

NOTE

Obligate bacterial endosymbionts of *Acanthamoeba* spp. related to the β -*Proteobacteria*: proposal of '*Candidatus Procabacter acanthamoebae*' gen. nov., sp. nov.

Matthias Horn,¹ Thomas R. Fritsche,² Tanja Linner,¹ Romesh K. Gautom,³ Marit D. Harzenetter¹ and Michael Wagner¹

¹ Lehrstuhl für Mikrobiologie, Technische Universität München, Am Hochanger 4, D-85350 Freising, Germany

² Dept of Laboratory Medicine, University of Washington, Seattle 98195, WA, USA

³ Washington State Public Health Laboratory, Seattle 98155, WA, USA

Author for correspondence: Michael Wagner. Tel: +49 8161 715444. Fax: +49 8161 715475. e-mail: wagner@mikro.biologie.tu-muenchen.de

All obligate bacterial endosymbionts of free-living amoebae currently described are affiliated with the α -*Proteobacteria*, the *Chlamydiales* or the phylum *Cytophaga-Flavobacterium-Bacteroides*. Here, six rod-shaped Gram-negative obligate bacterial endosymbionts of clinical and environmental isolates of *Acanthamoeba* spp. from the USA and Malaysia are reported. Comparative 16S rDNA sequence analysis demonstrated that these endosymbionts form a novel, monophyletic lineage within the β -*Proteobacteria*, showing less than 90% sequence similarity to all other recognized members of this subclass. 23S rDNA sequence analysis of two symbionts confirmed this affiliation and revealed the presence of uncommon putative intervening sequences of 146 bp within helix-25 that shared no sequence homology to any other bacterial rDNA. In addition, the 23S rRNA of these endosymbionts displayed one polymorphism at the target site of oligonucleotide probe BET42a that is conserved in all other sequenced β -*Proteobacteria*. Intra-cytoplasmic localization of the endosymbionts within the amoebal host cells was confirmed by electron microscopy and fluorescence *in situ* hybridization with a specific 16S rRNA-targeted oligonucleotide probe. Based on these findings, the provisional name '*Candidatus Procabacter acanthamoebae*' is proposed for classification of a representative of the six endosymbionts of *Acanthamoeba* spp. studied in this report. Comparative 18S rDNA sequence analysis of the *Acanthamoeba* host cells revealed their membership with either *Acanthamoeba* 18S rDNA sequence type T5 (*Acanthamoeba lenticulata*) or sequence type T4, which comprises the majority of all *Acanthamoeba* isolates.

Keywords: *Acanthamoeba*, free-living amoebae, endosymbiont, '*Candidatus Procabacter acanthamoebae*'

Rod-shaped bacterial endosymbionts within free-living amoebae of the genus *Acanthamoeba* which co-exist with their hosts without lysing the amoebal cell were first described by Proca-Ciobanu *et al.* (1975).

Abbreviations: FISH, fluorescence *in situ* hybridization; IVS, intervening sequence.

The EMBL/GenBank/DDBJ accession numbers for the 16S rDNA of '*Candidatus Procabacter acanthamoebae*' strain UWC12 is AF177427. See the text for details of other new sequence data determined in this study.

More recent studies have demonstrated that stable bacterial endosymbiosis can be observed in about 25% of *Acanthamoeba* isolates (Fritsche *et al.*, 1993) and also occurs in other free-living amoebae like *Hartmannella vermiformis* (Horn *et al.*, 2000). 16S rDNA sequence analysis has assigned all currently investigated endosymbionts either to the α -*Proteobacteria* (Fritsche *et al.*, 1999; Horn *et al.*, 1999; Birtles *et al.*, 2000), to novel genera within the *Chlamydiales* (Amann *et al.*, 1997; Fritsche *et al.*, 2000; Horn *et al.*,

2000) or to the phylum *Cytophaga-Flavobacterium-Bacteroides* (Horn *et al.*, 2001).

In this report, six Gram-negative, rod-shaped obligate bacterial endosymbionts of clinical and environmental isolates of *Acanthamoeba* spp. were investigated. The presence of endosymbionts in four of these *Acanthamoeba* strains has previously been observed, but no attempts were made to identify them (Fritsche *et al.*, 1993). All symbionts analysed in the present study co-existed with their respective *Acanthamoeba* host and did not cause apparent lysis of their host cells. Previous attempts to cultivate the investigated bacterial endosymbionts using various media and culturing conditions had failed (Fritsche *et al.*, 1993). Consequently, phylogenetic characterization of these endosymbionts was performed by the full-cycle rRNA approach, including comparative 16S rDNA sequence analysis and detection of endosymbionts within the amoebic host cell by fluorescence *in situ* hybridization (FISH) using specific 16S rRNA-targeted oligonucleotide probes. In addition, phylogenetic affiliation of the *Acanthamoeba* host cells was determined by comparative 18S rDNA sequence analysis.

Isolation of acanthamoebae

Six *Acanthamoeba* isolates (*Acanthamoeba polyphaga* strain Page 23 and *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226 and TUMSJ-341) containing rod-shaped bacterial endosymbionts were analysed in this study. *Acanthamoeba* sp. strains UWC6 and UWC12 have previously been isolated from corneal scrapings of *Acanthamoeba* keratitis patients (Fritsche *et al.*, 1993). *A. polyphaga* Page 23 was isolated from freshwater (Drummond, WI, USA; Page, 1967) and was obtained from the American Type Culture Collection (ATCC 30871). *Acanthamoeba* sp. strain UWE2 was recovered from a soil sample collected in Clearwater County, MN, USA, and *Acanthamoeba* sp. strains TUMSJ-226 and TUMSJ-341 were isolated in this study from a eutrophic lake sediment from Malaysia. Isolations and maintenance of amoebae were performed as described previously (Fritsche *et al.*, 1993). Prokaryotic endosymbionts of the *Acanthamoeba* isolates were readily detected under the microscope using Gram- and Giemsa- or DAPI-staining.

Morphological characterization of bacterial endosymbionts by electron microscopy (EM)

Examination of *Acanthamoeba* isolates by EM was performed as described elsewhere (Horn *et al.*, 1999). It was shown that the rod-shaped endosymbionts of *A. polyphaga* Page 23 and *Acanthamoeba* sp. strains UWC6, UWC12 and UWE2 were $0.3\text{--}0.5 \times 1.0\text{--}2.0 \mu\text{m}$ and had a Gram-negative type cell wall (Fig. 1). Electron-translucent spheroid structures were observed in some bacterial cells. Endosymbionts were found equally distributed within the cytoplasm of both

trophozoites and cysts and were not enclosed in vacuoles or embedded in slime layers, as reported for other symbionts of acanthamoebae (Fritsche *et al.*, 1998; Horn *et al.*, 1999).

16S and 23S rDNA-based phylogeny of bacterial endosymbionts

Simultaneous isolation of DNA from amoebae and their endosymbionts was performed using a previously described modification of the UNSET procedure (Hugo *et al.*, 1992; Fritsche *et al.*, 1998). Oligonucleotide primers targeting 16S rDNA and 23S rDNA signature regions that are highly conserved within the domain *Bacteria* were used for PCR to obtain near full-length bacterial 16S and 23S rRNA gene fragments, respectively. Nucleotide sequences of forward and reverse primers used for amplification of 16S rDNA were 5'-AGAGTTTGATYMTGGCTCAG-3' (*E. coli* 16S rDNA positions 8–27; Weisburg *et al.*, 1991) and 5'-CAKAAAGGAGGTGATCC-3' (*Escherichia coli* 16S rDNA positions 1529–1546). Nucleotide sequences of primers used for amplification of 23S rDNA were 5'-TCYGAATGGGGNAAC-3' (*E. coli* 23S rDNA positions 115–130) and 5'-CCGGTCCTCTCGTAC-3' (*E. coli* 23S rDNA positions 2654–2669). Amplification of near full-length amoeba 18S rRNA gene fragments was carried out using primers SSU1 (5'-AACCTGGTTGATCCTGCCAG-3') and SSU2 (5'-GATCCTTCTGCAGGTTCCATTAT-3'), complementary to conserved target regions at both ends of the 18S rDNA (Gast *et al.*, 1994). Amplified products were cloned into *E. coli* (TOPO TA kit; Invitrogen) and sequenced.

Near full-length 16S rRNA gene sequences (approx. 1400 bp) from bacterial endosymbionts of *A. polyphaga* Page 23 and *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226 and TUMSJ-341, as well as near full-length 23S rRNA gene sequences (approx. 2600 bp) from bacterial symbionts of *Acanthamoeba* sp. UWC6 and UWC12 were obtained and added to the rRNA sequence database of the Technische Universität München (encompassing about 15000 published and unpublished homologous small-subunit rRNA primary structures) using the ARB program package (program available at <http://www.mikro.biologie.tu-muenchen.de>). Alignment of retrieved rDNA sequences was performed using the ARB automated alignment tool and refined by visual inspection and secondary structure analysis. Comparative sequence analysis revealed that the 16S rRNA genes of the six endosymbionts were novel and showed highest, albeit moderate, similarities with members of the β -*Proteobacteria* (less than 90%), whereas 16S rDNA similarity values between the respective symbionts were much higher (95–99%). Analysis of 23S rRNA genes of bacterial endosymbionts of *Acanthamoeba* sp. UWC6 and UWC12 confirmed their membership with the β -*Proteobacteria*. Whereas 23S rDNA similarity values with all β -*Proteobacteria* deposited in

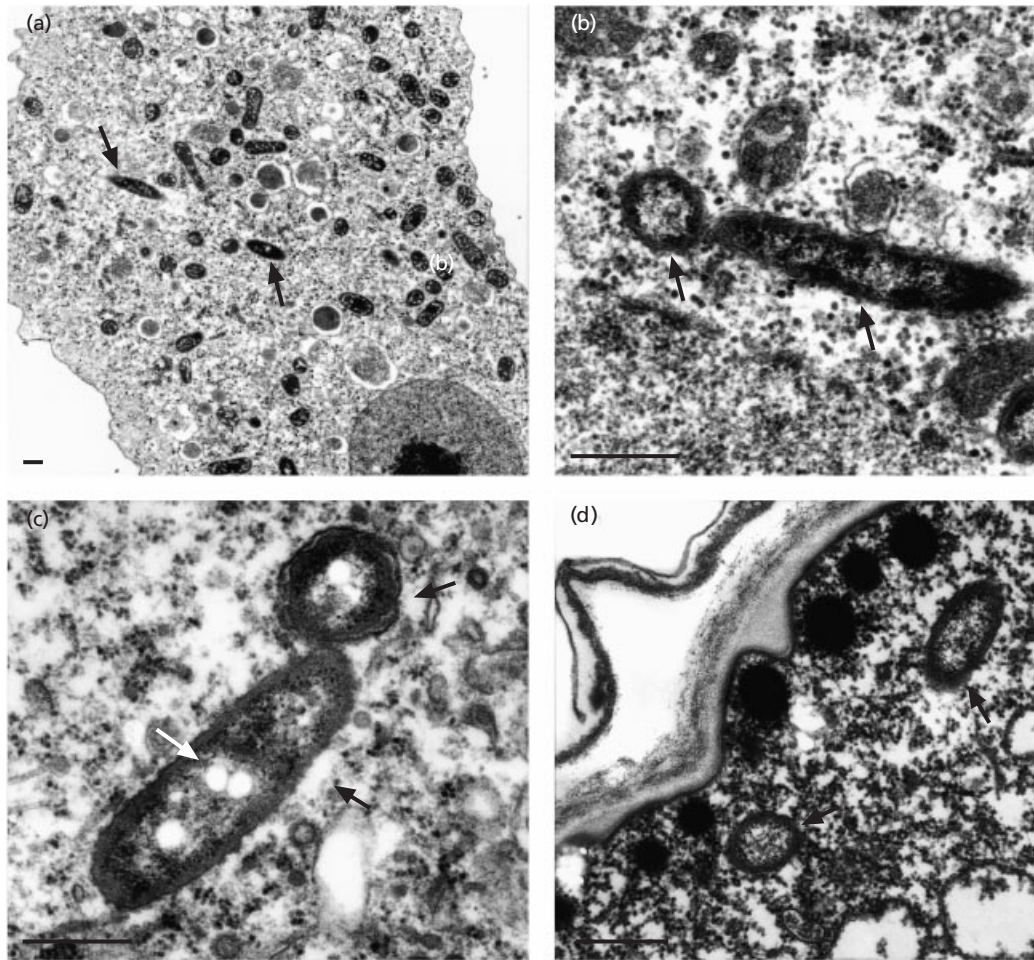


Fig. 1. Electron micrographs of: (a, b) '*Candidatus Procabacter acanthamoebae*' (endosymbiont of *Acanthamoeba* sp. UWC12); (c) the endosymbiont of *A. polyphaga* Page 23; and (d) the endosymbiont of *Acanthamoeba* sp. UWE2 showing their intracytoplasmic localization. Symbionts are marked with black arrows. Electron-translucent inclusions (white arrow) are present in some bacterial cells. Bars, 0.5 μ m.

public databases were below 88%, the 23S rDNA sequences of the two *Acanthamoeba* symbionts were almost identical (99% sequence similarity). Subsequent phylogenetic analyses, performed by applying the distance matrix, parsimony and maximum-likelihood methods implemented in ARB to different datasets (with and without filters excluding highly variable positions), consistently demonstrated for both 16S and 23S rDNA that the investigated *Acanthamoeba* endosymbionts form a novel and monophyletic cluster within the β -Proteobacteria (Fig. 2).

Sequence accession numbers. The EMBL/GenBank/DDBJ accession numbers for the 16S rDNA of '*Candidatus Procabacter acanthamoebae*' strain UWC12 is AF177427. Additional EMBL/GenBank/DDBJ accession numbers reported in this study are AF352393 (23S rDNA of '*Candidatus Procabacter acanthamoebae*' strain UWC12, endosymbiont of *Acanthamoeba* sp. UWC12), AF177424 (16S rDNA of the endosymbiont of *Acanthamoeba* sp. UWE2), AF177426 (16S

rDNA of the endosymbiont of *Acanthamoeba* sp. UWC6), AF352392 (23S rDNA of the endosymbiont of *Acanthamoeba* sp. UWC6), AF177425 (16S rDNA of the endosymbiont of *Acanthamoeba polyphaga* Page 23), AF352385 (16S rDNA of the endosymbiont of *Acanthamoeba* sp. TUMSJ-226) and AF352386 (16S rDNA of the endosymbiont of *Acanthamoeba* sp. TUMSJ-341). Amoebal 18S rDNA sequences obtained have accession numbers AF352390 (*Acanthamoeba* sp. UWC12), AF352388 (*Acanthamoeba* sp. UWE2), AF352387 (*Acanthamoeba* sp. UWC6), AF352389 (*Acanthamoeba* sp. TUMSJ-226) and AF352391 (*Acanthamoeba* sp. TUMSJ-341).

Putative intervening DNA sequences are present in the symbiont 23S rRNA genes

Analysis of the 23S rRNA genes of the β -proteobacterial endosymbionts of *Acanthamoeba* sp. UWC6 and UWC12 revealed the presence of 146 additional bases at position 550 (according to *E. coli* rRNA

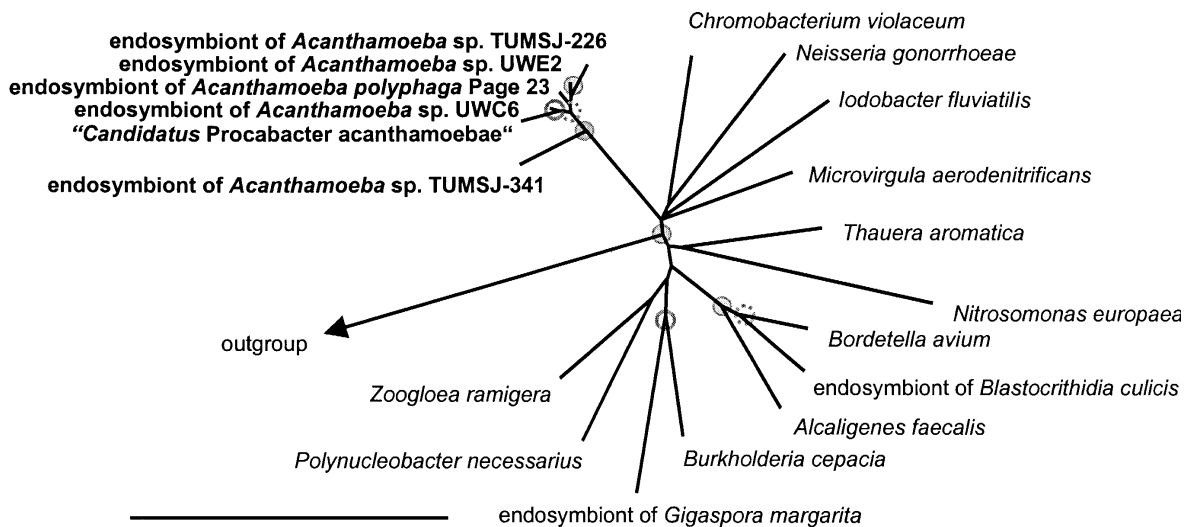


Fig. 2. 16S rDNA-based neighbour-joining trees reflecting the affiliation of endosymbionts of *A. polyphaga* Page 23 and *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226 and TUMSJ-341 with selected members of the β -Proteobacteria. Circles, parsimony bootstraps values (1000 replicates) of 100% (filled circles), > 80% (open circles) and > 50% (dashed circles). Bootstrap values less than 50% were omitted. Bar, 10% estimated evolutionary distance. Strains and accession numbers of the species shown in this tree: *Chromobacterium violaceum* ATCC 12472^T, M22510; *Neisseria gonorrhoeae* NCTC 83785, X07714; *Iodobacter fluviatilis* ATCC 33051^T, M22511; *Microvirgula aerodenitrificans* SGLY2^T, U89333; *Thauera aromatica* K 172^T, X77118; *Nitrosomonas europaea* C-31^T, M96399; *Bordetella avium* ATCC 35086^T, U04947; endosymbiont of *Blastocrithidia culicis*, L29265; *Alcaligenes faecalis* ATCC 8750^T, M22508; *Burkholderia cepacia* ATCC 25416^T, M22518; endosymbiont of *Gigaspora margarita*, X89727; *Polynucleobacter necessarius* ATCC 30859^T, X93019; *Zoogloea ramigera* ATCC 25935, X74914.

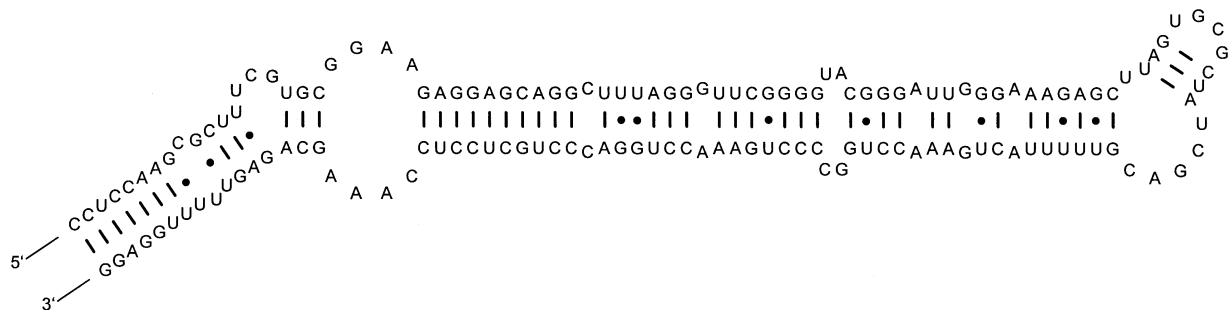


Fig. 3. Structure of the putative IVS present in the 23S rDNA sequence of 'Candidatus Procabacter acanthamoebae' (endosymbiont of *Acanthamoeba* sp. UWC12) and the endosymbiont of *Acanthamoeba* sp. UWC6. The predicted rRNA secondary structure was edited and visualized using the program RNAVIZ (De Rijk & De Wachter, 1997).

gene numbering) that are identical in both investigated symbionts. rRNA secondary structure prediction using the free energy minimization algorithm as implemented in the programs RNASTRUCTURE (Mathews *et al.*, 1999) and MFOLD (Walter *et al.*, 1994) demonstrated that these insertions form a stable stem-loop structure, replacing the tetraloops at helix-25 in the postulated 23S rRNA secondary structure of *E. coli* (Fig. 3) and thus can be regarded as putative intervening sequences (IVS) which are within the 23S rRNA genes typically found at helix-25 or helix-45 (Burgin *et al.*, 1990). The G + C content of these inserts

matches the overall G + C content of the symbiont 23S rRNA gene (51 mol%). Although a primary stem of at least 14 bp, which is the postulated cleavage site (Liiv & Remme, 1998), is present in the predicted symbiont IVS secondary structure, no significant sequence homology to any previously described rRNA gene or IVS could be found in public databases. To our knowledge, this is the first demonstration of putative IVS in the 23S rRNA gene of members of the β -Proteobacteria. The presence of IVS within rRNA genes has previously been reported for several bacterial genera of other phylogenetic groupings. IVS were, for example, re-

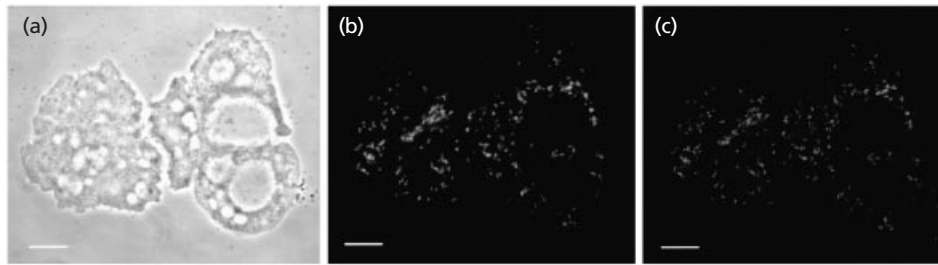


Fig. 4. FISH, demonstrating the intracellular localization of 'Candidatus Procabacter acanthamoebae' (endosymbiont of *Acanthamoeba* sp. UWC12) using bacterial probe EUB338 labelled with FLUOS (b) and endosymbiont-specific probe S-G-Proca-438-a-A-18 labelled with Cy3 (c). A phase-contrast image is shown in (a). Bar, 10 μ m.

ported in the 23S rDNA of *Haemophilus* (Song *et al.*, 1999), *Salmonella* (Pabbaraju & Sanderson, 2000) and *Leptospira* (Ralph & McClelland, 1993) and in the 16S rDNA of *Helicobacter* (Linton *et al.*, 1994) and 'Candidatus Blochmannia' (Sauer *et al.*, 2000). The highly variable extended stem-loop structures formed by the IVS are excised from the rRNA post-transcriptionally (Burgin *et al.*, 1990). Interestingly, the presence of IVS is not necessarily conserved in very closely related bacterial strains and IVS are not always present in all copies of the rDNA operons within the same bacterium (Pabbaraju & Sanderson, 2000). A more detailed analysis of the β -proteobacterial symbiont IVS is currently being performed, but was beyond the scope of the present study.

In situ detection of symbionts within *Acanthamoeba* host cells by FISH

Bacterial endosymbionts of *A. polyphaga* Page 23 and *Acanthamoeba* sp. UWC6, UWC12, UWE2, TUMSJ-226 and TUMSJ-341 were readily visualized by FISH using the bacterial probe EUB338 (5'-GCTGCCTCCCGTAGGAGT-3'; Amann *et al.*, 1990). Application of the 23S rRNA-targeted probe BET42a (5'-GCCTTCCCCTTCGTTT-3'; Manz *et al.*, 1992), previously designed to hybridize to all bacteria within the β -Proteobacteria, however, resulted in only weak fluorescent signals. Subsequent inspection of the 23S rRNA genes of *Acanthamoeba* sp. UWC6 and UWC12 confirmed the presence of a single central strong T-U mismatch at the target site of probe BET42a destabilizing the probe/rRNA hybrids. Consequently, oligonucleotide probe S-G-Proca-438-a-A-18 (probe designation according to Alm *et al.*, 1996) was designed to specifically hybridize to a complementary target region on the 16S rRNA of all analysed endosymbionts. This probe displays at least two mismatches to all other available 16S rRNA sequences. Optimal hybridization stringency for probe S-G-Proca-438-a-A-18 was observed with 20% formamide in the hybridization buffer. Using these stringent hybridization conditions, no detectable signal was obtained when activated sludge from a municipal sewage treatment plant, known to contain a high diversity of

bacteria (Snaidr *et al.*, 1997), was hybridized with the endosymbiont-specific probe S-G-Proca-438-a-A-18. Consequently, positive hybridization reactions of bacterial endosymbionts with specific probe S-G-Proca-438-a-A-18 demonstrated that the retrieved 16S rDNA sequences did originate from endosymbionts of *A. polyphaga* Page 23 and *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226 and TUMSJ-341 and, furthermore, confirmed their intracellular location (Fig. 4). In addition, simultaneous hybridization of the endosymbionts with probe S-G-Proca-438-a-A-18 and the bacterial probe EUB338 labelled with different dyes illustrated that all bacteria within the *Acanthamoeba* host cells were stained by both probes, demonstrating the absence of phylogenetically different symbionts within the respective amoebal host cells.

18S rDNA-based phylogeny of *Acanthamoeba* host cells

Comparative sequence analysis of 18S rRNA gene fragments retrieved from the amoeba host cells revealed highest sequence similarities with members of the genus *Acanthamoeba* (95–99%) and thus confirmed the morphology-based classification of the investigated host amoebae. 18S rDNA sequence similarities of more than 95% with members of the *Acanthamoeba* 18S rRNA sequence type T4 allowed the amoebal host cells *A. polyphaga* Page 23 and *Acanthamoeba* sp. strains UWC6, UWC12, UWE2 and TUMSJ-226 to be assigned to this sequence type, which comprises the majority of *Acanthamoeba* isolates including all clinical isolates (Stothard *et al.*, 1998). In contrast to these isolates, *Acanthamoeba* sp. TUMSJ-341 showed highest 18S rDNA similarity (95%) with members of sequence type T5, which is indicative for the species *Acanthamoeba lenticulata* (Stothard *et al.*, 1998). Like *A. lenticulata*, *Acanthamoeba* sp. TUMSJ-341 contained a larger 18S rDNA (3000 bp) than all other *Acanthamoeba* species (2200 bp), caused by a 640 bp insertion with similarities to the group I intron previously found in the small subunit ribosomal gene of *A. lenticulata* (Gast *et al.*, 1994). All applied phylogenetic treeing methods consistently confirmed the membership of the investigated

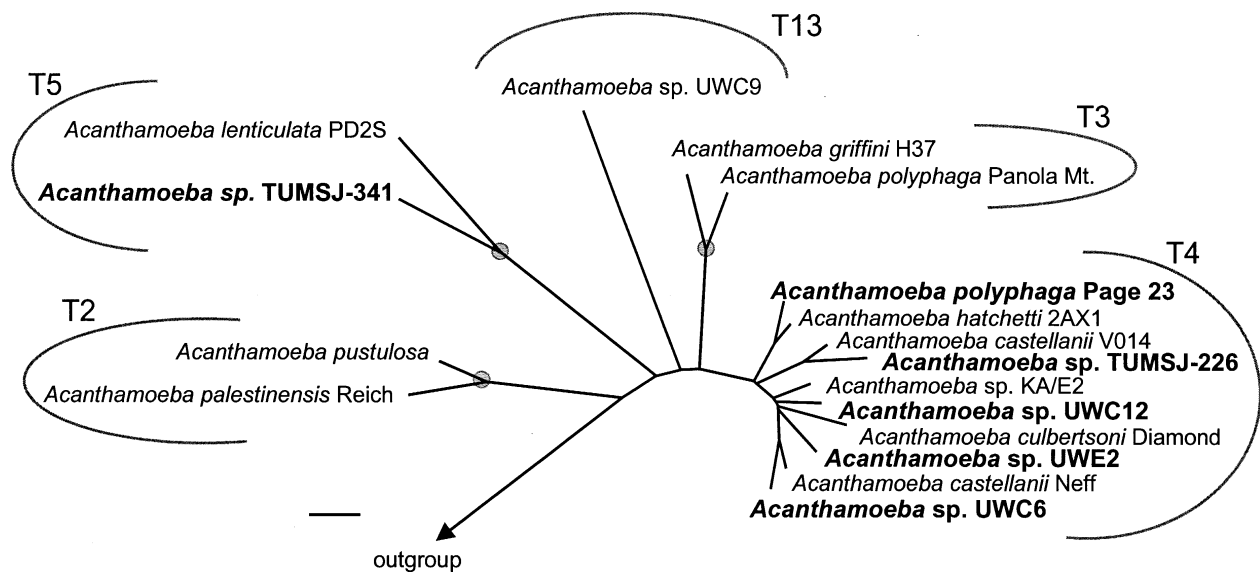


Fig. 5. 18S rDNA-based neighbour-joining tree reflecting the affiliation of amoebal host cells *A. polyphaga* Page 23 and *Acanthamoeba* sp. strains UWC6, UWC12, UWE2, TUMSJ-226 and TUMSJ-341. *Acanthamoeba* 18S rDNA sequence types are noted. Filled circles indicate parsimony bootstraps values (1000 replicates) of 100%. Bootstrap values less than 50% were omitted. Bar, 1% estimated evolutionary distance. Accession numbers of the species shown in this tree: *Acanthamoeba griffini*, S81337; *A. polyphaga* Panola, AF019052; *A. polyphaga* Page 23, AF019061; *Acanthamoeba hatchetti*, AF019060; *Acanthamoeba castellanii* V014, U07401; *Acanthamoeba* sp. KA/E2, AF005998; *Acanthamoeba culbertsoni*, AF019057; *A. castellanii* Neff, U07416; *Acanthamoeba palestinensis*, U07411; *Acanthamoeba pustulosa*, AF019050; *A. lenticulata*, U94741; and *Acanthamoeba* sp. UWC9, AF132134.

Acanthamoeba isolates with 18S rRNA sequence types T4 and T5 (Fig. 5).

Classification of the novel β -proteobacterial endosymbionts of acanthamoebae

While endosymbiosis of bacteria within protozoa, plants or animals is widespread in nature, the majority of described bacteria with this lifestyle are members of the α - or γ -*Proteobacteria*. Currently, only four endosymbiotic micro-organisms pertaining to the β -*Proteobacteria* have been reported: *Polynucleobacter necessarius*, an endosymbiont of the hypotrichous ciliate *Euplotes aediculatus* (Springer *et al.*, 1996); *Kinetoplastibacterium crithidii* and *Kinetoplastibacterium blastocrithidii*, which are obligate endosymbionts of the insect trypanosomatids *Crithidia* sp. and *Blastocrithidia culicis*, respectively (Du *et al.*, 1994); and endosymbionts of the arbuscular-mycorrhizal fungus *Gigaspora margarita* (Bianciotto *et al.*, 1996). The β -proteobacterial *Acanthamoeba* endosymbionts identified in this study and the other, distantly related currently recognized endosymbiotic bacteria within this subclass are members of different lines of descent (Fig. 2) suggesting that the capability to thrive within eukaryotic host cells has evolved several times within the β -*Proteobacteria*.

As the investigated endosymbionts of *Acanthamoeba* spp. showed a 16S rDNA sequence similarity with all other members of the β -*Proteobacteria* of less than 90% and thus could not be assigned to any recognized

taxon within this subclass, provisional classification of one representative, the endosymbiont of *Acanthamoeba* sp. UWC12, as '*Candidatus Procabacter acanthamoebae*' gen. nov., sp. nov. is proposed. The 16S rDNA sequence similarity of 95–99% for the endosymbionts of *A. polyphaga* strain Page 23 and *Acanthamoeba* sp. strains UWC6, UWE2, TUMSJ-226 and TUMSJ-341 with '*Candidatus Procabacter acanthamoebae*' suggests that they all belong to the tentative genus '*Procabacter*'.

Description of '*Candidatus Procabacter acanthamoebae*' gen. nov., sp. nov.

Procabacter acanthamoebae (Pro.ca.bac.ter. N.L. masc. n. *Procabacter* pertaining to the microbiologist M. Proca-Ciobanu, who was the first to report rod-shaped intracellular symbionts in *Acanthamoeba*; a'canth.a.moe.bae. L. gen. sing. n. *acanthamoebae* of *Acanthamoeba*, taxonomic name of a genus of *Acanthamoebidae*, pertaining to the name of the host amoeba, *Acanthamoeba* sp. strain UWC12, in which the organism was first discovered).

The short description is as follows: Gram-negative; rod-shaped morphology; 0.3–0.5 \times 1.0–2.0 μ m; basis of assignment, 16S rDNA sequence accession number AF177427, 23S rDNA sequence accession number AF352393, 16S rRNA-targeted nucleotide probe S-G-Proca-0438-a-A-18 (5'-CGATTCCTCCCRGACA-A-3'); not cultivated on cell-free media; obligate intracytoplasmic symbiont of *Acanthamoeba* sp.

strain UWC12 isolated from a corneal scrapings of *Acanthamoeba* keratitis patients at the University of Washington Medical Center, Seattle, WA, USA; artificial infection of amoebal host cells with 'Candidatus Procabacter acanthamoebae' enhances their *in vitro* cytopathogenicity (Fritsche *et al.*, 1998).

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