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The Genus *Caedibacter* Comprises Endosymbionts of *Paramecium* spp. Related to the *Rickettsiales* (*Alphaproteobacteria*) and to *Francisella tularensis* (*Gammaproteobacteria*)

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Obligate bacterial endosymbionts of paramecia able to form refractile inclusion bodies (R bodies), thereby conferring a killer trait upon their ciliate hosts, have traditionally been grouped into the genus *Caedibacter*. Of the six species described to date, only the *Paramecium caudatum* symbiont *Caedibacter caryophilus* has been phylogenetically characterized by its 16S rRNA gene sequence, and it was found to be a member of the *Alphaproteobacteria* related to the *Rickettsiales*. In this study, the *Caedibacter taeniospiralis* type strain, an R-body-producing cytoplasmatic symbiont of *Paramecium tetraurelia* strain 51k, was investigated by comparative 16S rRNA sequence analysis and fluorescence in situ hybridization with specific oligonucleotide probes. *C. taeniospiralis* is not closely related to *C. caryophilus* (80% 16S rRNA sequence similarity) but forms a novel evolutionary lineage within the *Gammaproteobacteria* with the family *Francisellaceae* as a sister group (87% 16S rRNA sequence similarity). These findings demonstrate that the genus *Caedibacter* is polyphyletic and comprises at least two phylogenetically different bacterial species belonging to two different classes of the *Proteobacteria*. Comparative phylogenetic analysis of *C. caryophilus*, five closely related *Acanthamoeba* endosymbionts (including one previously uncharacterized amoebal symbiont identified in this study), and their hosts suggests that the progenitor of the alphaproteobacterial *C. caryophilus* lived within acanthamoebae prior to the infection of paramecia.

The ability of certain paramecia to kill other paramecia was first described in 1938 by Sonneborn, who observed that sensitive paramecia exhibit distinct morphological symptoms upon ingestion of toxic "particles" released by the killer strain and ultimately die (52). In 1958, electron microscopy studies revealed that these heritable cytoplasmic particles are gram-negative rod-shaped prokaryotes (16) which eluded cultivation using standard laboratory media (30, 32) and that the toxic effect is associated with proteinaceous, refractile inclusion bodies (R bodies) found inside the bacterial endosymbionts (30, 32). Subsequently, all R-body-producing obligate intracellular symbionts of paramecia were combined into the genus *Caedibacter* and classified according to morphological, functional, and phenotypic properties (17, 32, 35). Within the genus *Caedibacter*, the six species *Caedibacter caryophilus*, *Caedibacter varicaedens*, *Caedibacter taeniospiralis*, *Caedibacter pseudomutans*, *Caedibacter paraconjugatus*, and *Caedibacter macronucleorum* (11, 38, 47) have been recognized. *C. caryophilus*, the only member of the genus *Caedibacter* that has been characterized by its 16S rRNA gene sequence, was found to be affiliated with the *Alphaproteobacteria* (57). Within the *Alphaproteobacteria*, *C. caryophilus* clusters together with obligate endosymbionts of acanthamoebae (6, 20) and the paramecium endosymbionts *Holospora obtusa* and *Holospora elegans* (3, 14).

In this study, the phylogenetic affiliation of the *Paramecium tetraurelia* symbiont *C. taeniospiralis* strain 51k (initially described by Beale et al. [5], named by Preer et al. [32], and validated by Preer and Preer [34]) was investigated by the full-cycle rRNA approach, including comparative 16S rRNA gene sequence analysis and detection of endosymbionts within their host cells by fluorescence in situ hybridization (FISH) using specific 16S rRNA-targeted oligonucleotide probes. These analyses showed that despite several consistent morphological and functional features of its members, the genus *Caedibacter* contains at least two only distantly related bacteria which are affiliated with different classes of the *Proteobacteria*. In addition, a previously uncharacterized *Acanthamoeba* endosymbiont was included in this study, as it was found to hybridize with a *C. caryophilus*-specific oligonucleotide probe, indicating close phylogenetic relatedness. Comparative analysis of the phylogeny of these alphaproteobacterial symbionts and their hosts suggested that the progenitor of *C. caryophilus* lived and coevolved within acanthamoebae prior to the infection of paramecia.

MATERIALS AND METHODS

Isolation and maintenance of protozoa. *P. tetraurelia* strain 51k containing endosymbiotic *C. taeniospiralis* was isolated previously in Spencer, Ind. (ATCC 30632). The morphology and fine structure of the symbiont, at that time designated the kappa particle, was described by Beale et al. (5), and the symbiont was later classified as the type strain of *C. taeniospiralis* (17). The paramecia were maintained over the years in lettuce medium or in a decoction of cereal leaves supplemented with living *Enterobacter aerogenes* cells at 23°C as described elsewhere (54). *Acanthamoeba* sp. strain TUMK-23 harboring rod-shaped endosymbionts was recovered in this study from activated sludge of a wastewater treatment plant connected to a rendering plant (Kraftisried, Germany), using the

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TABLE 1. Oligonucleotide probes used for FISH[a]

Probe name	Designation[b]	Sequence (5′-3′)	Target rRNA	Specificity	% FA[c]	Reference
Ctaenio998	S-S-Ctaenio-998-a-A-18	CTCTCTCGTCTTCTATGG	16S	*C. taeniospiralis* 51k	60	This study
Ctaenio129	S-S-Ctaenio-129-a-A-18	CCCTCTGTACGGCAGATT	16S	*C. taeniospiralis* 51k	70	This study
Ctaenio86	S-S-Ctaenio-86-a-A-18	GAAAGTGGAAGTCGAACC	16S	*C. taeniospiralis* 51k	ND	This study
EUB338		GCTGCCTCCCGTAGGAGT	16S	Almost all bacteria	0–50	3
EUB338-II		GCAGCCACCCGTAGGTGT	16S	*Planctomycetales*	0–50	8
EUB338-III		GCTGCCACCCGTAGGTGT	16S	*Verrucomicrobiales*	0–50	8
ALF1B		CGTTCGYTCTGAGCCAG	16S	*Alphaproteobacteria*	20	28
BET42a		GCCTTCCCACTTCGTTT	23S	*Betaproteobacteria*	35	28
GAM42a		GCCTTCCCACATCGTTT	23S	*Gammaproteobacteria*	35	28
CC23a		TTCCACTTTCCTCTCTCG	16S	*C. caryophilus*	ND	58

[a] Difference alignments are available at probeBase (http://www.probebase.net).
[b] According to Alm et al. (1).
[c] Optimal formamide (FA) concentration in the hybridization buffer. ND, not determined.

technique described by Visvesvara (58). Amoebal cultures were axenized as described previously (20) and maintained in trypticase-soy-yeast extract broth at 20°C.

Electron microscopy. *Acanthamoeba* sp. strain TUMK-23 was examined by electron microscopy using a modification of a previously published method (15). Briefly, aliquots of amoebae in broth were fixed with 2% glutaraldehyde in 0.1 M cacodylate. The fixed amoebae were then pelleted in agar and embedded. Thin sections were stained with uranyl acetate and lead citrate and examined with a Phillips CM-10 electron microscope. *C. taeniospiralis* was examined by electron microscopy as described for *C. caryophilus* by Schmidt et al. (47). Briefly, symbiont-bearing paramecia were fixed with glutaraldehyde, postfixed with OsO_4, and embedded in epoxid resin. Sections were stained with uranyl acetate and lead citrate and investigated with a Zeiss 10 electron microscope.

Orcein staining. Paramecia were fixed on a slide with osmium vapor for 5 to 10 s, postfixed with a drop of a mixture of ethanol and acetic acid (3:1), and stained with a drop of orcein dissolved in acetic and lactic acid as described by Beale and Jurand (4).

Killer tests. To determine whether *C. taeniospiralis*-bearing paramecia display killer activity, symbiont-free paramecia of the same strain were incubated together with symbiont-bearing cells in small wells of depression slides as described by Sonneborn (54). The wells were observed for deformed, spinning, or dying cells for up to 48 h (the descriptions of different killer traits are summarized in reference 32).

DNA isolation, PCR, cloning, and sequencing. In order to separate extracellular bacteria from the *C. taeniospiralis*-bearing paramecium cells, 2 ml of a dense xenically grown paramecium culture was filtered through a 0.45-μm-pore-size syringe filter (PALL Gelman Laboratory, Ann Arbor, Mich.) and washed twice with distilled water. Paramecia were recovered from the filter disk by reverse filtration with 2 ml of distilled water and subsequently harvested by centrifugation (21,910 × *g*; 3 min). Simultaneous DNA isolation from *P. tetraurelia* and its intracellular bacteria was performed using the DNeasy tissue kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Simultaneous isolation of DNA from *Acanthamoeba* sp. strain TUMK-23 and its endosymbionts was performed using a modified UNSET procedure (22) as described by Horn et al. (20).

PCR amplification of near-full-length bacterial 16S rRNA gene fragments was performed using primers targeting signature regions of the 16S rRNA gene that are highly conserved within the domain *Bacteria*. The nucleotide sequences of forward and reverse primers were 5′-AGAGTTTGATYMTGGCTCAG-3′ (*Escherichia coli* 16S ribosomal DNA [rDNA] positions 8 to 27 [7, 59]) and 5′-GGYTACCTTGTTACGACT-3′ (*E. coli* 16S rDNA positions 1492 to 1511) or 5′-CAKAAAGGAGGTGATCC-3′ (*E. coli* 16S rDNA positions 1529 to 1546). Amplification of near-full-length paramecium and amoeba 18S rRNA genes was carried out using primers SSU1 (5′-AACCTGGTTGATCCTGCCAG-3′) and SSU2 (5′-GATCCTTCTGCAGGTTCACCTAT-3′), complementary to conserved target regions at both ends of the 18S rRNA gene (12). 16S rRNA and 18S rRNA gene amplification reactions were performed separately using annealing temperatures of 54 (16S rRNA gene) and 55°C (18S rRNA gene), respectively. Negative controls without a DNA template were included in all PCRs. The presence and sizes of the amplification products were determined by agarose gel electrophoresis and ethidium bromide staining.

Amplified products were cloned into *E. coli* using the TOPO TA cloning kit (Invitrogen, Carlsbad, Calif.) following the instructions of the manufacturer. Nucleotide sequences of cloned rRNA gene fragments were determined by the dideoxynucleotide method (45) by cycle sequencing of purified plasmid preparations (Qiagen) using the Thermo Sequenase cycle-sequencing kit (Amersham Life Science, Little Chalfont, England) and an infrared automated DNA sequencer (Li-Cor Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers. For sequencing, the dye-labeled vector-specific primers M13/pUC V (5′-GTAAAACGACGGCCAGT-3′) and M13/pUC R (5′-GAAACAG CTATGACCATG-3′) were applied. Primer Ac1138 (5′-CTCTAAGAAGCAC GGACG-3′ [20]) was used to complete the 18S rRNA gene sequence of *Acanthamoeba* sp. strain TUMK-23.

Comparative sequence analysis. Sequence homology searches within the public databases DDBJ-EMBL-GenBank were performed using the BLASTn service available at the National Center for Biotechnology Information website (2). The 16S and 18S rRNA sequences obtained were added to the rRNA sequence database of the Technische Universität München (encompassing about 15,000 published and unpublished homologous small-subunit rRNA primary structures) by use of the program package ARB (available at http://www.arb-home.de). Alignment of retrieved the rRNA sequences was performed by using the ARB automated alignment tool (Fast Aligner version 1.03). The alignments were refined by visual inspection and by secondary-structure analysis (26). Phylogenetic analyses were performed by applying the distance matrix, parsimony, and maximum-likelihood methods implemented in ARB to different data sets. Bootstrap analysis (1,000 resamplings) was performed for the parsimony trees using the Phylip program package (10). To determine the robustness of the phylogenetic trees, analyses were performed with and without the application of filter sets excluding highly variable positions. In detail, the filters included only those positions that are conserved in at least 50% of all bacteria, all *Alphaproteobacteria*, and all *Gammaproteobacteria* within the 16S rRNA database. For the phylogenetic analysis of the protozoa, filters were constructed which considered only those 18S rRNA positions that are conserved in at least 50% of all eukaryotic or *Acanthamoeba* sequences present in the 18S rRNA database. Unless otherwise noted in the text, the bacterial nomenclature proposed in the taxonomic outline (release 1; April 2001) of the second edition of *Bergey's Manual of Systematic Bacteriology* (http://www.cme.msu.edu/bergeys/) was used.

Oligonucleotide probes. The oligonucleotide probes used in this study are listed in Table 1. New oligonucleotide probes were designed using the Probedesign and Probematch tools implemented in the ARB software package. In order to ensure probe specificity, all available rRNA sequences included in the ARB database were checked for the presence of the probe target sites. The oligonucleotides were synthesized and directly labeled with 5(6)-carboxyfluorescein-*N*-hydroxysuccinimide ester (FLUOS) or the hydrophilic sulfoindocyanine fluorescent dye Cy3 or Cy5 (Interactiva, Ulm, Germany). Newly designed oligonucleotide probe sequences were deposited at the oligonucleotide probe database probeBase (http://www.probebase.net).

FISH. For in situ hybridization, 10 μl of dense protozoal cultures was spotted on glass slides and dried at room temperature. Lysis of the paramecia was prevented by using a constant airflow to speed up the drying process. The immobilized specimens were fixed with 4% formaldehyde for 30 to 60 min at room temperature, washed twice with sterile water or Page's saline, and subsequently dehydrated in 50, 80, and 98% ethanol for 5 min each. The optimal hybridization and washing conditions for the endosymbiont-specific probes S-S-Ctaenio-998-a-A-18 and S-S-Ctaenio-129-a-A-18 were determined using the hybridization and washing buffers of Manz et al. (27) following procedures de-

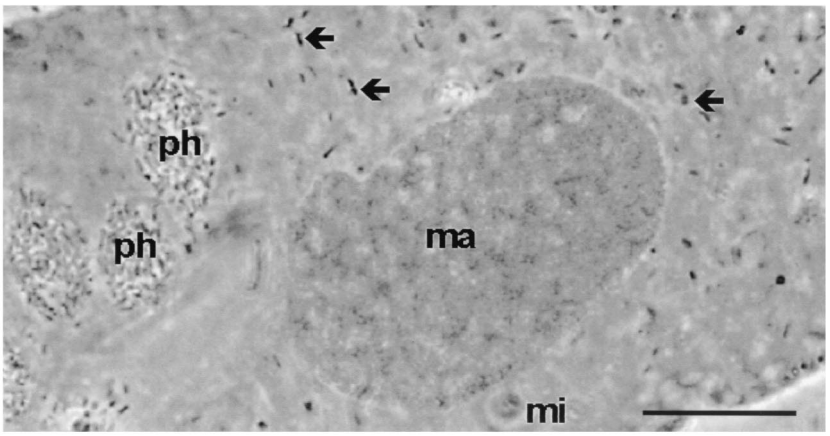

FIG. 1. Phase-contrast micrograph of *P. tetraurelia* strain 51k stained with orcein. Numerous symbionts (arrows) in the cytoplasm and bacteria in phagosomes (ph) are visible. ma, macronucleus; mi, micronucleus. Bar, 10 μm.

scribed previously (20). The intensity of the fluorescence signal remained the same with up to 60 (S-S-Ctaenio-998-a-A-18) or 70% (S-S-Ctaenio-129-a-A-18) formamide in the hybridization buffer. Both probes were subsequently used at 35% formamide in the hybridization buffer. In some experiments, the DNA-binding dye DAPI (4′,6-diamino-2-phenylindole; Sigma, Buchs, Switzerland) was used for the visualization of intracellular bacteria. Subsequent to FISH, the specimens were covered with 5 μl of DAPI solution (1 μg/μl), incubated for 5 min in the dark, washed with sterile water, and air dried.

Slides were examined using a laser scanning confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany). Images were recorded and processed using the standard software package delivered with the instrument (version 2.01).

Nucleotide sequence accession numbers. The recovered 16S and 18S rRNA gene sequences have been submitted to the DDBJ-EMBL-GenBank databases under accession numbers AY102612 (16S rRNA gene of *C. taeniospiralis* strain 51k), AY102613 (18S rRNA gene of *P. tetraurelia* strain 51k), AY102614 (16S rRNA gene of the endosymbiont of *Acanthamoeba* sp. strain TUMK-23), and AY102615 (18S rRNA gene of *Acanthamoeba* sp. strain TUMK-23).

RESULTS

Morphology, phylogeny, and in situ identification of *C. taeniospiralis* 51k. Bacterial endosymbionts were readily visualized within *P. tetraurelia* by orcein staining and phase-contrast light microscopy (Fig. 1). *C. taeniospiralis* 51k was formerly described as a cytoplasmic endosymbiont of *P. tetraurelia* able to produce type 51 R bodies (with R-body genes encoded on a plasmid) conferring a hump killer trait upon its paramecium host (5, 17). Consistent with this description, electron microscopy revealed that the rod-shaped endosymbionts of the investigated *P. tetraurelia* 51k measured 0.4 to 0.7 by 1.0 to 2.5 μm and possessed a gram-negative-type cell wall (Fig. 2). The bacteria were equally distributed within the cytoplasm and were surrounded by an electron-translucent layer (Fig. 2). Type 51 R bodies were observed within the investigated endosymbionts and were shown to confer a hump killer trait upon their *P. tetraurelia* hosts (9, 32, 56), inducing the formation of aboral blisters in sensitive (symbiont-free) paramecia.

Near-full-length 16S rRNA gene sequences (1,544 bp) of intracellular bacteria of *P. tetraurelia* were amplified, cloned, and sequenced. Out of 10 16S rRNA clones analyzed, 7 were found to have identical sequences. This sequence was considered to represent the endosymbionts of *P. teraurelia*, while the other three sequences were assumed to be derived from bacteria that were present in the food vacuoles of the xenically grown paramecium culture. This 16S rRNA gene sequence was novel and showed moderate 16S rRNA sequence similarity with members of the *Gammaproteobacteria*. Within the *Gammaproteobacteria*, the highest 16S rRNA similarity values were obtained with representatives of the *Francisella* group (86.5 to 87.3%). A significantly lower 16S rRNA similarity (80%) was observed with the alphaproteobacterial symbiont *C. caryophilus*. All applied treeing methods consistently confirmed the affiliation of the retrieved 16S rRNA gene sequences with the *Gammaproteobacteria* and demonstrated that they form a novel evolutionary lineage within this subgroup with the *Francisella* cluster as a sister group (Fig. 3).

Consistent with these findings, *C. taeniospiralis* was readily visualized within its *Paramecium* host cells by FISH using the probe GAM42a (targeting a signature region on the 23S rRNA of the *Gammaproteobacteria*) (Fig. 4), while no fluorescence

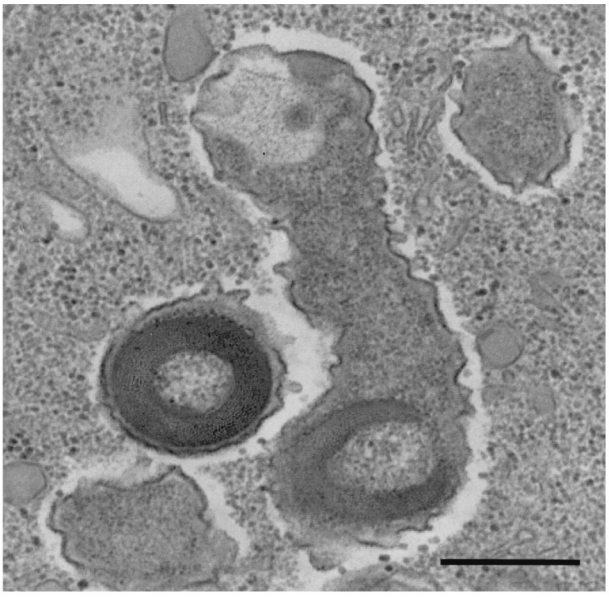

FIG. 2. Ultrastructure of *C. taeniospiralis* harboring type 51 R bodies within the cytoplasm of its host, *P. tetraurelia*. Bar, 0.5 μm.

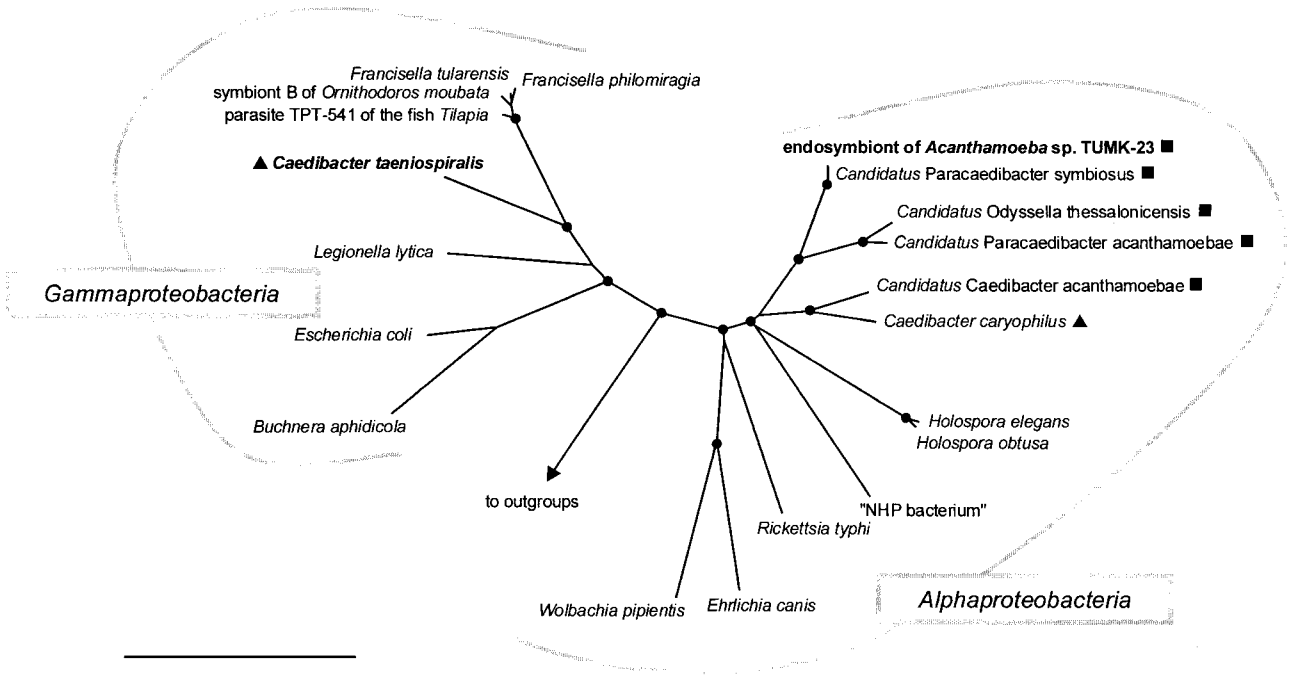

FIG. 3. 16S rRNA-based neighbor-joining tree showing (i) the phylogenetic affiliation of *C. taeniospiralis* 51k (endosymbiont of *P. tetraurelia*) with representative members of the *Gammaproteobacteria* and (ii) the relationship of the endosymbiont of *Acanthamoeba* sp. strain TUMK-23 with the alphaproteobacterial *C. caryophilus* (endosymbiont of *P. caudatum*) and other representative members of the *Alphaproteobacteria.* "*Candidatus* Caedibacter acanthamoebae," "*Candidatus* Paracaedibacter acanthamoebae," "*Candidatus* Paracaedibacter symbiosus," and "*Candidatus* Odyssella thessalonicensis" were recently described as endosymbionts of acanthamoebae (5, 18). The respective eukaryotic hosts are indicated by symbols: ▲, *Paramecium* sp.; ■, *Acanthamoeba* sp. Parsimony bootstrap values (1,000 resamplings) of >97% are indicated as solid circles. "NHP bacterium," shrimp pathogen causing necrotizing hepatopancreatitis. Bar, 10% estimated evolutionary distance.

was detected with the probes ALF1B and CC23a (specific for the *Alphaproteobacteria* and *C. caryophilus*, respectively; data not shown). Application of the newly designed probes S-S-Ctaenio-998-a-A-18 and S-S-Ctaenio-129-a-A-18, complementary to signature regions on the retrieved *Francisella*-related 16S rRNA sequence, demonstrated that this sequence originated from the intracytoplasmatic *P. tetraurelia* endosymbiont *C. taeniospiralis* (Fig. 4). Simultaneous hybridization with the probes S-S-Ctaenio-998-a-A-18 and S-S-Ctaenio-129-a-A-18 and the bacterial probe EUB338 labeled with different dyes illustrated that all detectable bacteria within the cytoplasm of *P. tetraurelia* were stained by all three probes, demonstrating the absence of phylogenetically different symbiotic bacteria within the host cells (Fig. 4).

The 16S rRNA gene of *C. taeniospiralis* contained a stretch of 97 additional base pairs replacing the tetraloop corresponding to helix 6 of the *E. coli* rRNA secondary structure (positions 82 to 87), which did not show significant similarity to any rRNA gene sequence deposited in public databases. This putative intervening sequence is able to form a stable stem-loop structure, as indicated by secondary-structure prediction using the software RNAstructure version 3.5 (28). The presence of

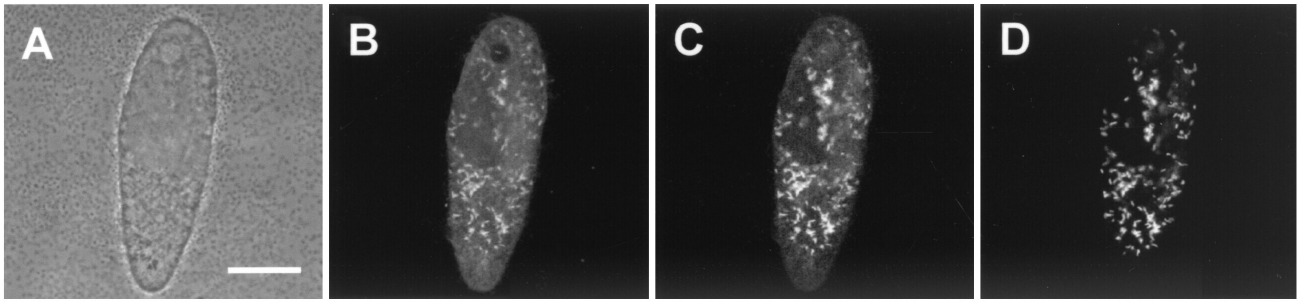

FIG. 4. In situ identification of *C. taeniospiralis* 51k within its host, *P. tetraurelia*. Identical microscopic fields are depicted. The fluorescence images (B to D) show a median section through the paramecium cell seen in the simulated phase-contrast image (A). Shown is FISH using the FLUOS-labeled oligonucleotide probe EUB338 (B), the Cy3-labeled endosymbiont-specific probe S-S-Ctaenio-129-a-A-18 (C), and the Cy5-labeled endosymbiont-specific probe S-S-Ctaenio-998-a-A-18 (D). Bar, 20 μm

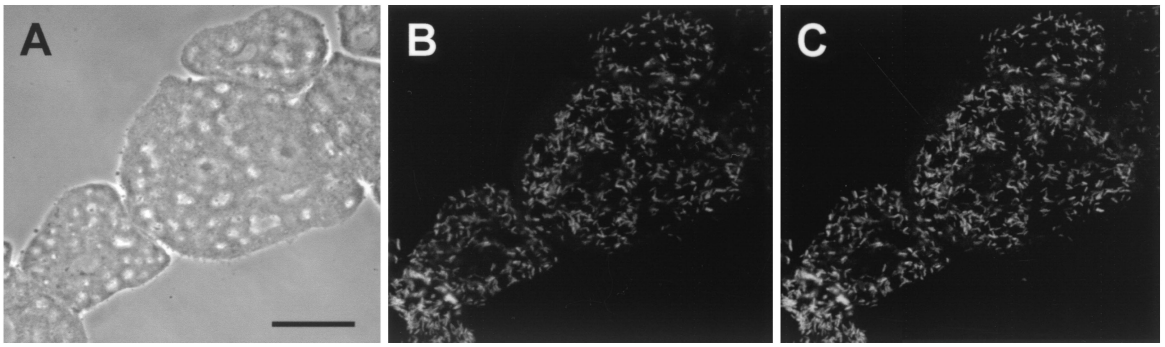

FIG. 5. In situ identification of the bacterial endosymbiont of *Acanthamoeba* sp. strain TUMK-23. Identical microscopic fields are depicted. (A) Phase-contrast image. (B and C) FISH using FLUOS-labeled oligonucleotide probe EUB338 (B) and Cy3-labeled *C. caryophilus*-specific probe CC23a (C). Bar, 10 μm.

such intervening sequences within rRNA genes has been reported previously for *C. caryophilus* and other intracellular bacteria (21, 25, 46, 57). However, a search within the publicly available 16S rRNA gene sequences revealed that <20 only distantly related sequences also possess additional base pairs in this region (for example, members of the *Firmicutes*, the *Actinobacteria*, the *Planctomycetes*, and the *Epsilonproteobacteria*). FISH using the probe S-S-Ctaenio-86-a-A-18, which targets the putative intervening sequence of *C. taeniospiralis*, resulted in no detectable signals, suggesting that this sequence stretch is either excised during maturation of the RNA or is simply not accessible due to the formation of a stable secondary structure.

Phylogeny and in situ identification of a *C. caryophilus*-related endosymbiont of *Acanthamoeba* sp. Previous studies demonstrated that bacteria closely related to the alphaproteobacterial *Paramecium caudatum* symbiont *C. caryophilus* also occur as endosmybionts of free-living amoebae (provisionally designated "*Candidatus* Paracaedibacter" and "*Candidatus* Odyssella" [6, 20]). Therefore, the *Acanthamoeba* isolate TUMK-23, which was found to contain bacterial endosymbionts that hybridize with the *C. caryophilus*-specific oligonucleotide probe CC23a (indicating a close relationship with *C. caryophilus*), was included in this study (Fig. 5). Electron microscopy revealed that this rod-shaped endosymbiont measured 0.2 to 0.3 by 1.3 to 1.7 μm and possessed a gram-negative-type cell wall (Fig. 6). The bacteria were equally distributed within the cytoplasm and were surrounded by an electron-translucent layer (Fig. 6). Consistent with previous descriptions of *C. caryophilus*-related symbionts of amoebae, no R-body-like structures were observed within these symbionts.

Near-full-length 16S rRNA gene sequences (1,464 bp) of the bacterial symbiont of *Acanthamoeba* sp. strain TUMK-23 were amplified, cloned, and sequenced. Comparative sequence analysis demonstrated that this endosymbiont shows the highest 16S rRNA sequence similarities to members of the *Alphaproteobacteria* and that it is closely affiliated with the *C. caryophilus*-related amoebal symbiont "*Candidatus* Paracaedibacter symbiosus" (99% 16S rRNA sequence similarity) (Fig. 3).

Phylogeny of the protozoan hosts of *Caedibacter* endosymbionts. Comparative analysis of 18S rRNA gene fragments (1,701 and 2,239 bp) retrieved from the *P. tetraurelia* and the *Acanthamoeba* sp. hosts revealed the highest sequence similarities to members of the genus *Paramecium* (98% with *P. tetraurelia* [50]) and *Acanthamoeba* (98% with *Acanthamoeba* sp. strain UWE39 [20]), respectively, and thus confirmed the morphology-based classification of these protozoa.

DISCUSSION

Ever since the cytoplasmic heritable killer trait of paramecia has been associated with endosymbiotic bacteria, several models for classification of these bacteria have been proposed (17, 32, 37, 38, 55). Initially, these endosymbionts were designated by Greek letters (for example, as kappa or mu particles) (32, 33, 53). While this codification is still in use, a binominal

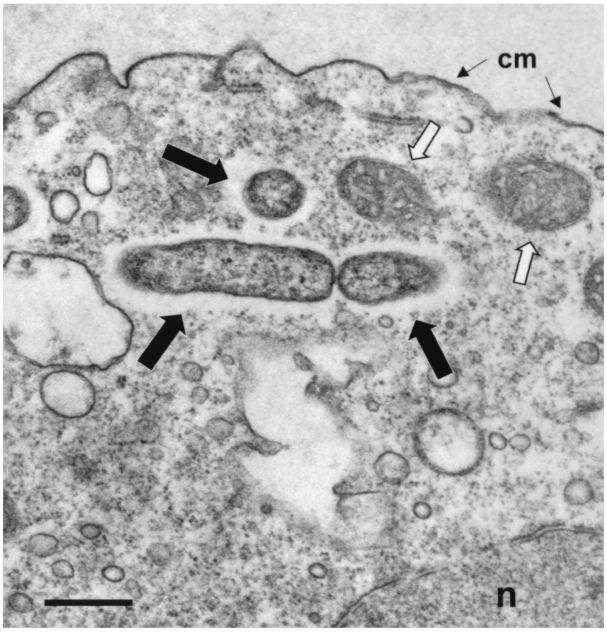

FIG. 6. Ultrastructure of the *C. caryophilus*-related endosymbiont (solid arrows) within the cytoplasm of its host, *Acanthamoeba* sp. strain TUMK-23. cm, amoebal cell membrane; n, nucleus. The open arrows indicate mitochondria. Bar, 0.5 μm.

nomenclature and the genus name *Caedibacter* (earlier designated *Caedobacter* [36]) were introduced in the 1970s (32). In brief, there is general agreement that the presence of R bodies, the host specificity of the symbionts, and the cell compartment in which the symbiont multiplies (macronucleus or cytoplasm) are the key taxonomic properties for classification of *Caedibacter* species. In addition, the different types of killing that the symbionts confer upon their hosts, the morphology of R bodies, the potential association with bacteriophages or bacteriophage-like structures, and the existence of different types of extrachromosomal elements were used for subclassification of the genus *Caedibacter* (30). However, taxonomic systems that are based on phenotypic properties do not necessarily reflect evolutionary history (29, 44, 61, 62). With respect to the *Caedibacter* endosymbionts of paramecia, significant differences in G+C content and low DNA-DNA hybridization values between different *Caedibacter* species provided initial indications that (i) this genus might contain genetically diverse bacteria and (ii) their common characteristic phenotype might be determined by genetically related extrachromosomal elements (40, 42, 43). The 16S rRNA-based phylogenetic analysis presented in this study substantiated this hypothesis and demonstrated that the genus *Caedibacter* is actually polyphyletic and comprises bacteria belonging to two different classes of the *Proteobacteria. C. taeniospiralis* forms a novel evolutionary lineage within the *Gammaproteobacteria* which groups together with other intracellular bacteria, namely, a symbiont of the tick *Ornithodorus mobuta*, a fish parasite, and the human pathogen *Francisella tularensis*. In contrast, *C. caryophilus* belongs to the alphaproteobacterial family *Holosporaceae* and is most closely related to amoebal endosymbionts (e.g., the endosymbiont of *Acanthamoeba* sp. strain TUMK-23 analyzed in this study) and the parasitic paramecium symbionts *H. obtusa* and *H. elegans* (3, 20).

One traditional key taxonomic criterion of the genus *Caedibacter* is the production of R bodies (13, 23, 30, 48). The findings reported here indicate that R-body production and the associated killer trait either resulted from convergent evolution (within different evolutionary lineages of the *Proteobacteria*) or evolved only once and were subsequently passed on by horizontal gene transfer. Supportive evidence for the latter hypothesis was provided by the discovery that some R-body proteins are encoded by transposable genetic elements like phages or plasmids (24, 31, 39, 40, 41). This scenario might also explain the otherwise peculiar existence of R-body-like structures in nonsymbiotic bacteria, like *Pseudomonas avenae* and *Pseudomonas taeniospiralis*, and their absence in the *Caedibacter*-related *Acanthamoeba* endosymbionts (20, 23, 24, 60). While comparative analysis of the genes necessary for R-body production (the genes necessary for type 51 R-body synthesis were cloned and heterologically expressed by Quackenbush and Burbach [19, 40]) may help to clarify their evolutionary history, our data clearly demonstrate that the presence of R bodies alone must not be used as a phylogenetically meaningful taxonomic marker.

Without considering R bodies as a key criterion for *Caedibacter* taxonomy, the alphaproteobacterial *C. caryophilus* phenotypically seems to differ significantly from all other validly described *Caedibacter* species. *C. caryophilus* is, for example, the only species that multiplies within *P. caudatum*, while nearly all other *Caedibacter* species, including *C. taeniospiralis*, thrive within members of the *Paramecium aurelia* species complex (11, 35, 47). The only exeption, *C. macronucleorum*, resides in *Paramecium duboscqui* (11). In addition, *C. caryophilus* and the phylogenetically uncharacterized *C. macronucleorum* live within the macronuclei of their hosts, whereas all other *Caedibacter* species are located directly within the host cytoplasm (11, 35, 47). In addition, it has been shown that *C. caryophilus* can lose its ability to produce R bodies (47, 49, 51) and switch from symbiotic to parasitic behavior under certain growth conditions (47). In summary, the phylogeny and the phenotypic properties of *C. caryophilus* are more similar to those of the closely related paramecium symbionts *Holospora* sp. and clearly separate *C. caryophilus* from other members of the genus *Caedibacter*.

Comparison of the evolutionary history of the alphaproteobacterial *C. caryophilus*, the closely related endosymbionts of acanthamoebae, and their respective eukaryotic host cells reveals a striking congruency of the branching patterns of their rRNA-based dendrograms (Fig. 7). This finding indicates coevolution between the symbiosis partners and suggests that the ancestor of the *C. caryophilus*-related endosymbionts lived within an amoebal progenitor and coevolved with its hosts during their diversification into the different *Acanthamoeba* sublineages. According to this scenario, *C. caryophilus*-related bacteria originally thrived in amoebal hosts and were relatively recently laterally transferred into a *Paramecium* host. Since the *Acanthamoeba* endosymbionts do not carry R bodies, the uptake of R-body-coding mobile genetic elements by the *C. caryophilus* symbiont might have taken place after the transfer into the new *Paramecium* host cell, for example, by coinfection of the host with different *Caedibacter* species (17). This hypothesis is also supported by the observation that R-body production is not essential for the *Caedibacter-Paramecium* symbiosis (47). A more robust inference of coevolution between alphaproteobacterial *Caedibacter* symbionts and their amoebal hosts, however, must await the isolation and comparative sequence analysis of further amoebae carrying *C. caryophilus*-related endosymbionts.

Clearly, additional rRNA sequence information from the other four validly described *Caedibacter* species and their host paramecia is necessary for a more detailed understanding of the phylogeny of these unique bacteria and the evolution of the *Caedibacter-Paramecium* symbiosis. We obtained several *Paramecium* strains from the American Type Culture Collection which had been described as carrying *Caedibacter* endosymbionts (*P. tetraurelia* stock 51m1k carrying *C. pseudomutans* ATCC 30633, *Paramecium biaurelia* stock 7K carrying *C. varicaedens* ATCC 30637, and *P. biaurelia* stock 570 carrying *C. paraconjugatus* ATCC 30638). Unfortunately, DAPI staining and FISH analysis revealed that those cultures had lost their *Caedibacter* endosymbionts, as had the stock cultures at the American Type Culture Collection (data not shown and N. Hetrick, personal communication). Consequently, these *Caedibacter*-type strains are no longer publicly available. Joint efforts of protozoologists and bacteriologists are required to reisolate paramecia carrying these *Caedibacter* species for a more comprehensive phylogenetic analysis. A comprehensive knowledge of the evolutionary history of *Caedibacter* species will help us to understand the evolution of this complex,

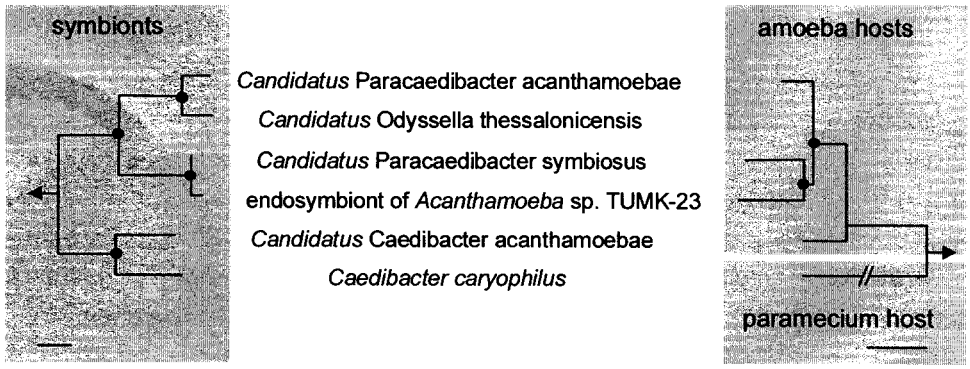

FIG. 7. Comparison of 16S and 18S rRNA-based neighbor-joining dendrograms of alphaproteobacterial *C. caryophilus*-related endosymbionts (left) and their *Acanthamoeba* or *Paramecium* host organisms (right) suggesting (i) coevolution between bacterial symbionts and *Acanthamoeba* hosts and (ii) relatively recent transfer of a *C. caryophilus*-like symbiont from *Acanthamoeba* to *Paramecium*. The 18S rRNA gene sequence of the amoebal host of "*Candidatus* Odyssella thessalonicensis" is not available (6). Parsimony bootstrap values (1,000 resamplings) higher than 99% are indicated as solid circles. The arrow points to outgroups. Bars, 10% estimated evolutionary distance.

unique symbiotic system in which plasmids or phages induce bacterial hosts to produce R bodies and associated toxins (30, 36) that provide their eukaryotic host cells a selective advantage against closely related ciliates.

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