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Novel Trypanosomatid-Bacterium Association: Evolution of Endosymbiosis in Action

Alexei Y. Kostygov,[a] Eva Dobáková,[b] Anastasiia Grybchuk-Ieremenko,[a] Dalibor Váhala,[a] Dmitri A. Maslov,[c] Jan Votýpka,[b,d] Julius Lukeš,[b,e,f] Vyacheslav Yurchenko[a,b,g]

Life Science Research Centre, Faculty of Science, University of Ostrava, Ostrava, Czech Republic[a]; Biology Centre, Institute of Parasitology, Czech Academy of Sciences, České Budějovice (Budweis), Czech Republic[b]; Department of Biology, University of California at Riverside, Riverside, California, USA[c]; Department of Parasitology, Faculty of Science, Charles University, Prague, Czech Republic[d]; Faculty of Sciences, University of South Bohemia, České Budějovice (Budweis), Czech Republic[e]; Canadian Institute for Advanced Research, Toronto, Ontario, Canada[f]; Department of Pathology, Albert Einstein College of Medicine, Bronx, New York, USA[g]

A.Y.K., E.D. and A.G.-I. contributed equally to this work.

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 symbiosis. 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 eukaryotic-prokaryotic relationships.

Address correspondence to Vyacheslav Yurchenko, vyacheslav.yurchenko@osu.cz, and Julius Lukeš, jula@paru.cas.cz.

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For at least 1.5 billion years, prokaryotes and eukaryotes co-evolved, and they have established numerous symbiotic associations. Even the very origin of eukaryotes would most likely be impossible without the acquisition of an endosymbiotic bacterium which became the omnipresent mitochondria of extant eukaryotic cells (1). Similarly, the rise of algae was associated with the incorporation of a cyanobacterium that eventually transformed into the photosynthetic organelle—the plastid. At the later stages of eukaryogenesis, various primary, secondary, and even tertiary endosymbiotic events added additional levels of complexity to eukaryotic cells (2).

More recent intracellular associations involving prokaryotes are relatively widespread among eukaryotic taxa and have considerable impacts on the ecology, physiology, and metabolism of both participants. The particular effects of such endosymbiotic associations can vary depending on whether they evolved toward mutualism, parasitism, or a range of intermediate forms. In the case of mutualism, endosymbionts provide some advantages to the host, from which they receive a sheltered environment rich in nutrients. However, if they are parasites, host defense mechanisms have to be overcome, and only one partner benefits from such a relationship. The host either benefits from the expanded metabolic capabilities derived from the endosymbiont, potentially allowing it to colonize new ecological niches, or experiences stress due to destructive effects of unwanted dwellers and the necessity to feed them.

The studies of endosymbiotic prokaryotes were for a long time impeded by the failure to cultivate most of them. Indeed, it was proposed recently that the majority of bacteria are uncultivable, as in nature they are part of complex communities, members of which depend on mutually exchanged metabolites (3). The study of uncultured bacteria has been aided by the advent of genomics and bioinformatics. These approaches have allowed a closer look at interactions between the partners of symbiotic associations and have uncovered common trends in the evolution of endosymbiont genomes (4–6). Recently, it was shown that some bacterial endosymbionts have reached an extreme level of genome reduction that is compensated by the balancing effect of collaborative networks among them (7).

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 *Gregarina garnhami* (11), the dinoflagellate *Hematodinium* sp. (12), the heterokont *Blastocystis* sp. (13), or the ciliate *Balantidium jocularum* (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 phosphatidylcholine 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). Trypanosomatids artificially 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-
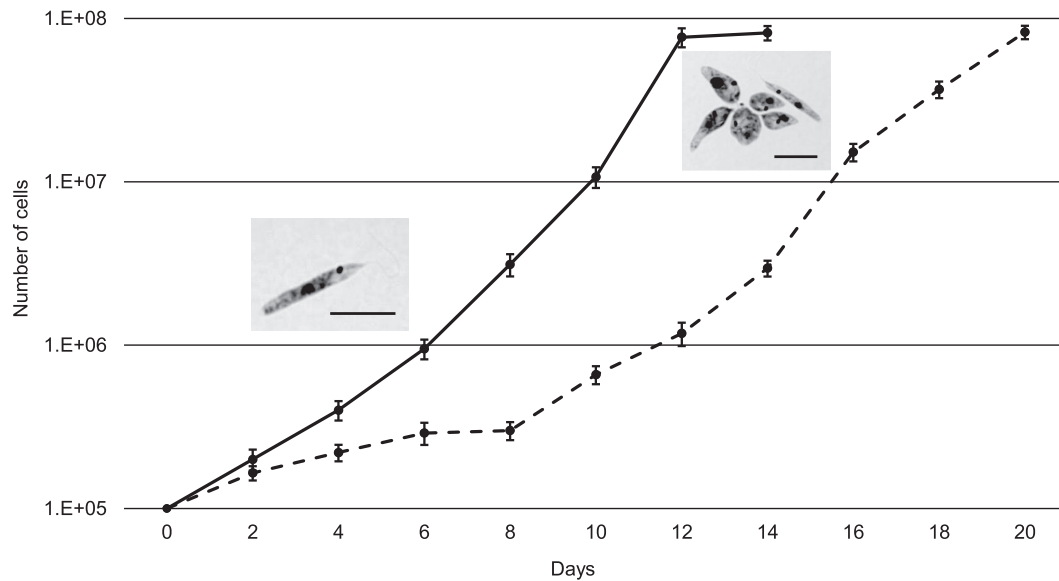
FIG 1 Growth curves of *Novymonas esmeraldas* (isolate E262AT.01). Prevailing morphotypes at the early/mid-log (promastigotes) and stationary (choano-mastigotes in rosettes) stages are shown in insets. Scale bars are 10 μm. Stationary-phase (dotted line) and early-log-phase (solid line) cultures were diluted to the same initial cell density and compared side by side. Data are from three independent biological replicates. The error bars indicate standard deviations.

rials and Methods), elimination of the intracellular bacteria from trypanosomatids did not occur. However, under these conditions, the cells divided considerably more slowly and amassed conspic-uously more bacteria than in the absence of antibiotics (data not shown). Importantly, no bacterium-free cells were observed un-der such growth conditions.

Detection of bacterial endosymbionts by FISH. Giemsa and 4′,6-diamidino-2-phenylindole (DAPI) staining allowed the de-tection 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, point-ing 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 drasti-cally. 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 supple-ments. 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 labora-tories in Ostrava, Prague, and České Budějovice (see Table S2 in the supplemental material). None of the 41 subclones obtained,
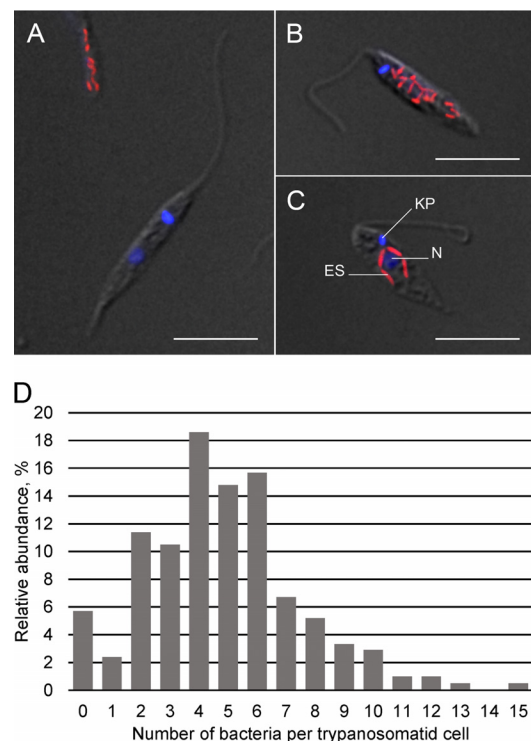

FIG 2 Bacterial endosymbionts in *Novymonas esmeraldas* (isolate E262AT.01) cells were detected by FISH. (A to C) DAPI- and Eub338-probed cells were ana-lyzed by differential interference contrast (DIC) combined with fluorescence mi-croscopy. Scale bars are 10 μm. (D) Relative abundances of the endosymbionts in host cells (*n* = 210). ES, endosymbiont; KP, kinetoplast; N, nucleus.
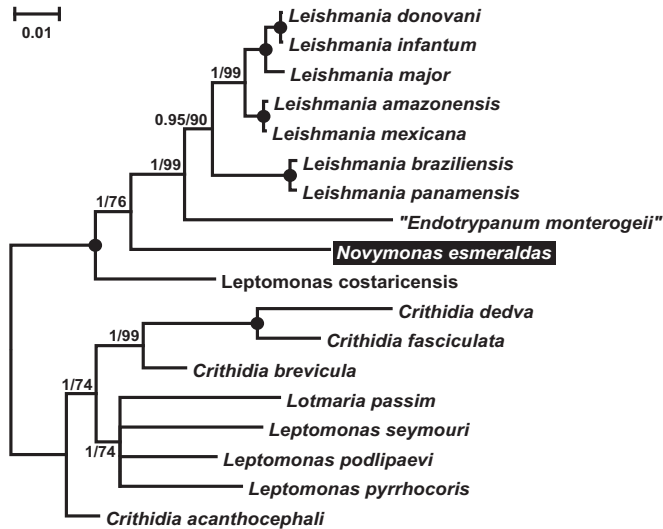
FIG 3 Phylogenetic tree for the trypanosomatid isolate studied in this work, inferred by the maximum-likelihood method using an 18S SSU rRNA, 28S LSU-α rRNA, and Hsp83 gene concatenated sequence set. Numbers at nodes indicate the posterior probability/bootstrap percentage. Nodes having 1.0 posterior probability and 100% bootstrap support are marked with black circles. The bar represents the number of substitutions per site. The name "*Endotrypanum monterogeii*" is enclosed in quotation marks since it is considered to be a misidentified member of the genus *Leishmania*. It is traditionally used for the corresponding culture. The species under study is highlighted.

which were analyzed in detail, was free of bacteria. Similarly to the original E262AT.01 culture, a fraction of cells lacked bacteria. Different clones exhibited different rates of cell division, but there was no correlation between their growth and the number of endosymbiotic bacteria per host cell.

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*. The phylogenetic position was supported by the highest posterior probability and only a moderate bootstrap percentage. The latter was higher when different subsets of taxa were tested; in particular, the removal of *Endotrypanum monterogeii* led to an increase of this value to 85 (data not shown).

Analysis of the SL RNA gene sequences (GenBank accession numbers KT944298 and KT944299) revealed that the new species belongs to typing unit 42 (*TU*42) (19, 41). It also includes environmental DNA isolates 104SI and CAR-B7 (GenBank accession number DQ864304), isolated in Ecuador and Central African Republic from the predatory true bug *Zelus* sp. (Heteroptera) and the biting midge *Culicoides* cf. *fulvithorax* (Diptera), respectively (41, 43). Moreover, the 18S rRNA gene sequences of isolates CAR-B7, E262AT, and GAB3 (the latter collected from *Culicoides* cf. *distinctipennis* in Gabon) were identical (GenBank accession numbers KT944308 and KT944309).

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 freezing followed by transmission electron microscopy (HPF-TEM) (Fig. 5 and 6). SEM analysis confirmed the presence of all of the main morphotypes (promastigotes, choanomastigotes, and amastigote-like cells) identified by light microscopy (Fig. 5A and B; also data not shown). Upon prolonged cultivation, the prevailing choanomastigotes 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 perpendicular 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).
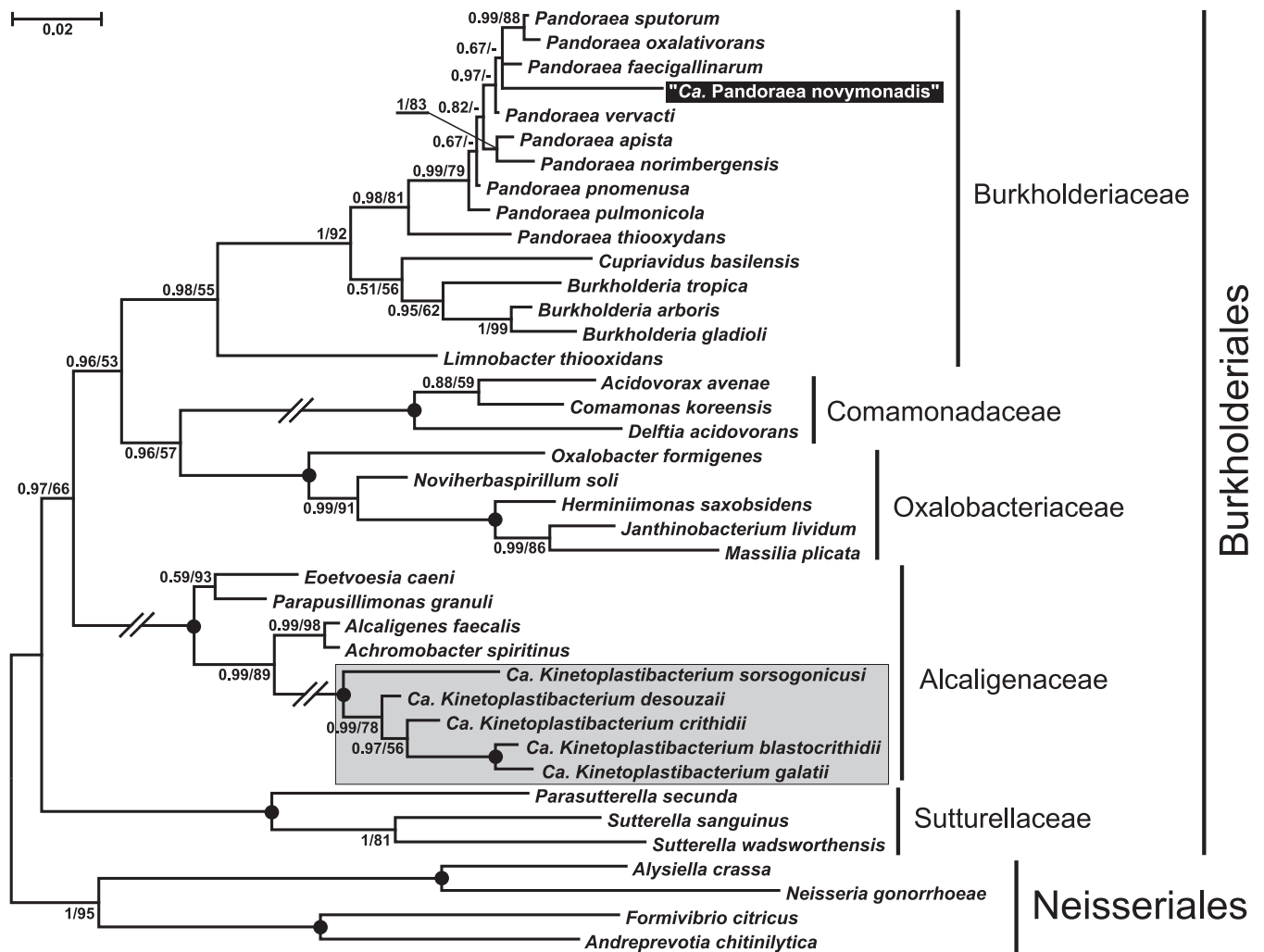
FIG 4 16S rRNA-based Bayesian phylogenetic tree of bacterial endosymbionts of trypanosomatids. Names of species for sequences retrieved from GenBank are indicated. The species newly described in this work is highlighted. "*Candidatus* Kinetoplastibacterium" spp. (family *Alcaligenaceae*) are boxed and shaded. Bayesian posterior probabilities and bootstrap percentages for maximum likelihood analysis are shown at the nodes. Slashed branches are at 50% of their original lengths. Dashes indicate bootstrap support below 50% or different maximum-likelihood topology. Black dots represent 100% bootstrap support and Bayesian posterior probability of 1.0. The tree was rooted with sequences of four species of the order *Neisseriales*. The scale bar denotes the number of substitutions per site.

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 Leishmaniinae. 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)

Species diagnosis and description: The species is identified by its distinct phylogenetic position on the 18S rRNA and other gene trees, as well as by its unique SL
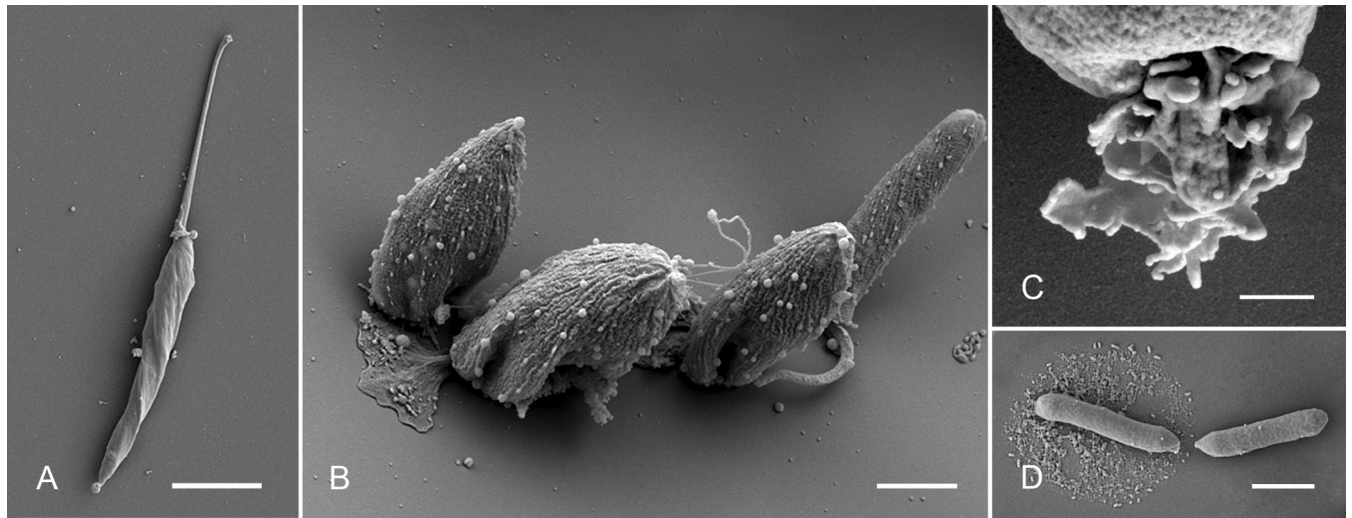
FIG 5 Scanning electron microscopy (SEM) images of *Novymonas esmeraldas* sp. nov. and "*Candidatus* Pandoraea novymonadis" sp. nov. (A) Free-swimming promastigote; (B) sessile forms (both pro- and choanomastigote-shaped, with or without modified flagellum); (C) prominently modified flagellum of a sessile choanomastigote; (D) bacilli in the axenic culture of "*Ca.* Pandoraea novymonadis." Scale bars are 5 μm (A), 2 μm (B), 400 nm (C), and 1 μm (D).

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 μm in length and from 1.3 to 4.8 μm in width. The length of the flagellum varies from 7.8 to 19.5 μm for elongated promastigotes. Spherical choanomastigotes are 4.5 to 9.7 μm long and 2.8 to 6.4 μm wide, with the flagellum ranging between 8.6 and 20.4 μm. 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. *distinctipennis* 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°53′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, Grybchuk-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 μm in width, fitting the genus description (45). They are cultivable axenically and motile. The species is identified by its
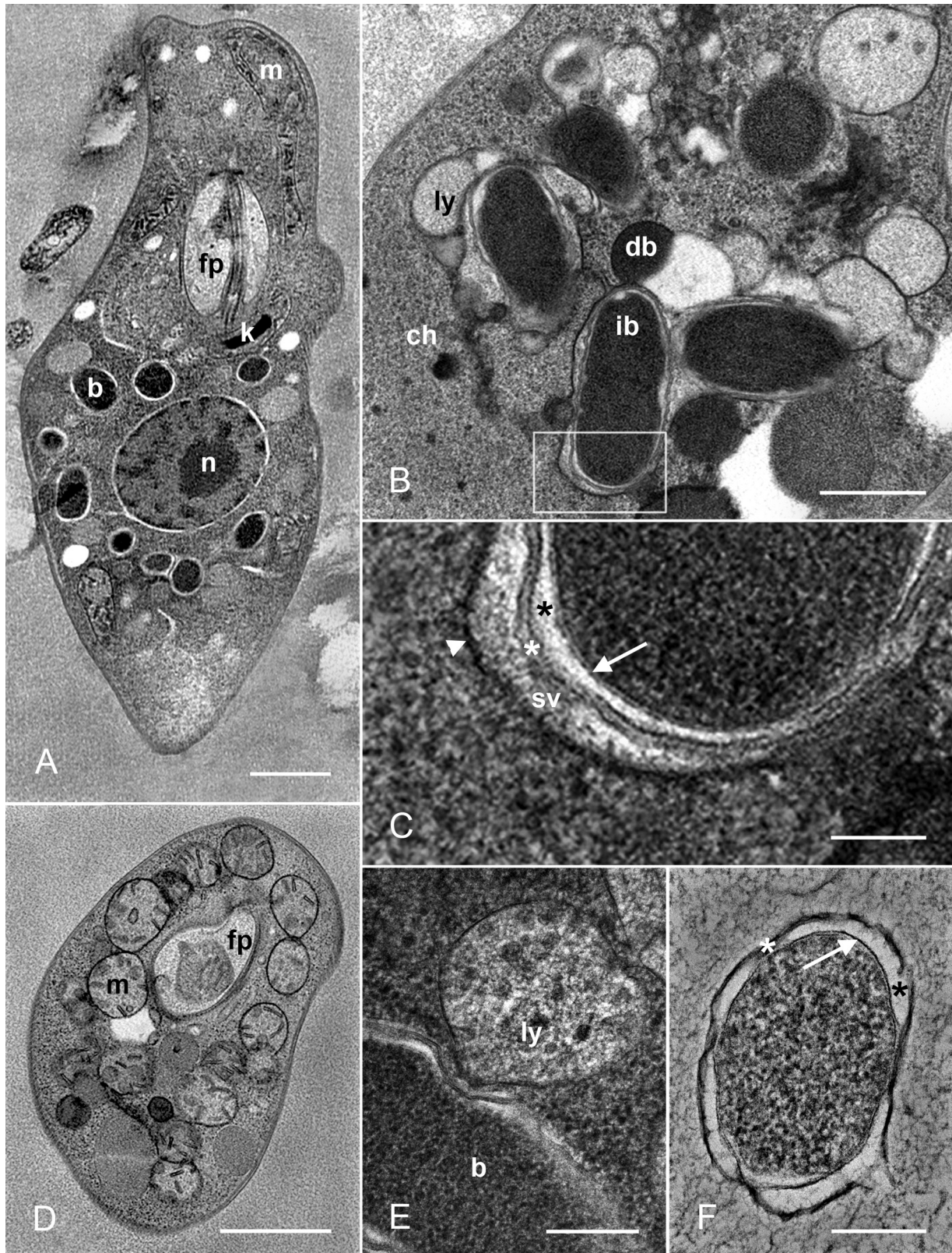
FIG 6 Transmission electron microscopy (TEM) images of *Novymonas esmeraldas* sp. nov. and "*Candidatus* Pandoraea novymonadis" sp. nov. (A) General view of *Novymonas* cell showing typical features of trypanosomatids such as the nucleus (n), kinetoplast (k), mitochondrion (m), and flagellar pocket (fp), 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 Life Science Research Centre in Ostrava and the Institute of Parasitology, České Budějovice, Czech Republic (accession 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 dixenous 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 Leishmaniinae.

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 *Alcaligenaceae* (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 aposymbiotic 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

(arrow). (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).

nership 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 additives as described above, pH 7.4; (ii) M199 medium with additives 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 endosymbionts (see Table S2 in the supplemental material).

Isolation of axenic endosymbiont culture. 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 (EMSIS 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′) labeled on the 5′ end with Cy3 fluorescent dye (63). E262AT.01 cells were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature and processed as described elsewhere (64, 65). Slides were mounted in ProLong gold antifade reagent with DAPI (Life Technologies) and observed with the Axioplan 2 fluorescence microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), and images were captured using cellSens imaging software version 1.11 (Olympus Life Science, Tokyo, Japan). The numbers of endosymbiotic bacteria were counted for 210 randomly selected trypanosomatid cells.

DNA isolation, PCR amplification, cloning, and sequencing. Total genomic DNA of the trypanosomatids or bacteria was isolated from the axenically grown culture (5 ml for trypanosomatids and 1 ml for bacteria) using the DNeasy blood & tissue kit (Qiagen, Hilden, Germany) or GenElute bacterial genomic DNA kit (Sigma-Aldrich), respectively, according to the manufacturers' protocols. The 18S rRNA gene was amplified using primers S762 and S763 and sequenced directly, as described previously (66, 67). Hsp83 gene amplification was performed using the primers 100XF (5′-CAGCTGATGTCCCTGATCATYAAYACNTTYTA-3′) and 970XR (5′-TCGAGGGAGAGRCCNARCTTRATC-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 GAAGCGCAACAACATCAAGC-3′) and XR2 (5′-GCACAGRTCCTCR CAGTTGTCCA-3′). The LSU α-segment of the 28S rRNA gene was amplified using primers LSF (5′-ACAGACCTGAGTGTGGCAGGACTA C-3′) and LMR (5′-CCCACATGCAATTTCTTTTTGGA-3′) and sequenced with oligonucleotides LSIF (5′-CGAAAGGTGGTGAACTATGC CTGAACA-3′) and LSIR (5′-CCAGCTACTAGGTGGTTCGATGAGT C-3′). For amplification of the spliced leader (SL) RNA gene, primers M167 and M168 were used (69). The resulting PCR products were cloned using the InstTA PCR cloning kit (Thermo Fischer Scientific, Waltham, MA) and sequenced as described previously (57, 70). To amplify the complete 16S rRNA 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 glycosomal 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 3.8.3.1 (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 + GI, GTR + GI, 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 (*Crithidia dedva* Hsp83), KT944292 (*Crithidia brevicula* Hsp83), KT944293 (*Novymonas esmeraldas* Hsp83), KT944294 (*Leptomonas costaricensis* Hsp83), KT944295 (*Leptomonas podlipaevi* Hsp83), KT944296 (*Leptomonas pyrrhocoris* Hsp83), KT944297 (*Leptomonas seymouri* Hsp83), KT944298 and KT944299 (*N. esmeraldas* SL RNA), KT944300 (*N. esmeraldas* gGAPDH), KT944301 (*C. brevicula* 28S rRNA), KT944302 (*C. dedva* 28S rRNA), KT944303 (*N. esmeraldas* 28S rRNA), KT944304 (*L. costaricensis* 28S rRNA), KT944305 (*L. podlipaevi* 28S rRNA), KT944306 (*L. pyrrhocoris* 28S rRNA), KT944307 (*L. seymouri* 28S rRNA), KT944308 (*Trypanosomatidae* sp. CAR-B7 18S rRNA), KT944309 (*N. esmeraldas* 18S rRNA), and KT944310 ("*Candidatus* Pandoraea novymonadis" 16S rRNA).

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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