Phylogeny of *Prostheco bacter*, the Fusiform Caulobacters: Members of a Recently Discovered Division of the *Bacteria*

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Prostheco bacter fusiformis is morphologically similar to caulobacters; however, it lacks a dimorphic life cycle. To determine the relatedness of the genus *Prostheco bacter* to dimorphic caulobacters and other prosthecate members of the α subgroup of the *Proteobacteria* (α -*Proteobacteria*), we isolated and sequenced 16S rRNA genes from four *Prostheco bacter* strains. Surprisingly, the results of phylogenetic analyses placed the fusiform caulobacters in a deeply rooted division of the *Bacteria* that was most closely affiliated with the *Planctomycetes-Chlamydia* group and only distantly related to the α -*Proteobacteria*. The genus *Prostheco bacter* shares a common lineage in this division with *Verrucomicrobium spinosum*, a polyprosthecate, heterotrophic bacterium. Consistent with this phylogenetic placement, menaquinones were isolated from *Prostheco bacter* strains and menaquinones have been isolated from *Verrucomicrobium* strains and planctomycetes but not from members of the α -*Proteobacteria*. Thus, the genus *Prostheco bacter* is a second genus in the recently described order *Verrucomicrobiales*. Members of the genus *Prostheco bacter* are susceptible to β -lactam antibiotics and contain meso-diaminopimelic acid, indicating that they, unlike members of the *Planctomycetales* or *Chlamydiales*, have peptidoglycan cell walls. This major phenotypic difference, together with the phylogenetic independence of the verrucomicrobia, indicates that these bacteria and the sources of related 16S ribosomal DNAs obtained from soils, freshwater, and the marine pelagic environment represent an unrecognized division of the *Bacteria*.

Prosthecae are narrow extensions of the cell wall that contain cytoplasm (49). The most widely known prosthecate bacteria are the caulobacters. Collectively, the caulobacters were defined as rod-shaped or vibrioid heterotrophs that possess single, polar, unbranched prosthecae and divide by transverse fission (19). Prototypical caulobacters have a dimorphic life cycle (53). Each cell divides asymmetrically, producing a non-prosthecate, motile swarmer cell and a sessile, prosthecate cell; the swarmer cell develops a prostheca, there is a concomitant loss of motility, and the cell subsequently divides. These bacteria are placed in the genera *Caulobacter* and *Asticcacaulis* on the basis of whether the cells have polar or subpolar prosthecae (34, 36).

In contrast, a caulobacter that Henrici and Johnson called the fusiform caulobacter divides symmetrically, and each resulting cell possesses a prostheca at the time of division (19). Furthermore, neither cell is motile. The cells of this caulobacter remain attached at the divisional pole following fission; thus, the daughter cells form mirror images of one another.

Whereas dimorphic caulobacters were isolated as early as 1905 (21) and 1914 (33), pure cultures of the fusiform caulobacter were not obtained until the 1970s. The following four strains were isolated: FC1 (= ATCC 27091), FC2, FC3, and FC4^T (= ATCC 25309^T) (T = type strain) (8, 51). Although all of these organisms are freshwater isolates, they were isolated from geographically distant regions of the United States and from both oligotrophic (strain FC4^T) and eutrophic (strain FC1) environments. Other attempts to isolate fusiform caulobacters from enrichment cultures with media containing plant extracts (62), from Antarctic sea ice (30), or from other freshwater sources (38) have failed, perhaps because of the complex nutritional requirements of these bacteria.

Nonetheless, pure-culture studies performed with the four fusiform caulobacter strains led to greater understanding of the differences between these organisms and the dimorphic caulobacters. In addition to the obvious developmental differences, morphological, nutritional, and genetic differences were identified (29, 51); together, the differences were striking enough to place the fusiform caulobacters in a separate genus, the genus *Prostheco bacter*, which contains a single described species, *Prostheco bacter fusiformis* (type strain, FC4) (51).

Phylogenetically, the dimorphic caulobacters, the genera *Caulobacter* and *Asticcacaulis*, belong to the alpha subdivision of the *Proteobacteria* (α -*Proteobacteria*) (46, 48). These organisms share a common lineage in this division with other heterotrophic prosthecate bacteria, including the genera *Hyphomicrobium*, *Pedomicrobium*, *Filomicrobium*, *Hyphomonas* (46), *Prosthecomicrobium*, *Ancalomicrobium* (43), *Stella* (13), and *Hirschia* (42). The phylogenetic position of the fusiform caulobacters has never been determined (50). To assess whether the genus *Prostheco bacter* is related to prosthecate members of the α -*Proteobacteria*, we determined the phylogenetic positions of all four *Prostheco bacter* strains by performing a 16S ribosomal DNA (rDNA) sequence analysis. We supplemented the results of the phylogenetic analysis with data from comparisons of chemotaxonomic markers which have been used to classify prosthecate bacteria (45).

MATERIALS AND METHODS

Electron microscopy. Cells were shadowed with platinum-palladium and were viewed with a model JEM-100B electron microscope operated at 60 kV.

16S rDNA sequencing and phylogenetic analysis. Prior to the phylogenetic analysis, *Prostheco bacter* 16S rDNA was purified and sequenced. Genomic DNA was isolated from late-exponential-phase cells by using an InstaGene kit (Bio-Rad, Hercules, Calif.). 16S rDNA was amplified by PCR by using universal primers (39) and the following parameters: 32 cycles consisting of 1.5 min at 94°C, 1 min at 42°C, and 4 min at 72°C, with the last step of the last cycle continuing for 10 min (16). For strains FC1 and FC3, the PCR product was digested with *NotI*, isolated by agarose gel electrophoresis, and purified by using a Prep-A-Gene kit (Bio-Rad). The purified product was cloned into pBluescript II KS⁺ (Stratagene, La Jolla, Calif.) by using standard techniques (40). Multiple clones were pooled and sequenced together by using a *Taq* DyeDeoxy terminator

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TABLE 1. 16S rDNA sequences and nucleotide positions used in this study

Organism or clone	GenBank accession no.	Division ^a	Nucleotide positions ^b
<i>Thermotoga maritima</i> MSB8 (= DSM 3109)	M21774	2.2, <i>Thermotogales</i>	27-1491
<i>Chloroflexus aurantia</i> J-10-F1 (= ATCC 29366)	M34116	2.4, Green nonsulfur bacteria	27-1483
<i>Cytophaga flevensis</i> ATCC 27944	M58767	2.6, <i>Flexibacter-Cytophaga-Bacteroides</i>	27-1490
<i>Chlorobium vibrioforme</i> 6030 (= DSM 260)	M62791, M27804	2.7, Green sulfur bacteria	27-1491
<i>Pirellula staleyi</i> ATCC 27277 ^T	M34126	2.9.1, <i>Planctomyces-Planctomyces</i>	27-1491
<i>Chlamydia psittaci</i> 6BC (= ATCC VR125)	M13769	2.9.2, <i>Planctomyces-Chlamydia</i>	27-1491
<i>Verrucomicrobium spinosum</i> IFAM 1439	X90515	2.9.3, <i>Planctomyces-Verrucomicrobium</i>	31-1481
Clone env.MC15	X64382	2.9.3, <i>Planctomyces-Verrucomicrobium</i>	27-996, 1224-1223
Clone env.MC17	X64381	2.9.3, <i>Planctomyces-Verrucomicrobium</i>	27-996, 1224-1223
Clone env.MC18	X64374	2.9.3, <i>Planctomyces-Verrucomicrobium</i>	27-1240
Clone env.MC31	X64380	2.9.3, <i>Planctomyces-Verrucomicrobium</i>	70-996, 1224-1223
<i>Nostoc muscorum</i>	X59559	2.10, Cyanobacteria plus chloroplasts	27-1491
<i>Fibrobacter succinogenes</i> A3C (= ATCC 51219)	M62683	2.11, <i>Fibrobacter</i> phylum	27-1377
<i>Spirochaeta halophila</i> RS1 ^T (= ATCC 29478 ^T)	M88722, M34262	2.12, Spirochetes and their relatives	27-1377
<i>Caulobacter crescentus</i> CB2A	M83799	2.13.1, Purple bacteria, α - <i>Proteobacteria</i>	27-1479
<i>Caulobacter subvibrioides</i> CB81	M83797	2.13.1, Purple bacteria, α - <i>Proteobacteria</i>	27-1481
<i>Neisseria gonorrhoeae</i> B5025 ^T (= ATCC 19424 ^T)	X07714	2.13.2, Purple bacteria, β - <i>Proteobacteria</i>	27-1491
<i>Escherichia coli</i>	J01695	2.13.3, Purple bacteria, γ - <i>Proteobacteria</i>	27-1491
<i>Desulfovibrio desulfuricans</i> ATCC 27774	M34113	2.13.4, Purple bacteria, δ - <i>Proteobacteria</i>	27-1491
<i>Fusobacterium gonidiformans</i> ATCC 25563	M58679	2.14, Fusobacteria and their relatives	27-1489
<i>Streptomyces lividans</i> TK21	Y00484	2.15.1, Gram positive high G+C content	27-1491
<i>Clostridium pasteurianum</i> ATCC 6013	M23930	2.15.2, Gram positive low G+C content	27-1489

^a Divisions as described in Ribosomal Database Project version 5.0 (23).

^b Nucleotide positions corresponding to the positions on the *E. coli* 16S rRNA gene (4). The following nucleotide positions were deleted because nucleotide alignment was uncertain: *E. coli* positions 69 to 101, 163 to 169, 182 to 226, 433 to 504, 838 to 853, and 997 to 1044 (4).

cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and 16S rDNA-specific forward and reverse primers (10).

The PCR products of FC2 and FC4^T were purified by using a Prep-A-Gene kit and were sequenced directly. As a result of the fact that multiple 16S rDNA copies from each organism were sequenced, interperon sequence variations, if they existed, were reported as ambiguous nucleotides or as the nucleotides that clearly predominated among copies of the gene (5).

Because of a sequence discrepancy between *Prostheco bacter* 16S rRNA genes and sequencing primers, the following three additional primers were required to complete the sequence analysis: SP3Rprosth (GGCTGCTGCCTCCCGTAGG; *Escherichia coli* positions 357 to 341 [4]), SP7Rgamma (TACCGGGGTATCTA ATCC; *E. coli* positions 802 to 785 [4]), and SP13Rgamma (ACGGGCGGTG TGTACAA; *E. coli* positions 1406 to 1390 [4]).

16S rDNA sequences were assembled by using the SeqApp program (15) and were initially aligned with similar sequences by using the Ribosomal Database Project version 5.0 ALIGN_SEQUENCE program (23). Additional sequences used in the phylogenetic analyses were obtained from the Ribosomal Database Project (23) or from GenBank (Table 1). Manual adjustments of the aligned data set were made. In addition, *Prostheco bacter* sequences were projected onto the *Pirellula staleyi* 16S rRNA secondary-structure model (17) to check for correct alignment of homologous nucleotides and to allow for comparisons of secondary-structure motifs. Since the data set included sequences from taxa belonging to many divisions of the *Bacteria*, certain regions of the 16S rRNA gene could not be confidently aligned. As comparisons of nucleotides which are not evolutionary counterparts can misguide phylogenetic analyses (32), we deleted these nucleotides (*E. coli* positions 69 to 101, 163 to 169, 182 to 226, 433 to 504, 838 to 853, and 997 to 1044 [4]) from all of the sequences, as has been done by other workers (3).

The single data set without nucleotides whose alignment was uncertain was analyzed by using several phylogenetic methods. Maximum-likelihood and parsimony analyses were performed by using fastDNAm1 (11, 31) and PAUP3.0s (54), respectively. The programs used in the neighbor-joining analysis were obtained from the PHYLIP package, version 3.2 (12).

Quinone analysis. Two-liter batch cultures of *Prostheco bacter* sp. strains FC1

and FC4^T were grown with rapid shaking on MMB, a dilute peptone-yeast extract medium amended with glucose (52). As *Prostheco bacter* strains do not require exogenously added vitamins, the vitamin solution was omitted. Cells were harvested during the late exponential phase of growth and lyophilized. The lyophilized cells were extracted, and ubiquinone and menaquinone fractions were purified by thin-layer chromatography (6). The quinone bands were scraped from the plate, eluted with chloroform, dried under N₂, and stored at 4°C in the dark until they were analyzed.

Final separation and identification of individual ubiquinones and menaquinones were performed by reverse-phase high-performance liquid chromatography with a Waters model 510 pump as described previously (6). The following bacteria whose quinone compositions have been published previously (7) were treated in parallel and were used as standards: *Rhodococcus erythropolis* ATCC 4277, *E. coli*, and *Pseudomonas putida* ATCC 29607. To identify the *Prostheco bacter* menaquinone, the numbers of isoprene units in standard menaquinone homologs were plotted against the logarithm of the net retention time (quinone retention time – solvent peak retention time) to form a straight line (7). This line was extrapolated from MK-8(H₂) and MK-7(H₂) of *R. erythropolis* through MK-6(H₂) of *Prostheco bacter* strains.

meso-Diaminopimelic acid isolation. *Prostheco bacter* strains and *R. erythropolis* and *E. coli* reference strains were grown and harvested. Cells were hydrolyzed with 6 N HCl by autoclaving them for 15 min as described previously (18). Hydrolysates were spotted onto Sigma type 100 cellulose thin-layer chromatography plates along with LL-, DD-, and meso-diaminopimelic acid standards (Sigma Chemical Co., St. Louis, Mo.), and the plates were developed (18). Diamino acids were visualized with ninhydrin reagent.

Determination of antibiotic MICs. Antibiotic MICs were determined as follows: 1 × 10⁷ to 3 × 10⁷ late-exponential-phase cells of *Prostheco bacter* sp. strain FC1 or FC2 were added to defined media (51) containing serial dilutions of penicillin G or ampicillin in microtiter wells, and growth was scored by measuring the increase in turbidity at 600 nm with a model EL311sx automated microplate reader (BIO-TEK, Winooski, Vt.) and a Delta Soft II apparatus (BioMetallics, Princeton, N.J.) after incubation for 3 or 5 days at room temperature. The MIC

FIG. 1. Shadowed electron micrograph of *Prosthecobacter* sp. strain FC2. Note the numerous radiating fimbriae and the distinctive bulbs at the prostheca tips (arrow). Bar = 2 μ m.

was defined as the lowest antibiotic concentration which completely prevented growth.

Nucleotide sequence accession numbers. The 16S rDNA sequences of *Prosthecobacter* sp. strains FC1, FC2, FC3, and FC4^T have been deposited in the GenBank database under accession numbers U60012, U60013, U60014, and U60015, respectively.

RESULTS

Electron microscopy. An electron micrograph of *Prosthecobacter* sp. strain FC2 (Fig. 1) shows that the cells of this organism are heavily fimbriate; this feature is absent from prosthecate dimorphic caulobacter surfaces (34). In addition, *Prosthecobacter* prosthecae have bulbous tips which are unique among bacteria. A close examination of *Prosthecobacter* prosthecae failed to reveal cross bands, which are characteristic of *Caulobacter* appendages (34, 36, 37).

Phylogenetics. The sequences of the four *Prosthecobacter* 16S rDNAs were determined and compared with each other and with the most closely related sequences obtained from the Ribosomal Database Project (Table 2). The 16S rDNA sequences of the *Prosthecobacter* strains exhibited higher levels of homology with each other than with any other sequence, indicating that the genus *Prosthecobacter* is a phylogenetically coherent group. When ambiguous nucleotides were excluded, the levels of 16S rDNA sequence similarity among the prostheco-bacters ranged from 98.3 to 94.8%. Generally, 16S rDNA sequence divergence values between 1 and 2% correspond to 50% DNA-DNA hybridization (2, 9, 47, 48); correspondingly, organisms that exhibit less than 70% DNA-DNA hybridization

qualify as separate species (59). Thus, the levels of 16S rDNA sequence difference between *Prosthecobacter* strains are at or above the level which separates bacterial species. This is consistent with the results of DNA-DNA hybridization experiments performed with strain FC4^T and the other *Prosthecobacter* strains; the levels of DNA-DNA hybridization between strain FC4^T and strains FC1, FC2, and FC3 were 5, 1, and 3%, respectively (29).

The *Prosthecobacter* sequences exhibited high levels of homology (89.8 to 90.2%) with the 16S rDNA sequence of only one cultured bacterium, *Verrucomicrobium spinosum* (1, 58). *V. spinosum* is a polyprosthecate, heterotrophic bacterium that was isolated from a eutrophic, alkaline lake in Germany (41). Phylogenetically, *V. spinosum* is separate from the α -*Proteobacteria* (1). Thus, the genus *Prosthecobacter* belongs to the recently described order *Verrucomicrobiales* (58) and is not closely related to the dimorphic caulobacters, with which it was previously associated (19). This result is surprising considering the gross morphological similarity between *Prosthecobacter* strains and *Caulobacter* strains and since this is only the second example of a heterotrophic, prosthecate organism outside the well-defined cluster of prosthecate bacteria within the α -*Proteobacteria*.

As determined by levels of sequence similarity, the genus *Prosthecobacter* is also linked to 16S rDNA clones representing uncultured bacteria obtained from several environments. These clones include clones obtained from a paddy field (env.PAD7, env.PAD18, and env.PAD50) (55), a clone obtained from a soybean field (env.FIE19) (55), a clone obtained from

TABLE 2. Levels of 16S rDNA homology between *Prostheco bacter* sequences and sequences of organisms representing main lines of descent within the *Bacteria*^a

Organism	% Homology with the following <i>Prostheco bacter</i> strains:			
	FC1	FC2	FC3	FC4 ^T
<i>Chlorobium vibrioforme</i>	78.9	78.3	78.5	78.9
<i>Nostoc muscorum</i>	78.8	78.1	78.0	78.5
<i>Fusobacterium goniodiformans</i>	76.7	76.3	76.5	75.9
<i>Streptomyces lividans</i>	80.6	81.1	80.8	80.5
<i>Clostridium pasteurii</i>	80.7	80.0	80.4	80.5
<i>Cytophaga flevens</i>	76.2	76.0	76.0	76.1
<i>Caulobacter crescentus</i>	79.8	79.5	79.2	79.6
<i>Neisseria gonorrhoeae</i> ATCC 19424 ^T	78.6	78.5	77.9	78.4
<i>Escherichia coli</i>	79.1	79.0	78.9	78.8
<i>Desulfovibrio desulfuricans</i>	81.9	81.5	81.0	81.5
<i>Fibrobacter succinogenes</i>	77.3	77.2	77.6	77.4
<i>Spirochaeta halophila</i> ATCC 29478 ^T	77.5	77.5	77.0	77.2
<i>Thermotoga maritima</i>	77.3	77.5	76.8	77.1
<i>Chloroflexus aurantia</i>	76.7	77.0	75.9	76.5
<i>Pirellula staleyi</i> ATCC 27277 ^T	75.1	75.3	74.9	74.9
<i>Chlamydia psittaci</i>	78.8	78.4	78.6	78.6

^a The nucleotide positions used in the analysis are shown in Table 1.

soil (EA25) (24), cluster III MC clones obtained from Australian forest soil (env.MC15, env.MC17, env.MC18, and env.MC31) (25), clones obtained from Adirondack lakes (Ack-M6, Ack-M10, Ack-DE36, Ack-DE41, Ack-DH1, and Ack-DH7) (20), clones obtained from a Carolina bay (RB35, RB31, RB02, RB24, RB14, RB01, and RB22) (60), and a clone obtained from a depth of 100 m in the Atlantic Ocean (env.25-19) (14). Therefore, relatives of the genus *Prostheco bacter* are prevalent in the environment even though they (probably) have not been cultured. Unfortunately, many sequences representing uncultured bacteria consisted of less than 25% of the 16S rRNA gene; therefore, it was not possible to determine the precise phylogenetic relationships between the genus *Prostheco bacter* and the bacteria from which the rDNA clones were obtained.

We compared the *Prostheco bacter* sequences with the 16S rDNA sequences of several representatives of each of the major bacterial phyla (61). The resulting levels of homology were between 68.2 and 76.1%, values which were at or below the values that separate members of different divisions (data not shown). To demonstrate that these values were not artifactual because of poor sequence alignment, we repeated this analysis with a data set from which positions of uncertain alignment were excluded. Still, the levels of homology were as low as the levels of homology between members of different divisions. Thus, on the basis of distance alone, the position of the *Prostheco bacter-Verrucomicrobium* group within the existing bacterial divisions is uncertain. Representative data are shown in Table 2.

To substantiate the phylogenetic relationship between the genus *Prostheco bacter* and *V. spinosum* and the cluster III MC clones, we searched for signature nucleotides which have been reported to be unique to the sequences of these organisms (25, 58). Indeed, three of four nucleotide signatures (at *E. coli* positions 768 [G], 811 [A], and 819 [G] [4]) of the *V. spinosum* and cluster III MC clone sequences were found in all four *Prostheco bacter* strains. The fourth nucleotide signature (at *E. coli* positions 826 and 874 [U · A]) was found in FC2; however, the other *Prostheco bacter* strains had the more common base pair, C · G. This analysis also revealed the presence of

four *Planctomyces* nucleotide signatures (at *E. coli* positions 47 and 394 [G · G], 48 [A], 53 and 539 [G · C], and 955 [C] [61]), each of which is also present in the *V. spinosum* and cluster III MC clone sequences (25, 58). The presence of these nucleotide signatures supports the placement of the *Prostheco bacter* group in the *Planctomyces-Chlamydia* supergroup. The majority of the *Planctomycetales* signatures, however, are not present in *Prostheco bacter* strains, which reflects the independence of the genus *Prostheco bacter* from the *Planctomyces* phylum. Interestingly, on the basis of sequence homology data alone, the prosthecobacters are only distantly related to the planctomycetes (Table 2).

Phylogenetic trees were created to determine relationships within and around the *Prostheco bacter-Verrucomicrobium* radiation. All of the trees were constructed by using data sets restricted to nucleotides whose alignment was certain. A data set consisting of *Prostheco bacter*, *V. spinosum*, and cluster III MC clone 16S rDNA sequences along with sequences representing some of the major bacterial phyla was analyzed to find the most parsimonious trees (54). The resulting four trees, which had equal total branch lengths, were analyzed by using MacClade 3.05 (27). The four trees had an average transition-to-transversion ratio of 1.05.

The results of a maximum-likelihood analysis (11, 31) of the data set with the transition-to-transversion ratio adjusted to 1.05 confirmed the phylogenetic coherence of the prosthecobacters and of the *Prostheco bacter-V. spinosum*-cluster III MC clone cluster (Fig. 2). The maximum-likelihood tree also suggested that the *Prostheco bacter* group diverged at an early point from a common ancestor of the genera *Planctomyces* and

FIG. 2. Maximum-likelihood (11, 31) analysis of 16S rDNA sequences from *Prostheco bacter* strains, *Verrucomicrobium* strains, and cluster III MC environmental clones, as well as bacteria representing established phyla. The numbers above the nodes are bootstrap values (based on 100 resamplings). Only bootstrap values greater than 50% are shown. Scale bar = 0.1 average change per nucleotide position. The nucleotide positions used in the analysis are shown in Table 1.

FIG. 3. Cladogram showing the results of bootstrap parsimony analyses. Heuristic searches were conducted by using PAUP3.0s (47) with a random input order and 10 replications per bootstrap. The numbers above the nodes are bootstrap percentages (based on 100 resamplings) obtained by the unweighted parsimony method. The numbers below the nodes are bootstrap percentages obtained from an identical analysis in which only transversions were counted. Only bootstrap values greater than 50% are shown. The nucleotide positions used in the analyses are shown in Table 1.

Chlamydia. However, support from 100 bootstrap resamplings failed to favor any particular relationship between the major divisions of the *Bacteria*. In particular, there was less than 50% support for a specific relationship between the *Prosthecobacter* group and the *Planctomycetes* or *Chlamydia* group.

The same data set was analyzed by DNADIST (12), using a Kimura two-parameter correction, a random input order, and a transition-to-transversion ratio of 1.05. When analyzed by NEIGHBOR (12), the resulting distance matrix produced a tree whose topology was similar to that of the maximum-likelihood tree (Fig. 2); the only difference was the relative positions of env.MC17 and env.MC31 (data not shown). Again, however, the bootstrap values supporting the early divergence

of the *Prosthecobacter* group from the *Planctomycetes-Chlamydia* lineage were less than 50%.

Finally, the unweighted data set was analyzed by using PAUP3.0s (54). A heuristic search with 10 different random input orders (54) produced a tree similar to the trees created by the other methods (Fig. 3). Again, however, the same method and 100 bootstrap resamplings failed to associate *Prosthecobacter* and related sequences with any established division. Importantly, a low, but significant bootstrap value supported grouping the planctomycetes and chlamydiae without the genus *Prosthecobacter*. This finding supports the hypothesis that the verrucomicrobia diverged from the *Planctomycetes-Chlamydia* superphylum at an early point. An identical analysis in which only transversions were weighted yielded a similar tree with similar bootstrap values (Fig. 3).

Peptidoglycan analysis. We isolated *m*-diaminopimelic acid from *Prosthecobacter* extracts. In addition, *Prosthecobacter* strains were susceptible to penicillin G (MIC, 10 µg/ml) and ampicillin (MIC, 0.3 µg/ml). Taken together, these data indicate that the members of the genus *Prosthecobacter* have peptidoglycan cell walls. Since all known members of the *Planctomycetes* and *Chlamydia* phyla lack peptidoglycan cell walls (22), the presence of peptidoglycan in the genus *Prosthecobacter* supports a phylogenetic branching scheme in which the genus *Prosthecobacter* diverged before the genera *Planctomycetes* and *Chlamydia* diverged.

Quinone analysis. Quinone profiles have recently been used to group prosthecate bacteria (45). Although ubiquinones are the predominant quinones in aerobically cultivated prosthecate members of the *Proteobacteria*, menaquinones are found in *Verrucomicrobium* strains. We isolated both menaquinones and ubiquinones from aerobically grown prosthecobacters. However, menaquinone MK-6(H₂) was the predominant quinone in extracts of both FC1 and FC4^T, providing further evidence that the genera *Prosthecobacter* and *Verrucomicrobium* are related (Table 3). Interestingly, menaquinone MK-6 (H₂) is also the dominant respiratory quinone in planctomycetes (45); this supports the hypothesis that the genus *Prosthecobacter* is more closely related to the planctomycetes and chlamydiae than to the members of the *Proteobacteria*.

DISCUSSION

Prosthecae greatly increase the surface area of cells, and thus it has been proposed that these structures confer several advantages to aerobic heterotrophic bacteria. These advantages include enhanced respiration and nutrient uptake (44),

TABLE 3. Phenotypic characteristics of representatives of two phylogenetically distinct groups of heterotrophic prosthecate bacteria

Genus	Prosthecae		G+C content of DNA (mol%)	Fimbriae	Motility	Fermen- tative	Utilization of:				Major quinone(s)
	Distribution	No./cell					Carbo- hydrates	Amino acids	Other organic acids	Primary alcohols	
<i>Verrucomicrobiales</i> group											
<i>Prosthecobacter</i> ^a	Polar	1	54.6–60.1	+ ^b	–	–	+	–	–	–	MK-6(H ₂), Q-n
<i>Verrucomicrobium</i> ^c	Radiating	5-30	57.9–59.3	+	–	+	+	–	–	–	MK-10, MK-6
<i>α-Proteobacteria</i> group											
<i>Caulobacter</i> ^d	Polar	1	62–67	D	+	–	+	+	D	–	Q-10
<i>Prosthecomicrobium</i> ^e	Radiating	10-30	65–70	–	D	–	D	+	D	+	Q-10

^a Data from reference 51 and this study.

^b +, 90% or more of the strains are positive; D, 10 to 90% of the strains are positive; –, 10% or fewer of the strains are positive.

^c Data from references 41 and 45.

^d Data from references 37 and 45.

^e Data from references 45 and 50.

as well as decreased sedimentation in aquatic environments (35, 53). In addition, holdfasts on the tips of some prosthecae allow bacteria to attach to solid substrates in favorable local environments (28). All things considered, it is not surprising that examples of heterotrophic prosthecae bacteria are found in more than one major division of the *Bacteria*.

All heterotrophic prosthecae bacteria that have been isolated and thoroughly characterized to date belong to one of two phylogenetically distinct groups, the *Verrucomicrobiales* and the α -*Proteobacteria*. Members of these two groups are similar in many ways; however, there appear to be three distinct phenotypic traits of members of the genus *Prostheco-bacter* that are found only in members of the *Verrucomicrobiales* (Table 3). First, both *Verrucomicrobium* strains and *Prostheco-bacter* strains are heavily fimbriate (41, 51), whereas no heavily fimbriate, prosthecae members of the α -*Proteobacteria* have been isolated in pure culture. Second, the verrucomicrobia contain menaquinones rather than ubiquinones as their predominant respiratory quinones (45). Third, the known members of the *Verrucomicrobiales* utilize only a narrow range of carbohydrates as sole sources of carbon (41, 51); some members of the α -*Proteobacteria* utilize a much wider range of substrates (37, 50). These criteria should prove to be useful in classifying prosthecae heterotrophic bacteria in the future.

It has been proposed previously that the order *Verrucomicrobiales* and related 16S rDNA clones represent a unique division of the *Bacteria* (1, 25, 58). We support this proposal on the basis of genetic data as well as phenotypic data. Phylogenetically, members of the *Verrucomicrobiales* are excluded from the planctomyces and chlamydia divisions by the three criteria used by Woese and his collaborators (61) to define the phyla of the *Bacteria* (in this study we regard the phyla defined by Woese [61] as divisions). First, as determined by a distance analysis, the members of the genera *Prostheco-bacter* and *Verrucomicrobium* (1) exhibit equally low levels of 16S rDNA homology with representatives of each of the established divisions of the *Bacteria* (61). Second, although *Verrucomicrobium* sequences contain four planctomyces nucleotide signatures, the majority of the signatures used to define the planctomyces phylum are absent. Too few *Chlamydia* sequences exist to define the signatures of this group. Third, the predicted secondary-structure features of *Verrucomicrobium* 16S rRNA molecules do not closely resemble *Planctomycetales* or *Chlamydia* features (data not shown). For example, *Prostheco-bacter* 16S rRNAs contain a short helix at *E. coli* positions 184 to 193 (4) and a long helix at *E. coli* positions 198 to 219 (4). Both of these higher-order structures are rare among bacteria and occur together only in the β - and γ -*Proteobacteria* and not in the planctomyces or the chlamydiae (17, 61). Therefore, in the absence of a more rigorous definition for a bacterial division, the *Verrucomicrobiales* must be considered a novel division of the *Bacteria*.

Correspondingly, although the *Verrucomicrobiales* seems to be associated with the planctomycetes and chlamydiae, each of three phylogenetic methods used failed to place the verrucomicrobia in any previously described division of the *Bacteria*. Instead, the genus *Prostheco-bacter*, *V. spinosum*, and related clones formed an isolated, robust clade that was supported by 100% of the bootstrap values in each phylogenetic analysis. Of particular significance was the fact that the unweighted parsimony method excluded the verrucomicrobia from the planctomycetes-chlamydia group, implying that the branch point for the verrucomicrobia was deep. However, the exact verrucomicrobium-planctomycetes-chlamydia branching order should not be accepted as fact since the results obtained with other phylogenetic methods failed to support any particular order with

strong bootstrap values and since the order of division level divergence is, in general, uncertain (26, 56). Finally, since the lack of peptidoglycan is a defining characteristic of both the planctomyces and the chlamydiae, the presence of peptidoglycan cell walls in members of the *Verrucomicrobiales* strongly supports the proposal that these bacteria are separate from the planctomyces and chlamydiae (61).

The fact that large taxonomic units like the verrucomicrobium division are still being added to the *Bacteria* is a testament to the vast microbial diversity yet to be discovered. Indeed, microbial diversity is still an exciting frontier in biology. To date, the division which contains the *Verrucomicrobiales* consists of only two genera of freshwater bacteria; however, molecular ecology studies have revealed that these bacteria are ubiquitous. Thus, the majority of the diversity within and around the *Verrucomicrobiales* remains totally uncharacterized. If the correct culturing approach is taken, a better understanding of the diversity within this group can be attained, as has recently been done for the *Planctomycetales* (57).

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