 4 kV. High-pressure freezing followed by transmission electron microscopy (HPF-TEM) was performed essentially as described elsewhere (61). Images were captured on a JEOL JEM-1010 microscope (JEOL) using a Mega View III camera (EM-SIS GmbH, Münster, Germany). Kinetoplasts were measured after HPF-TEM as described previously (62).

FISH. Bacterial endosymbionts were visualized by fluorescent in situ hybridization (FISH) using the bacterium-specific probe Eub338 (5′-GC TGCCTCCCGTAGGAGT-3′) and LMR (5′-CGGAGGAGGRCCCNTRACAC-3′) and LMR (5′-GCAAGRGCTCTCR AAGGGTAAGCT-3′). For amplification of the spliced leader (SL) RNA gene, primers S762 and S763 were used. To amplify the 18S rRNA gene, we used primers S762 and S763 and sequenced directly, as described previously (66, 67). Hsp83 gene amplification was performed using the primers 500F (5′-CAGCTGATGTCCCTATTGCATAYAAYACNTYTAT-3′) and 970XR (5′-CTGAGGAGCTGCCCNTRATAC-3′) as described elsewhere (68). The PCR products were sequenced directly with the amplification primers, as well as with two internal oligonucleotides, XF2 (5′-AA GAAGCCCAACAATCATGC-3′) and XR2 (5′-GCAACGRTCTCR AAGGGTAAGCT-3′). The LSU α-segment of the 28S rRNA gene was amplified using primers LS5 (5′-ACAGACCTGATTGTTGCGAGCCTA C-3′) and LM5 (5′-CCCACTGAAACCTTCTTTTTGA-3′) and sequenced with oligonucleotides LSIF (5′-CGAAGGGTTTGAACAATGTC GTGACA-3′) and LSIR (5′-CGACTCTAAGTTGGTCGTAGT C-3′). Amplification of the mitochondrial leader (SL) RNA gene, primers M167 and M168 were used (69). The resulting PCR products were cloned using the InstaClone PCR cloning kit (Thermo Fischer Scientific, Waltham, MA) and sequenced as described previously (57, 70). To amplify the complete 16S RNA sequence of the bacterial endosymbiont, we used the primers P1seq and 1486R (23). The internal transcribed spacer (ITS) region between the 16S and 23S rRNA genes was amplified with primers P3Seq and P23sRev (22). The PCR products were sequenced directly. We also amplified and sequenced the glyceraldehyde-3-phosphate dehydrogenase (gGAPDH) gene, which is widely used as a phylogenetic marker (62, 67, 71). However, we did not apply it to phylogenetic inference since it has been demonstrated to produce serious artefacts (20).

Phylogenetic analyses. The 18S small subunit (SSU) rRNA, 28S LSU-α rRNA, and Hsp83 gene sequences of 18 species of trypanosomatids (including isolate E262AT.01) were aligned using Muscle version 3.8.31 (72). The resulting alignments were refined manually using BioEdit version 7.2.5 (73), and ambiguously aligned positions from 18S and 28S sequences were removed prior to concatenation using Gblocks software (74) as described previously (75). The resulting data set, containing 5,782 (2,125 + 1,749 + 1,908) positions, was used for phylogenetic inference under a partitioned model with maximum-likelihood criterion and a Bayesian approach in Treefinder version 03.2011 (http://www.treefinder.de) and MrBayes version 3.2.5 (76). Analysis in Treefinder was performed with the following parameters: the TN + G model for the 18S rRNA gene, GTR + G for the 28S rRNA gene, and J3 + G, GTR + G, and J3 + G, respectively, for the three codon positions of the Hsp83 gene (as selected by the built-in
model selector of Treefinder using the Akaike information criterion); 5 gamma categories; and optimized substitution rates, nucleotide frequencies, and partition rates. Edge support was estimated by the bootstrap method with 1,000 replicates. Bayesian inference of phylogeny was accomplished with an analysis run for 5 million generations under the GTR + I + G substitution model (5 gamma categories) with the nucleotide frequencies, substitution rates, partition rates, and parameters of rate heterogeneity among sites unlinked for all 4 partitions (defined as described above). Other analysis parameters were left at their default states. Rooting of the tree obtained was done according to the previously published phylogenetic reconstructions that demonstrate subdivision of the subfamily Leishmaniinae into two clades (60, 77).

Reconstruction of the bacterial phylogeny was performed in a similar way, with a few alterations as specified below. Since the alignment of the 16S rRNA gene sequences was more accurate than the sequence alignment described above, no positions were removed from the alignment (except for end trimming). The data set contained 39 taxa and 1,446 nucleotide positions. The phylogenetic inference under the maximum-likelihood criterion was done in RAxML version 8.0 (78) under the GTR + G + I model as selected in jModelTest 2.1.4 (79). Edge reliability was estimated with 1,000 “slow” (as defined by the software) replicas of bootstrap resampling. No partitioning was applied in either maximum-likelihood or Bayesian analysis.

The accession numbers of the sequences used in all of the analyses described above are available from the authors upon request.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the new sequences determined in this work are KT944291 (Cerithia dextra Hsp83), KT944292 (Cerithia brevicula Hsp83), KT944293 (Nomyosoma esmeralda Hsp83), KT944294 (Leptonomas costaricensis Hsp83), KT944295 (Leptonomas podilpaevi Hsp83), KT944296 (Leptonomas pyrrhocoris Hsp83), KT944297 (Leptonomas seymouri Hsp83), KT944298 and KT944299 (N. esmeralda SL RNA), KT944300 (N. esmeralda gGAPDH), KT944301 (C. brevicula 28S RNA), KT944302 (C. dextra 28S RNA), KT944303 (N. esmeralda 28S RNA), KT944304 (L. costaricensis 28S RNA), KT944305 (L. podilpaevi 28S RNA), KT944306 (L. pyrrhocoris 28S RNA), KT944307 (L. seymouri 28S RNA), KT944308 (Trypanosomatidae sp. CAR-B7 18S RNA), KT944309 (N. esmeralda 18S RNA), and KT944310 (“Candidatus Pandorea novomonaldis” 16S RNA).

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org lookup/suppl doi:10.1128/mBio.01985-15/-DCSupplemental.

Table S1, PDF file, 0.2 MB.
Table S2, PDF file, 0.2 MB.

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