Comparative phylogenetic analyses of members of the order *Planctomycetales* and the division *Verrucomicrobia*: 23S rRNA gene sequence analysis supports the 16S rRNA gene sequence-derived phylogeny

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Almost complete 23S rRNA gene sequences were obtained from 13 planctomycete strains, the fimbriated, prosthecate bacterium *Verrucomicrobium spinosum* and two strains of the genus *Prosthecobacter*. The 23S rRNA genes were amplified by the PCR, using modified primers. The majority of the planctomycete strains investigated were shown to have 23S rRNA genes that were not linked to the 16S rRNA genes. Amplification of the 5′-termini of these genes was achieved using a novel primer-design strategy. Comparative phylogenetic analyses were performed using the 23S rRNA gene sequences determined in this study and previously determined 16S rRNA gene sequences. The phylogenetic dendrograms constructed from both datasets showed that the planctomycetes form a coherent group and distinct lineage within the domain *Bacteria*. Analysis of 23S rRNA gene sequences of *Verrucomicrobium spinosum*, *Prosthecobacter fusiformis* and *Prosthecobacter* sp. strain FC-2 showed that these organisms cluster together, as was also shown here and previously by analysis of 16S rRNA gene sequences. The distinct phylogenetic position of the division *Verrucomicrobia* was also supported by analysis of the 23S rRNA gene sequences, and no statistically significant phylogenetic relationship between the division *Verrucomicrobia* and the planctomycetes was found. The analyses presented in this study also provide further evidence that the chlamydiae are no more related to members of the order *Planctomycetales* and the division *Verrucomicrobia* than they are to members of other bacterial lineages.

Keywords: 23S rRNA gene, phylogeny, *Planctomycetales*, *Verrucomicrobia*

INTRODUCTION

Comparative phylogenetic analyses using the large-subunit rRNA (Ludwig & Schleifer, 1994), highly conserved proteins, such as elongation factors and ATPase subunits (Ludwig *et al.*, 1993), and RNA polymerases (Pühler *et al.*, 1989) generally correlate well with those performed with small-subunit rRNA data, supporting the phylogenetic distinctness of the

Bacteria and *Archaea* and recovering similar phylogenies within the domain *Bacteria*. In particular, it has been demonstrated that the 23S rRNA can serve as a phylogenetic marker and that the trees reconstructed from 16S and 23S rRNA sequence data are generally congruent (Leffers *et al.*, 1987; Höpfl *et al.*, 1989; Schleifer & Ludwig, 1989; Ludwig *et al.*, 1993; Ludwig & Schleifer, 1994).

Analysis of 16S rRNA gene sequences has been shown to be a useful method for determining the extent of phylogenetic diversity within diverse groups of bacteria, including a group of planctomycete isolates

(Ward *et al.*, 1995; Griepenburg *et al.*, 1999). However, in these studies, the addition of a large number of new planctomycete 16S rRNA gene sequences did not allow a more accurate determination of the phylogenetic position of the planctomycete lineage in relation to other lineages within the domain *Bacteria*. Although the phylogenetic distinctness and coherence of the planctomycetes is evident in 16S rRNA gene sequence analyses, the relationship between the planctomycete lineage and other main lines, particularly members of the order *Chlamydiales* and the division *Verrucomicrobia*, remains unclear (Ward-Rainey *et al.*, 1995). Given the recent expansion in size of the division *Verrucomicrobia* (which includes the single-species genus *Verrucomicrobium spinosum*, four strains of the genus *Prosthecobacter* and an ever-increasing number of environmental clone sequences; Hedlund *et al.*, 1996), it is important to be able to elucidate the interphylum relationships.

To determine whether the use of an alternative molecular chronometer could resolve the uncertain branching points, sequence data were obtained for the genes encoding 23S rRNA (*rrl*) from 13 planctomycete strains, *V. spinosum*, *Prosthecobacter fusiformis* and *Prosthecobacter* sp. strain FC-2. The 23S rRNA gene sequences were determined for the type strains of all of the validly described species. The phylogenies reconstructed from the sequence data for these molecules were compared with those obtained from 16S rRNA gene sequence analyses.

METHODS

Bacterial strains and culture conditions. Strains for which sequence data were obtained are listed in Table 1. The type strains of the validly described planctomycete species were obtained from the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and cultivated as described previously (Ward-Rainey *et al.*, 1997). Planctomycete strains 139, 140, 449, 639, 658, 670 and *V. spinosum* DSM 4136[T] were provided by Heinz Schlesner (University of Kiel, Kiel, Germany). *Prosthecobacter fusiformis* DSM 8960[T] and *Prosthecobacter* sp. strain FC-2 (ATCC 700199) were from the laboratory collection of James Staley (University of Washington, Seattle, WA, USA).

Isolation and purification of genomic DNA. Genomic DNA was isolated and purified as described previously (Rainey *et al.*, 1996).

PCR-mediated amplification of 23S rRNA genes. Amplification of 23S rRNA genes was performed using combinations of the PCR primers listed in Table 2. All other PCR parameters are as described for 16S rRNA gene sequencing in Rainey *et al.* (1996).

Sequencing of 23S rRNA gene PCR products. The 23S rRNA gene PCR products were sequenced directly as described previously (Rainey *et al.*, 1996), using the primers listed in Tables 2 and 3.

Sequence alignments

16S rRNA gene sequence alignment. Comparative phylogenetic analyses using 16S rRNA gene sequences were performed using the following reference sequences (strains are indicated where available and EMBL accession numbers are provided in parentheses): *Alcaligenes faecalis* (M22508), *Bacillus subtilis* NCDO 1769 (X60646), *Borrelia burgdorferi* strain B31 (X57404), *Bradyrhizobium japonicum* LMG 6138 (D12781), *Chlamydophila* (formerly *Chlamydia*) *psittaci* strain 6 BC (M13769), *Chlorobium limicola* strain 8327 (M31769), *Chloroflexus aurantiacus* strain 1892 (M34116), *Clostridium botulinum* G (M59087), *Escherichia coli* (J01695), *Flavobacterium odoratum* (M58777), *Gemmata obscuriglobus* DSM 5831[T] (AJ231191), *Isosphaera pallida* DSM 9630[T] (AJ231195), *Micrococcus luteus* (M38242),

Table 1. Strains for which 23S rRNA gene sequence data were obtained, and the primer pairs used to amplify the 5′-regions of the 23S rRNA genes (other PCR primer combinations used were 255f-2053r and 1948f-25r)

Strain	Culture collection no.	Sequence accession no.	Primer pair
Planctomyces brasiliensis	DSM 5305[T]	AF245364	23Sfplan-803r
Planctomyces limnophilus	DSM 3776[T]	AF245365	23Sfplan-803r
Planctomyces maris	DSM 8797[T]	AF245366	23SfPlmar-803r
Pirellula marina	DSM 3645[T]	AF245367	23Sfplan-803r
Pirellula staleyi	DSM 6068[T]	AF245368	23Sfplan-803r
Gemmata obscuriglobus	DSM 5831[T]	AF245369	23SfGemm-803r
Isosphaera pallida	DSM 9630[T]	AF245370	1241fplan-803r
Planctomycete strain 139	—	AF245371	23Sf139–803r
Planctomycete strain 140	—	AF245372	23Sf140–803r
Planctomycete strain 449	—	AF245373	23Sf449–803r
Planctomycete strain 639	—	AF245374	23Sf639–803r
Planctomycete strain 658	—	AF245375	23Sf658–803r
Planctomycete strain 670	—	AF245376	23Sf670–803r
Verrucomicrobium spinosum	DSM 4136[T]	AF245377	1248fplan-803r
Prosthecobacter fusiformis	DSM 8960[T]	AF245378	1100f–803r
Prosthecobacter sp. strain FC-2	ATCC 700199	AF245379	1100f-803r

Table 2. Primers tested for amplification of 23S rRNA gene

Primer name	Primer sequence (5′–3′)	Binding position*	Primer reference
1241fplan	CTG CAC ACG T(G/C)C TAC AAT G	1223–1241 (16S)	This study
23Sfplan	AAT (C/A)TA (A/T)GT G(A/G)(T/C) CAA GC	(−1)†–16	This study
23SfPlmar	AAA TC(A/C) AAA CGG CTA AGC	(−2)–16	This study
23SfGemm	GGG GAG TGC G(C/G)C CAA GC	(−1)–16	This study
23SfIso	AAT CAG GGT GGT CAA GC	(−1)–16	This study
23Sf139	AAG TAG TGA TGG TCA A(A/G)C	(−2)–16	This study
23Sf140	GAT ATC GGT GGC CAA (G/A)C	(−1)–16	This study
23Sf449	CAA GAT ACC AAT GGT CAA G	(−4)–15	This study
23Sf639	TAG CAC AAA GTG GTC AAG	(−3)–15	This study
23Sf658	AGA ATT TAA ATG GTC AAG C	(−3)–16	This study
23Sf670	GAT CAA CGT GGT CAA GC	(−1)–16	This study
241r	(GT)TT CGC TCG CC(A/G) CTA C	241–256	Lane (1991)
255f	AGT AG(C/T) GGC GAG CGA A	241–255	Lane (1991)
559r	CAT T(A/C)T (A/G)CA AAA GG(C/T) ACG C	559–577	This study, based on Lane (1991)
803r	TTC GG(A/G) GAG AAC (G/C)AG (A/C)TA	803–822	This study, based on Ludwig *et al.* (1992)
1091r	AGT (A/G)AG CT(A/G) TTA CGC	1091–1105	This study, based on Lane (1991)
1358r	TAC GC(C/T) T(G/T)T C(A/G)G CCT C	1358–1373	This study
1685r	CCT T(A/C)T C(C/G)C GAA (C/G)TT ACG G	1685–1703	This study, based on Ludwig *et al.* (1992)
1948f	GTA GCG AA(A/G) TTC CTT GTC G	1930–1948	This study, based on Ludwig *et al.* (1992)
2053r	CA(C/T) (A/G)GG GTC TTT C(C/T)G TC	2053–2069	This study, based on Lane (1991)
2498r	GAG (C/T)CG ACA TCG AGG	2498–2512	Lane (1991)
2603f	A(A/G)A (A/C)CG TCG TGA GAC AG	2587–2603	This study, based on van Camp *et al.* (1993)
25r	(C/G)(C/T)G TTC GG(A/G) A(A/T)G GGA	25–49 (5S)	Ludwig *et al.* (1992)

* *Escherichia coli* nomenclature (Brosius *et al.*, 1978, 1980).

† Negative numbers denote positions upstream of *E. coli* position 1.

Table 3. Additional primers used to sequence the 23S rRNA gene

Primer name	Primer sequence (5′–3′)	Binding position*	Primer reference
559rmod	CAT T(A/T)T (A/G)CA AAA GGC A(G/T/C)G C	559–577	This study, based on Lane (1991)
559rplan	CAT TAT (G/T)CA A(T/A)A GGC AC(G/A) C	559–577	This study, based on Lane (1991)
577fplan	GCG TGC C(T/A/T) TTG (C/A)AT AAT G	559–577	This study, based on Lane (1991)
975r	TCT GGG (T/C)TG TT(T/C) CCC T	975–990	Ludwig *et al.* (1992)
990f	AGG G(A/G)A ACA (A/G)CC CAG A	975–990	This study, based on Ludwig *et al.* (1992)
1200f	GGT AG(A/G) (A/G)GA GCG TTC	1186–1200	This study
1373f	GAG GCC GA(A/C) A(A/G)G CGT A	1358–1373	This study
1608r	C(C/T)A CCT GTG (A/T)C(A/G) GTT T	1608–1623	This study, based on Lane (1991)
2069f	GAC (A/G)GA AAG ACC CC(A/G) TG	2053–2069	This study, based on Lane (1991)
2744r	CTT AGA TGC (C/T)TT CAG C	2744–2759	Ludwig *et al.* (1992)
2744rplan	CTT A(T/G)A TGC (C/T)TT CAG C	2744–2759	This study, based on Ludwig *et al.* (1992)

* *E. coli* nomenclature (Brosius *et al.*, 1980).

Nannocystis exedens strain Na e1 (M94279), *Pirellula marina* IFAM 1313 (X62912), *Pirellula staleyi* DSM 6068ᵀ (AJ231183), *Planctomyces brasiliensis* DSM 5305ᵀ (AJ231190), *Planctomyces limnophilus* IFAM 1008 (X62911), *Planctomyces maris* DSM 8797ᵀ (AJ231184), *Prosthecobacter fusiformis* DSM 8960ᵀ (U60015), *Prostheco-*

bacter sp. strain FC-2 ATCC 700199 (U60013), *Staphylococcus aureus* ATCC 12600 (X68417), planctomycete strains 139 (X81945), 140 (X81939), 449 (AJ231177), 639 (X81950), 658 (X81954) and 670 (X81948), and *Streptomyces griseus* ATCC 10137 (X55435), *Thermus thermophilus* strain HB8 (X07998), *Thermotoga maritima* strain MSB8 (M21774) and *Verrucomicrobium spinosum* DSM 4136^T (X90515). Sequences were aligned manually using the ae2 sequence editor (Maidak *et al.*, 1999).

23S rRNA gene sequence alignment. 23S rRNA gene sequences were aligned using the alignment facility of the ARB package (W. Ludwig & O. Strunk, http://www.biol.chemie.tu-muenchen.de/pub/ARB/). The reference sequences used in phylogenetic analyses included the following (strains are indicated where available and sequence accession numbers are provided in parentheses): *A. faecalis* DSM 30030^T (X87282), *Bacillus subtilis* (D11460), *Borrelia burgdorferi* (M88330), *Bradyrhizobium japonicum* DSM 30131 (X71840), *Chlamydia* sp. strain BN9 (Y07555), *Chlorobium limicola* (M62805), *Clostridium botulinum* NCTC 7272 (X65602), *E. coli* (J01965), *F. odoratum* (M62807), *M. luteus* DSM 20030 (X06484), *N. exedens* DSM 71^T (X87286), *Staphylococcus aureus* ATCC 12600 (X68425), *Streptomyces griseus* (M76388), *Thermus thermophilus* HB8 (X12612) and *Thermotoga maritima* (M67498).

Phylogenetic analyses. Phylogenetic analyses of 16S rRNA and 23S rRNA gene sequences were performed using the programs DNADIST, DNAPARS, NEIGHBOR and CONSENSE contained in the PHYLIP package (Felsenstein, 1993). Bootstrap analysis was performed using SEQBOOT (Felsenstein, 1993) and 1000 bootstrapped datasets.

The dendrograms presented here were reconstructed by the neighbour-joining method (Saitou & Nei, 1987) from distance matrices (Jukes & Cantor, 1969).

RESULTS AND DISCUSSION

16S rRNA gene sequence analyses

Phylogenetic analyses of 16S rRNA gene sequences were performed on a dataset consisting of 1145 nucleotide positions (*E. coli* positions 40–1470). The 16S rRNA gene sequence analysis (Fig. 1a) demonstrated the phylogenetic distinctness of the planctomycete lineage. Members of the order *Planctomycetales* shared only 68·1–76·4% 16S rRNA gene sequence similarity to members of other lineages, while the intragroup similarity values ranged from 77·2 to 92·0%. The distinct phylogenetic position of, and phylogenetic depth within, the planctomycete lineage has been shown previously (Stackebrandt *et al.*, 1984, 1986a, b; Liesack *et al.*, 1992; Ward *et al.*, 1995; Griepenburg *et al.*, 1999). The phylogenetic coherence of the members of the order *Planctomycetales* was indicated by the recovery of this cluster in all trees generated from the bootstrapped dataset (Fig. 1a).

The fimbriate, prosthecate bacterium *V. spinosum* and the two strains of the genus *Prosthecobacter* formed a distinct phylogenetic lineage (Fig. 1a), as has been shown previously (Ward-Rainey *et al.*, 1995; Hedlund *et al.*, 1996), and clustered together in 100% of trees generated by bootstrap analysis. The intragroup 16S

rRNA gene sequence similarities ranged from 92·8 to 96·6%. These three members of the division *Verrucomicrobia* showed low levels of 16S rRNA gene sequence similarity to members of other lineages (69·3–77·9%). In this dendrogram there is no specific relationship between the planctomycete lineage and those of the chlamydiae and the division *Verrucomicrobia*. A common branching point is shared by the division *Verrucomicrobia* and the chlamydiae, but is not supported by bootstrap analysis (66%). Neighbour-joining analysis using the maximum-likelihood correction recovered the same tree topology as was found using the Jukes & Cantor correction, while maximum-parsimony did not recover any relationship between the planctomycetes, chlamydiae and *Verrucomicrobia* (data not shown). The 16S rRNA gene sequence similarity values between the *Verrucomicrobia*, the planctomycetes and the chlamydiae are not any higher than those between the *Verrucomicrobia* and members of other lineages.
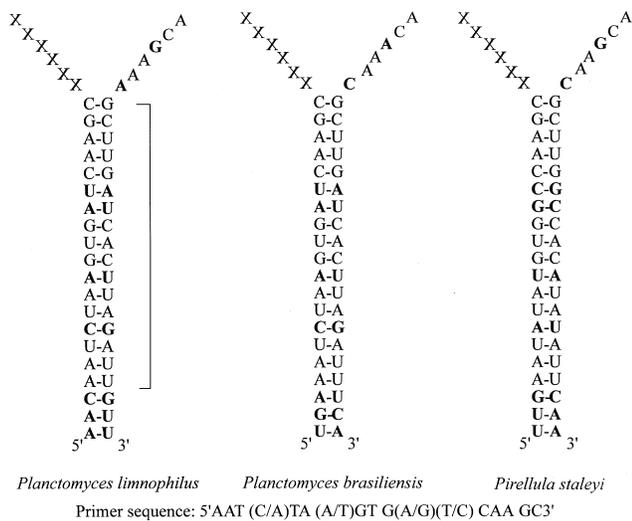
23S rRNA gene sequence analyses

PCR amplification. The PCR primers used in this study included those from previous 23S rRNA gene sequence-based phylogenetic studies (Lane, 1991; Ludwig & Schleifer, 1994). In some cases, the primers were modified on the basis of the 23S rRNA gene sequence of *Pirellula marina* (Liesack *et al.*, 1988).

The 23S rRNA gene could be amplified from all strains in three overlapping PCR reactions (Table 1). The 5′-region of the 23S rRNA gene was amplified successfully from *I. pallida* and *V. spinosum* using the primer combinations 1230fplan (binding to the 16S rRNA gene) and 803r (binding to the 23S rRNA gene). The 5′ 23S rRNA gene regions of *Prosthecobacter fusiformis* and *Prosthecobacter* sp. strain FC-2 were amplified using primers 1100f and 803r. However, this could not be achieved for the other strains investigated, as the 23S rRNA gene was found not to be linked to the 16S rRNA gene, making it impossible to use the 3′-end of the 16S rRNA gene as a target site for a PCR primer. Unlinked rRNA genes have been previously reported for *Pirellula marina* (Liesack & Stackebrandt, 1989), *Planctomyces limnophilus* and *G. obscuriglobus* (Menke *et al.*, 1991). The results of this study suggest that this arrangement is the most common one in planctomycetes, with *I. pallida* providing an exception to the rule. Linked 16S and 23S rRNA genes have been demonstrated by the PCR in strain 657 (data not shown), which is phylogenetically related to *I. pallida* (Ward *et al.*, 1995). The 16S and 23S rRNA genes of *V. spinosum* had been shown previously, by ribotyping, to be linked together (Menke *et al.*, 1991).

To obtain sequence data from the 5′-end of the 23S rRNA gene for the remainder of the strains, an alternative strategy was employed. Using the sequence data from the 3′-end of the molecule as a template, the processing stem that separates the 23S rRNA gene
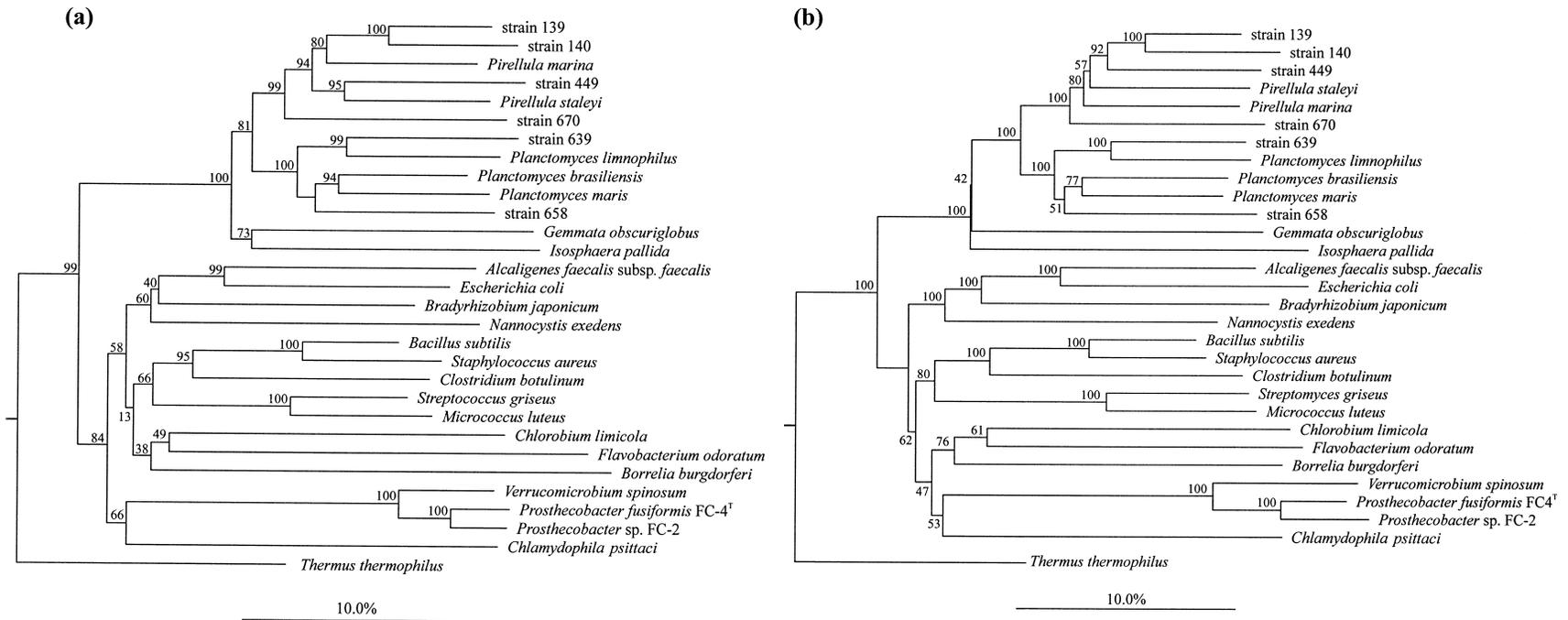
Fig. 1. (a) 16S rRNA gene and (b) 23S rRNA gene sequence-based phylogenetic dendrograms, indicating the phylogenetic relationships between members of the order *Planctomycetales*, the division *Verrucomicrobia* and representatives of the main lineages within the domain *Bacteria*. Roots were determined by using the 16S rRNA or 23S rRNA gene sequences of *Thermotoga maritima* as outgroups. Bars, 10 inferred nucleotide substitutions per 100 nucleotides (as determined by measuring the length of the horizontal lines joining any two organisms). Numbers at nodes refer to bootstrap values.

Planctomyces limnophilus *Planctomyces brasiliensis* *Pirellula staleyi*

Primer sequence: 5'AAT (C/A)TA (A/T)GT G(A/G)(T/C) CAA GC3'

Fig. 2. Schematic illustration of the strategy used to design PCR primers to amplify the 5′-region of the 23S rRNA gene of planctomycete strains. The stems formed by the base pairing of the 5′- and 3′-termini of the 23S rRNA are shown for three strains. The right-hand side of each stem represents the 3′ 23S rRNA gene sequence determined empirically. The left-hand side of each stem represents the deduced sequence of the 5′-terminus. Variable residues are depicted in bold type. The region used for the design of the PCR primers is bracketed and the sequence of the PCR primer designed for these three strains is provided at the bottom.

from flanking DNA was reconstructed (Fig. 2). Perfect Watson–Crick base-pairing in this region was assumed and the result was used to design a PCR primer that could be used for the amplification of the 5′-region of the gene. The high sequence variability in this region made it necessary to design nine different primers (Table 2). With the exception of strains 139, 140 and 449, the 5′-region of the 23S rRNA gene was amplified successfully using these primers and the PCR conditions described previously. In the case of strains 139, 140 and 449, the PCR reaction consistently produced additional non-specific bands even when highly stringent PCR conditions were used. This problem was circumvented by excising the target band from a preparative agarose gel of the PCR product, purifying the DNA and using it as template for another round of PCR. In this way, a PCR product of the correct size was obtained.

The 3′-end of the 23S rRNA gene proved much easier to obtain, as the 5S rRNA gene was found to be linked to the 23S rRNA in all strains investigated. Thus the 3′-end of the 23S rRNA gene could be amplified using a primer binding to the 5S rRNA gene.

Sequence analyses. Complete 23S rRNA gene sequences, from position 1 to position 2904 (*E. coli* nomenclature; Brosius *et al.*, 1980) were obtained for 13 planctomycete strains, *V. spinosum* and the two *Prosthecobacter* strains. To obtain the sequence data, many of the previously described 23S rRNA gene-sequencing primers had to be modified (Table 3) because of

mismatches between the primers and the new 23S rRNA gene sequences.

The 23S rRNA gene sequence dataset used for analyses contained all of the 23S rRNA gene sequences determined in this study and several reference sequences. Comparison of complete 23S rRNA gene sequences was not possible because of the inclusion of reference sequences lacking the 3′-terminus of the molecule. Regions of ambiguous alignment were excluded from the analysis, resulting in a dataset comprising 2550 nucleotide positions (*E. coli* positions 1–136, 161–268, 286–362, 371–542, 552–651, 655–1163, 1188–1414, 1420–1466, 1546–1577, 1587–1712, 1750–2204 and 2208–2768).

The 23S rRNA gene sequence data were used to reconstruct phylogenetic dendrograms (Fig. 1b) showing the relationships of members of the order *Planctomycetales* and the division *Verrucomicrobia* with respect to representatives of the major lines of descent within the domain *Bacteria*. Fig. 1b, which was generated by the neighbour-joining method, shows the planctomycetes and the division *Verrucomicrobia* to be unrelated to members of any other lineages within the domain *Bacteria*, as has been shown previously by 23S rRNA gene sequence analysis of *Pirellula marina* (Ludwig & Schleifer, 1994). Comparison of a larger number of planctomycete 23S rRNA gene sequences confirmed the isolated phylogenetic position suggested by the topology of the tree presented in Ludwig & Schleifer (1994). The phylogenetic coherence of the planctomycete lineage was also demonstrated by a 100 % bootstrap value for the node representing the common ancestor of this group.

The 23S rRNA gene sequence similarity between the planctomycetes and members of other lineages within the domain *Bacteria* ranged from 68·0 to 76·2 %, whereas the intragroup similarities were between 75·8 and 91·2 %. Members of the division *Verrucomicrobia* shared 68·0–74·0 % 23S rRNA gene sequence similarity with members of other lineages and were shown to be a phylogenetically coherent group recovered in 100 % of trees generated by bootstrap analysis. The *Verrucomicrobia* intragroup similarity values ranged from 89·3 to 94·2 %. The 23S rRNA gene sequence similarity values are in very close agreement with the similarity values obtained by comparison of 16S rRNA gene sequences (see above). On the basis of 23S rRNA gene sequence analyses, no relationship between the planctomycetes, the division *Verrucomicrobia* and the chlamydiae was observed (Fig. 1b).

Comparison of the intra phylum relationships within the order *Planctomycetales* revealed by 16S and 23S rRNA gene sequence analyses (Fig. 1a, b) shows some similarities. A relationship between *G. obscuriglobus* and *I. pallida* is observed in the 16S rRNA gene sequence-based tree (Fig. 1a), but is not supported by bootstrap analysis, as has been reported previously in a 16S rRNA gene sequence-based phylogenetic study of planctomycetes (Liesack *et al.*, 1992). The instability

of the *Gemmata–Isosphaera* branching point is also demonstrated by a bootstrap value of 42% for this node in the 23S rRNA gene sequence-based tree.

Analysis of 23S rRNA gene sequences showed the same relationship between members of the genus *Planctomyces* and strains 639 and 658 (Fig. 1b) as that seen with 16S rRNA gene sequence analysis (Fig. 1a), and the *Planctomyces* cluster is recovered in all trees in both cases. Some rearrangements in branching order between the 16S and 23S rRNA gene trees are seen within the group containing members of the genus *Pirellula* and the strains 139, 140 and 449. Examination of the phylogenetic relationships of all members of this group (Ward *et al.*, 1995; Griepenburg *et al.*, 1999) showed that the branching points within the cluster are rather unstable, so some discrepancies in internal structure when 16S and 23S rRNA genes are used is not surprising. This cluster is recovered in 94% of trees generated using 16S rRNA gene sequences but in only 80% of 23S rRNA gene sequence-based trees (Fig. 1). The group containing *Planctomyces* and *Pirellula* is supported by a 100% bootstrap value for 23S rRNA gene sequence analysis (Fig. 1b) but is recovered in only 81% of 16S rRNA gene sequence-based trees (Fig. 1a).

Phylogenetic analyses of the 23S rRNA gene sequence dataset obtained using the neighbour-joining method with the maximum-likelihood correction (Felsenstein, 1981) produced the same tree topology as that found using the correction of Jukes & Cantor (1969) (data not shown). Phylogenetic analysis by maximum parsimony also produced the same tree as that obtained by neighbour-joining, except that members of the division *Verrucomicrobia* were found to group with the planctomycetes, whereas the low- and high-G+C Gram-positive bacteria did not cluster together.

Deletions in the 23S rRNA genes of some planctomycetes and *V. spinosum*. The 23S rRNA genes of *Planctomyces brasiliensis*, *Planctomyces limnophilus*, *G. obscuriglobus*, *V. spinosum*, *Prosthecobacter fusiformis*, *Prosthecobacter* sp. strain FC-2, strain 639 and strain 670 were shown to carry a deletion of approximately 50 nucleotides in domain III, which encompasses helix 58. Deletion of helix 58 has been reported only in members of the α-subclass of the *Proteobacteria* and has been suggested to be a signature for members of this group (Ludwig & Schleifer, 1994). The presence of helix 58 in some planctomycete strains indicates that this signature is not exclusive to the α-*Proteobacteria*. The fact that the deletion does not occur in all planctomycete strains means that it cannot be considered as a signature for the planctomycete lineage and probably indicates that its phylogenetic meaning is restricted to lower taxonomic levels.

Conclusions

Almost complete 23S rRNA gene sequences were obtained from 13 planctomycete strains, *V. spinosum*, *Prosthecobacter fusiformis* and *Prosthecobacter* sp.

strain FC-2. The modified 23S rRNA gene PCR primer set provides a useful resource for future 23S rRNA gene sequence analysis of additional planctomycete strains and members of the division *Verrucomicrobia*.

The phylogenetic position of the planctomycetes and members of the division *Verrucomicrobia* was determined using 16S rRNA and 23S rRNA gene sequences. Comparative phylogenetic analyses were performed using 16S and 23S rRNA gene reference sequences from the same species. Phylogenetic analyses using both genes showed that the planctomycete strains investigated form a phylogenetically distinct lineage within the domain *Bacteria*. These analyses using multiple planctomycete sequences, including members of as yet undescribed taxa confirmed the results previously obtained with the 23S rRNA gene sequence of the single planctomycete species *Pirellula marina* (Liesack *et al.*, 1988; Ludwig & Schleifer, 1994). Analysis of multiple planctomycete sequences confirmed the phylogenetic coherence of the planctomycete lineage, which was previously suggested by the results of 16S rRNA gene sequence analyses (Stackebrandt *et al.*, 1986a, b; Liesack *et al.*, 1992; Ward *et al.*, 1995; Griepenburg *et al.*, 1999). The phylogenetic distinctness, coherence and depth of the planctomycete lineage were evident from the results of three different methods of phylogenetic analysis and were supported by bootstrap analyses.

The phylogenetic distinctness of the division *Verrucomicrobia* was confirmed in this study by analysis of complete 16S and 23S rRNA gene sequences and was observed in the dendrograms produced from all methods of analysis. However, the phylogenetic position of members of the division *Verrucomicrobia* in relation to other members of the domain *Bacteria* was shown to depend on the numbers of planctomycete sequences included in the analyses and the method of analysis chosen. The branching-point instability of the *Verrucomicrobia* lineage is reflected in the low level of statistical significance assigned to any grouping involving these taxa.

In conclusion, phylogenetic analyses using two different molecular chronometers have confirmed the phylogenetic distinctness of the planctomycete and *Verrucomicrobia* lineages and have indicated that there is no statistically significant relationship between the members of these two lineages and the chlamydiae.

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