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

Cora L. Beier,¹ Matthias Horn,¹* Rolf Michel,² Michael Schweikert,³ Hans-Dieter Görtz,³ and Michael Wagner¹

Lehrstuhl für Mikrobiologie, Technische Universität München, D-85350 Freising,¹ Central Institute of the Federal Armed Forces Medical Service, D-56065 Koblenz,² and Biologisches Institut, Universität Stuttgart, D-70569 Stuttgart,³ Germany

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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 Franciscellaceae 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 suggested 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 pseudo-mutans, Caedibacter paraconjugatus, and Caedibacter macro-nucleorum (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 (34). 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 (Kraftsried, Germany), using the
According to the instructions of the manufacturer, simultaneous DNA isolation from intracellular bacteria was performed using the DNeasy tissue kit (Qiagen, Hilden, Germany) following the modification of a previously published method (15). Bacteria were xenically grown paramecium culture was 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 Zeiss 10 electron microscope. The wells were observed for deformed, spinning, or dying activity, symbiont-free paramecia of the same strain were incubated to- wards to prevent detection of endosymbionts. The nucleotide sequences of 16S ribosomal DNA [rDNA] positions 8 to 27 [7, 59] and 16S rDNA positions 1529 to 1546. Ampliuria were synthesized and directly labeled with 5(6)-carboxyfluorescent dye Cy3 or Cy5 (Interactiva, Ulm, Germany). Newly designed oligonucleotide probes. The nucleotide sequences of C. taeniospiralis sp. strain TUMK-23 and its endo- symbionts were performed using a modified UNSET procedure (22) described previously (20) and maintained in trypticase-soy-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). Bacteria were xenically grown paramecium culture was 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 Philips CM-10 electron microscope. The nucleotide sequences of C. taeniospiralis sp. strain TUMK-23 and its endosymbionts were performed using a modified UNSET procedure (22) described previously (20) and maintained in trypticase-soy-extract broth at 20°C.

**Oocyte 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 oorein 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 to- gether 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 extracel- lular 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 centrifuga- tion (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) following the instructions of the manufacturer. Simulta- neous isolation of DNA from *Acanthamoeba* sp. strain TUMK-23 and its endo- symbionts was performed using a modified UNSET procedure (22) described as performed 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'-AGAGTTTGATCTGGCCTGAC-3' (Eschschit a coli 16S ribosomal DNA [rDNA] positions 8 to 27 [7, 59]) and 5'-GGAATTCCTAGGATCC-3' (E. coli 16S DNA positions 1492 to 1511) or 5'-CAAGAGGATCC-3' (E. coli 16S DNA positions 1529 to 1546). Amplification of near-full-length parsimony and amoeba 18S rRNA genes was carried out using primers SSU1 (5'-CATCCAGAGGATCC-3') and SSU2 (5'-GGTTGAGGATCC-3') (E. coli 16S rDNA positions 12). 16S rRNA and 18S rRNA gene sequences were amplified 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 PCR reactions. 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 prep- aration (Qiagen) using the Thermo Sequenase cycle-sequencing kit (Amersham Life Science, Little Chalfont, England) and an infrared automated DNA se- quencer (Li-Cor Inc., Lincoln, Nebr.) under conditions recommended by the manufacturers. For sequencing, the dye-labeled vector-specific primers M13/ pUC V (5'-GAAAACGCGCCAGCTG-3') and M13pUC R (5'-GGAAACGCGCCAGCTG-3') were used. Primer Ace1138 (5'-CTCTAGAAGCGCACGACGGAACG-3') was used to complete the 18S rRNA gene sequence of C. taeniospiralis sp. strain TUMK-23.

**Comparative sequence analysis.** Sequence homology searches within the pub- lic databases DDBJ-EMLB-GenBank were performed using the BLASTn se- rvice 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 (comprising 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 with the ARB automated alignment tool (Fast Aligner version 1.03). The alignments were refined by visual inspection and by secondary-structure analysis (26). Phyloge- netic analyses were performed by applying the distance matrix, parsimony, and maximum-likelihood methods implemented in ARB to different data sets. Boot- strap analysis (1,000 resamplings) was performed for the parsimony trees using the Phylip program package (10). To determine the robustness of the phyloge- netic 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 *Acanthamoeba* bac- teria, 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 eukary- otic or *Acanthamoeba* sequences present in the 18S rRNA database. Unless otherwise noted in the text, the bacterial nomenclature proposed in the taxo- nomic 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 Probede- sign and Probedemat tools implemented in the ARB software package. In order to probe sequence specificity, all available rRNA sequences included in the ARB database were checked for the presence of the probe target sites. The oligo- nucleotides were synthesized and directly labeled with 5(6)-carboxyfluores- cein-N-hydroxysuccinimide ester (FLUOS) or the hydrophilic sulfoindocyanine fluorescent dye Cy3 or Cy5 (Interactiva, Ulm, Germany). Newly designed oligo- nucleotide probe sequences were deposited at the oligonucleotide probe data- base 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 air flow 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 subse- quently dehydrated in 50, 80, and 96% 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 hy- bridization and washing buffers of Manz et al. (27) following procedures de-
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).

**RESULTS**

**Morphology, phylogeny, and in situ identification of* C. taeniospiralis* 51k.** Bacterial endosymbions 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 endosymbions of the investigated* P. tetraurelia* strain 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 endosymbions 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 endosymbions of* P. tetraurelia*, 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. caryophillus*. 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. 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.](image)

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

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**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 alpaproteobacterial C. caryophilus (endosymbiont of P. caudatum) and other representative members of the Alphaproteobacteria. “Candidatus Caedibacter acanthamoebae,” “Candidatus Paracaedibacter acanthamoebae,” “Candidatus Paracaedibacter symbiosus,” and “Candidatus Odysella 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.

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**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
such intervening sequences within rRNA genes has been reported previously for \textit{C. caryophilus} and other intracellular bacteria (21, 25, 46, 57). However, a search within the publicly available 16S rRNA gene sequences revealed that only distantly related sequences also possess additional base pairs in this region (for example, members of the \textit{Firmicutes}, the \textit{Actinobacteria}, the \textit{Planctomycetes}, and the \textit{Epsilonproteobacteria}).

FISH using the probe S-S-Ctaenio-86-a-A-18, which targets the putative intervening sequence of \textit{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 \textit{C. caryophilus}-related endosymbiont of \textit{Acanthamoeba} \textit{sp.} Previous studies demonstrated that bacteria closely related to the alphaproteobacterial \textit{Paramecium caudatum} symbiont \textit{C. caryophilus} also occur as endosymbionts of free-living amoebae provisionally designated “\textit{Candidatus} Paracaedibacter” and “\textit{Candidatus} Odyssellinga” [6, 20]). Therefore, the \textit{Acanthamoeba} isolate TUMK-23, which was found to contain bacterial endosymbionts that hybridize with the \textit{C. caryophilus}-specific oligonucleotide probe CC23a (indicating a close relationship with \textit{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 \textmu m 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 \textit{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 \textit{Acanthamoeba} \textit{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 genus \textit{Paramecium} (98\% with \textit{P. tetraurelia} [50]) and \textit{Acanthamoeba} (98\% with \textit{Acanthamoeba} \textit{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
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 bacteri- sponds confer upon their hosts, the morphology of R bod- ies, the potential association with bacteriophages or bacteri- ophage-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 Caed- ibacter endosymbionts of paramecia, significant differences in G+C content and low DNA-DNA hybridization values be- tween different Caedibacter species provided initial indications that (i) this genus might contain genetically diverse bacteria and (ii) their common characteristic phenotype might be de- termined by genetically related extrachromosomal elements (40, 42, 43). The 16S rRNA-based phylogenetic analysis pre- sented in this study substantiated this hypothesis and demon- strated that the genus Caedibacter is actually polyphyletic and comprises bacteria belonging to two different classes of the Proteobacteria. C. tennispaliralis forms a novel evolutionary lin- eage within the Gammaproteobacteria which groups together with other intracellular bacteria, namely, a symbiont of the tick Ornithodoros moubata, 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 Caedi- bacter 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 evolu- tion (within different evolutionary lineages of the Proteobacte- ria) 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 avenueae and Pseudomonas tennispaliralis, and their absence in the Caedi- bacter-related Acanthamoeba endosymbions (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 meaning- ful taxonomic marker.

Without considering R bodies as a key criterion for Caedibacter taxonomy, the alphaproteobacterial C. caryophilus pheno- typically seems to differ significantly from all other validly described Caedibacter species. C. caryophilus is, for example, the only species that multiplex within P. caudatum, while nearly all other Caedibacter species, including C. tennispaliralis, thrive within members of the Paramecium aurelia species complex (11, 35, 47). The only expection, 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 cyto- plasm (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 symbions Holospora sp. and clearly separate C. caryophilus from other members of the genus Caedibacter.

Comparison of the evolutionary history of the alphaproteo- bacterial C. caryophilus, the closely related endosymbions 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 co- evolution between the symbiosis partners and suggests that the ancestor of the C. caryophilus-related endosymbions 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 horizontally transferred into a Paramecium host. Since the Acanthamoeba endosymbions do not carry R bodies, the up- take 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 hypothe- sios is also supported by the observation that R-body produc- tion is not essential for the Caedibacter-Paramecium symbiosis (47). A more robust inference of coevolution between alpha- proteobacterial Caedibacter symbions and their amoebal hosts, however, must await the isolation and comparative se- quence 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 biurelia stock 7K carrying C. vari- caedens ATCC 30637, and P. biurelia stock 570 carrying C. paraconjugatus ATCC 30638). Unfortunately, DAPI staining and FISH analysis revealed that those cultures had lost their Caedibacter endosymbions, 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 ef- forts 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,
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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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 Odysella thessalonicensis” is not available (6). Parsimony bootstrap values (1,000 resamplings) higher than 99% are indicated as solid circles. Bars, 10% estimated evolutionary distance.