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Divergent members of the bacterial division *Verrucomicrobiales* in a temperate freshwater lake

Gabriël Zwart [a,*], Raymond Huismans [a], Miranda P. van Agterveld [a], Yves Van de Peer [b], Peter De Rijk [b], Hugo Eenhoorn [a], Gerard Muyzer [c], Erik J. van Hannen [a], Herman J. Gons [a], Hendrikus J. Laanbroek [a]

[a] *Department of Microbial Ecology, Centre for Limnology, Netherlands Institute of Ecology, Rijksstraatweg 6, 3631 AC Nieuwersluis, The Netherlands*

[b] *Department of Biochemistry, University of Antwerp (UIA), Universiteitsplein 1, 2610 Antwerp, Belgium*

[c] *Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany*

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Abstract

Bacterial diversity in the water column of a freshwater lake in the Netherlands was investigated by analysis of 16S rRNA gene sequences recovered through PCR amplification from total community DNA. Among 23 unique cloned sequences, two appeared to belong to the recently described bacterial division *Verrucomicrobiales*. One of the two sequences was most similar to a group of environmental clones that form a distinct lineage within the division. The other sequence was divergent (less than 85% similarity) from all 16S rRNA gene sequences, both from cultivated species and from environmental clones, known in this division to date. Analysis by denaturing gradient gel electrophoresis (DGGE) and sequencing of DNA recovered through excision from the DGGE gel showed that the two sequence types were present in the lake throughout the year. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

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1. Introduction

Until recently, our knowledge of natural bacterioplankton diversity has largely been based on cultivation studies (see for instance [1]). However, cultivation methods have been shown to recover only a small subset of the species present in environmental samples [2–5], thus generating a distorted view of bacterial community composition. These limitations and the difficulties with discriminating morphologically similar bacteria have compelled microbial ecologists to use molecular techniques to investigate the species composition of bacterial communities [6–8]. These techniques allow the direct sampling of the genetic diversity of microbial communities, without the need for cultivation, through the recovery of

* Corresponding author. Tel.: +31 (294) 239315; Fax: +31 (294) 232224; E-mail: zwart@cl.nioo.knaw.nl

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evolutionary marker molecules such as the ribosomal RNA (rRNA) genes. Although not completely without bias itself [9,10], this molecular approach has successfully been employed to survey the diversity of planktonic bacteria in the world's oceans [11–14]. In contrast, freshwater ecosystems have rarely been the focus of such direct molecular investigations of microbial diversity.

A common type of freshwater ecosystem in the Netherlands is the eutrophic cyanobacterially dominated lake. With the ultimate goal of understanding the structure and function of the microbial community in such a lake, we used molecular techniques to investigate bacterioplankton diversity in Lake Loosdrecht (The Netherlands). This shallow lake (1.9 m mean depth) is still eutrophic despite dephosphorization of inlet water since 1983 [15]. Its water is turbid throughout the year as a result of wind resuspension of sedimented detritus and the abundant presence of filamentous cyanobacteria [16]. The bacterial diversity was investigated by analysis of 16S rRNA gene (16S rDNA) sequences which were recovered from water samples through PCR amplification. In the analysis we followed a dual approach. On the one hand, nearly full-length amplified genes were cloned and individual clones were sequenced. On the other hand, amplified DNA from the lake was directly analyzed using denaturing gradient gel electrophoresis (DGGE) [17]. The comparison of clones and the direct amplification pattern in DGGE allowed the identification of particular bands from the lake sample. Using this approach, we identified two divergent members of the recently proposed bacterial division *Verrucomicrobiales* [18–20]. In this division only cultivated species from two genera have been described: *Verrucomicrobium* and *Prosthecobacter*.

2. Materials and methods

2.1. Sampling and DNA extraction

Water samples were collected immediately below the surface in sterile bottles, from a jetty on the north-east bank of Lake Loosdrecht, and kept dark at 4°C. Within 2 h after sampling, 50 ml of each sample was filtered over a polycarbonate membrane filter (0.2 μm pore size, 2.5 cm diameter, BA83 Schleicher and Schuell, Dassel, Germany). The filter was cut in two with a sterile scalpel and each half was stored in a microcentrifuge tube at −80°C until further processing. Sampling dates were 19 April 1995, 30 August 1995 and 19 January 1996, with lake water temperatures of 10, 16 and 0°C, respectively.

To lyse the cells, 0.5 g of zirconium beads (0.1 mm diameter), 0.5 ml TE buffer (10 mM Tris, pH 7.6, 1 mM EDTA) and 0.5 ml buffered phenol (pH 7–8) were added to the tubes containing the filter and the tubes were vigorously shaken (5000 rpm) on a Mini Beadbeater (Biospec Products, Bartlesville, OK, USA) for 2 min with intermittent cooling on ice. The tubes were then centrifuged for 5 min at $10\,000 \times g$ and the upper (aqueous) phase was collected and extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1) by brief shaking on a vortex mixer and separation of the aqueous and the organic phase through centrifugation. Then, the DNA was precipitated by adding 1/10 volume of 3 M sodium acetate (pH 5) and two volumes of 96% (v/v) ethanol and centrifuging for 30 min at $14\,000 \times g$. Subsequently, the DNA was dissolved in TE, incubated in the presence of RNase A (2 mg ml^{-1}) at 70°C for 10 min and purified on a Wizard column (Promega, Madison, WI, USA) according to the manufacturer's recommendations.

2.2. Clone library construction

To generate near full-length 16S rDNA clones, the extracted DNA was subjected to PCR amplification with the following primers: F27 (5′-AGAGTTTGATCMTGGCTCAG-3′ [21]) which is specific for most Bacteria and R1492 (5′-GRTACCTTGTTACGACTT-3′) which is specific for most Bacteria and Archaea. Numbering refers to the *Escherichia coli* 16S rRNA gene position corresponding to the 3′ end of the primers. PCR amplification was performed using a PE480 thermocycler (Perkin-Elmer, Foster City, CA, USA) in a 50 μl reaction mixture containing approximately 100 ng of purified DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.01% (w/v) gelatine, 200 μM of each deoxynucleotide, 1.5 mM $MgCl_2$, 2.5 units of Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany) and 0.5 μM

of each primer. The temperature cycling conditions were as follows. First, a preincubation at 94°C for 5 min; then 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min; and finally, an incubation at 72°C for 5 min.

The PCR products were excised from an agarose gel (1.6%) and purified with QIAquick spin columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The product was ligated into pGEM-T vector (Promega) and transfected through heat shock to Epicurian Coli XL1-Blue MRF′ supercompetent *E. coli* cells (Stratagene, La Jolla, CA, USA). The presence of inserts was determined by performing a PCR directly on a bacterial sample from white (positive) colonies, using primers flanking the cloning site on the vector. Plasmids were purified from successfully transfected clones using the High Pure Plasmid isolation kit of Boehringer Mannheim.

To determine the sequences of plasmid inserts, cycle sequence reactions were performed using Thermosequenase (Amersham, Little Chalfont, UK) according to the manufacturer's instructions. Fragment separation, detection and base calling was done using a Vistra DNA Sequencer 725 (Amersham). The sequences were determined in two directions with two vector-specific primers flanking the cloning site: M13 (−21) forward (5′-TGTAAAACGACGGCCAGT-3′) and M13 (−26) reverse (5′-GAAACAGCTATGACCATG-3′) which were labelled with Texas red, and five primers binding at sites in the 16S rDNA: F357 (5′-CCTACGGGAGGCAGCAG-3′), R1053 (5′-AGCTGACGACAGCCATGC-3′) and R1221 (5′-CATTGTAGCACGTGTGTAGCC-3′) which were Texas red-labelled, and F797plus (5′-GCGTTCTTCATCGTTGCGAG-CAAACRGGATTAGATACCC-3′) and R518plus (5′-GCGTTCTTCATCGTTGCGAG-ATTACCGCGGCTGCTGG-3′). The latter two unlabelled primers (in combination with R1492 and F27, respectively) were used to generate PCR products from the plasmids which subsequently were subjected to cycle sequencing. The Texas red-labelled primer used for sequencing of these PCR products (Stef1Tex, 5′-GCGTTCTTCATCGTTGCGAG-3′) binds specifically to the site introduced in the PCR reaction through the extensions at the 5′ end of F797plus and R518plus.

2.3. DGGE profiling

The DNA extracted from the water samples was amplified for DGGE analysis using the PCR procedure essentially as described by Muyzer et al. [22]. The PCR primers were F357GC (5′-CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCCCTACGGGAGGCAGCAG-3′), which contains a GC-rich 'clamp' and is specific for most Bacteria, and R518 (5′-ATTACCGCGGCTGCTGG-3′) which is specific for most Bacteria, Archaea and Eukarya. The temperature cycling conditions were as follows. After a preincubation at 94°C for 5 min, a total of 25 cycles were performed of 94°C for 1 min, T_A for 1 min and 72°C for 1 min. In the first 20 cycles, T_A decreased by 1°C, stepwise every two cycles, from 65°C in the first cycle to 56°C in the twentieth. This 'touch-down' procedure was followed to decrease aspecific amplification [23]. In the last five cycles T_A was 55°C. Cycling was followed by 5 min incubation at 72°C. The magnesium chloride concentration was 3.0 mM. Other PCR reaction conditions were as described above.

For analysis of cloned sequences in DGGE, the same approximately 200-bp region of the 16S rDNA was amplified from the plasmids using the primers F357GC and R518. With the exception of the magnesium chloride concentration (1.5 mM), reaction conditions and thermocycling were as described above.

DGGE was performed essentially as described by Muyzer et al. [22]. Briefly, similarly sized PCR-products were separated on a 1.5 mm thick, vertical gel containing 8% (w/v) polyacrylamide (37.5:1 acrylamide:bisacrylamide) and a linear gradient of the denaturants urea and formamide, increasing from 30% at the top of the gel to 60% at the bottom. Here, 100% denaturant is defined as 7 M urea and 40% (v/v) formamide. Electrophoresis was performed in a buffer containing 40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 7.6 (0.5×TAE) for 16 h at 100 V. The gel was stained for 1 h in 0.5×TAE containing 0.5 μg ml^{-1} ethidium bromide followed by 20 min destaining in 0.5×TAE. The gel was recorded with a CCD camera system (The Imager, Appligene, Illkirch, France). Processing of the image was limited to improvement of contrast and applied solely on the image as a whole, with the help of the

program Aldus Photostyler 2.0 (Aldus corporation, Seattle, WA, USA).

2.4. Sequencing of DNA from DGGE bands

A small block of gel from the middle of the target band was excised from the DGGE gel with a surgical knife. The block was placed into a 2-ml screw-cap tube and 0.5 g of beads and 0.5 ml TE were added. To elute the DNA from the gel, the tube was shaken with a Mini Beadbeater for 2 min at 5000 rpm and thereafter incubated overnight at 4°C. To determine the migration characteristics of the eluted DNA, 5 μl of the supernatant was subjected to PCR with the primers F357GC and R518 as described above and analyzed by DGGE. For sequence determination, we followed two approaches. First, eluted DNA was directly sequenced by reamplifying 5 μl of the supernatant with the primers F357 (no GC-clamp) and R518plus, and AmpliTaq Gold (Perkin-Elmer) using the PCR conditions described above. The amplification product (50 μl) was excised from an agarose gel (2% agarose, 0.5×TAE) and purified using QIAquick spin columns (Qiagen). 5 μl out of the 30 μl of spin column eluate was subjected to sequence reactions with the primer Stef1Tex as described above. Second, DNA eluted from the DGGE gel was cloned and sequenced by reamplifying 5 μl of the supernatant with the primers F357 (no GC-clamp) and R518, and Taq DNA polymerase (Boehringer Mannheim) using the PCR conditions described above. The cloning of the PCR product into pGEM-T and the sequence reactions with the primer M13 (−26) reverse were performed as described above.

2.5. Phylogenetic analysis

The 16S rDNA sequences recovered from Lake Loosdrecht water were screened against sequences in the Ribosomal Database Project [24] using the program Similarity Rank (http://rdpgopher.life.uiuc.edu/RDP/commands/rank.html), and against GenBank/EMBL sequences using the programs Fasta [25,26] and Blast [27] (via http://www.ddbj.nig.ac.jp/E-mail/homology.html). Then, the recovered sequences and the sequences with highest similarity from the above procedures were aligned to reference sequences from all described bacterial phyla obtained from the SSU rRNA database [28]. This alignment was made on the basis of comparison of secondary structural elements in the ribosomal RNA using the Dedicated Comparative Sequence Editor (DCSE [29]). Similarity values were computed on the basis of this alignment using the program package TREECON [30]. No corrections were made for multiple substitutions and gaps were not taken into account.

Phylogenetic trees were constructed using two different methods. First, neighbor-joining analysis was performed with the program package TREECON in which evolutionary distances were corrected for multiple substitutions according to the algorithm of Jukes and Cantor [31]. Gaps were taken into account as single mutational events regardless of their length [32]. The resulting distance matrix was used to construct an evolutionary tree using the neighbor-joining method [33]. The consistency of the tree branches was assessed by bootstrap analysis from resampled data [34]. Second, maximum likelihood analysis was performed using the test version 4.0d59 for DOS of the program PAUP* developed by David L. Swofford (Laboratory of Molecular Systematics, Smithsonian Institution, Washington, DC, USA). Nucleotide frequencies and transition to transversion ratios were estimated from the data. Nucleotide substitution rates were assumed to follow a gamma distribution with shape parameter=0.5 with settings according to the HKY model [35].

2.6. Nucleotide sequence accession numbers

Sequences of LD19 and LD29 have been deposited with EMBL under the accession numbers AF009974 and AF009975, respectively.

3. Results

We obtained 16S rDNA sequences from a single sample taken from Lake Loosdrecht in January 1996. The DNA extracted from this sample was amplified in two ways. First, a primer set was used to amplify approximately 200 bp (*E. coli* positions 338–538) of the 16S rDNA. This amplification product was subjected to DGGE to separate the different sequence variants, thus generating a 'direct amplifi-

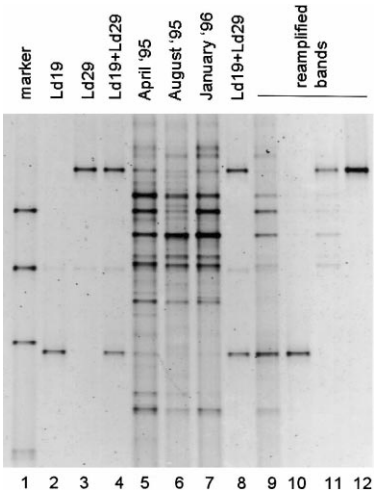

Fig. 1. Denaturing gradient gel (30–60% denaturants) of clones LD19 and LD29 and natural samples from Lake Loosdrecht. Lane 1, marker lane; top to bottom: PCR products from *Lactococcus lactis lactis*, *Escherichia coli*, *Micrococcus luteus*. Lanes 2 and 3, PCR products from clone LD19 and clone LD29, respectively. Lanes 4 and 8, a mixture of PCR products from clones LD19 and LD29. Lanes 5–7, PCR products of natural samples from Lake Loosdrecht collected April 1995, August 1995 and January 1996, respectively. Lanes 9 and 11, reamplified products from the January profile, bands 'LD19' and 'LD29', respectively. Lanes 10 and 12, second reamplifications of bands shown in lanes 9 and 11, respectively (see text).

cation pattern' (Fig. 1, lane 7). Second, a primer set was used to amplify almost the entire 16S rRNA gene (*E. coli* positions 7–1512). This amplification product was cloned into plasmids. For DGGE analysis, however, the entire 16S rRNA gene is too large. Therefore, the aforementioned 200-bp primer set was used to generate amplification products from the cloned 16S rDNA sequences, which could then be analyzed using DGGE. In this manner, it was possible to analyze the diversity among the cloned 16S rDNAs and furthermore, it was possible to compare the gel positions of the 200-bp PCR products of the clones with the direct amplification pattern from the natural sample.

Among the 97 clones analyzed, we could distinguish 23 bands migrating to different positions in the DGGE gel (data not shown). Partial sequence analysis of representative clones from each of these positions revealed that 12 sequences clustered with the cyanobacteria or plastids, five clones clustered with the flavobacteria and four clones clustered with the proteobacteria (unpublished results). Sequences from two clones, LD19 and LD29, however, did not cluster within any of the bacterial phyla described by Woese (reviewed in [36]).

The 200-bp PCR products of clones LD19 and LD29 appear in lanes 2–4 of the DGGE gel in Fig. 1, next to the amplification products of lake samples taken in April and August of 1995 and January of 1996 (lanes 5–7). In all three lake samples, low intensity bands are visible at the exact position of the clones. To investigate if the amplified DNA of the bands from the lake sample indeed exhibited the same sequence as the clones, we excised gel material from the 'LD19' and 'LD29' bands of the January sample and reamplified the DNA with the 200-bp primer set. Although we took pains to excise a very small piece of DGGE gel from the middle of the band, the reamplification products showed not only the targeted band, but also some of the more intense bands from the original pattern (lanes 9 and 11). Apparently, small amounts of DNA from all sequence types were present throughout the gel, possibly in the form of single stranded molecules. The low intensity of the targeted bands may have increased the chance of amplifying these background products. To further enrich the 'LD19' and 'LD29' DNA, we then excised the target bands from the reamplification patterns and once more reamplified the DNA. The products of this second reamplification each showed only one visible band at the position of clone LD19 and LD29, respectively (lanes 10 and 12). This reamplified DNA was analyzed by 'direct' sequencing and by sequencing clones from the reamplified DNA. The direct sequences of the reamplified 'LD19' and 'LD29' bands exhibited 98.9% and 99.5% sequence similarity, respectively, to the original clones LD19 and LD29, over approximately 190 unambiguous nucleotides. All clones derived from the reamplified DNA (two for 'LD19' and four for 'LD29') also displayed a high degree of sequence similarity (99–100%) to the corresponding original clones. The observed mismatches were probably due to polymerase misincorporations during the PCR reamplification procedures.

To investigate their phylogenetic placement, clones LD19 and LD29 were further sequenced to obtain the complete cloned 16S rDNA nucleotide sequence. Comparison of the aligned LD19 and LD29 sequen-

ces to over 2000 nearly full-length database sequences showed that they had a similarity of less than 76% to any of the entries from the established bacterial divisions. The closest database sequences were from molecular clones recovered from geographically disparate and diverse environments. These include clones from a forest soil in Australia (termed MC cluster III clones, prefix MC) [18], a Washington State grassland soil (clone EA25) [37], an Amazonian mature forest soil and an Amazonian pasture land soil (prefixes M and P, respectively) [38], clones from the sediment of a freshwater lake in South Carolina, USA (prefix RB) [39] and clones from the water column of freshwater lakes in the Adirondack mountains, New York State, USA (prefix ACK) [40]. These environmental clones belong to the new bacterial division *Verrucomicrobiales* [19] which also comprises cultivated species from the genera *Verrucomicrobium* [19] and *Prosthecobacter* [20] and three cultivated isolates from an Italian rice paddy soil (VeCb1, VeGlc2 and VeSm13) [41]. Table 1 shows selected similarities of LD19 and LD29 to representatives of the established phyla as well as to environmental clones. LD29 is most similar (approximately 92%) to clones MC17 and EA25. One of the clones from the Adirondack mountain lakes, ACK-DE41, has a similarity of 93% to LD29, but this comparison includes only 308 nucleotides. LD19 is more divergent with a similarity to the closest sequences, MC17 and EA25, of approximately 83%.

Adherence of clones LD19 and LD29 to the proposed division of *Verrucomicrobiales* is supported by phylogenetic trees shown in Figs. 2 and 3. Fig. 2 shows the result of neighbor-joining analysis on approximately 300 bases of the 16S rDNA. This limited region was chosen to include the Adirondack mountain lake clones. In this analysis we observed four distinct radiations, hereafter referred to as groups A–D. Group A, which contains only cultivated species of the genera *Verrucomicrobium* and *Prosthecobacter*, is most closely related to group B, which contains only environmental sequences including clone LD29. The consistency of each of the groups A, B and D is supported by bootstrap values greater

Table 1
Percent 16S rDNA sequence similarity of clones LD19 and LD29 to representatives from major bacterial divisions and selected environmental clones

Species or clone	Similarity (%)			
	Bases 49–1041		Bases 531–1373	
	LD19	LD29	LD19	LD29
Thermus aquaticus	73	72	76	76
Chloroflexus aurantiacus	70	72	73	73
Bacillus subtilis	69	71	73	74
Microcystis wesenbergii	71	74	74	75
Escherichia coli	71	71	74	75
Spirochaeta aurantia	69	69	74	75
Flavobacterium aquatile	68	68	71	71
Chlamydia pneumoniae	69	71	75	76
Planctomyces limnophilus	68	69	72	74
Prosthecobacter fusiformis	80	86	82	87
Verrucomicrobium spinosum	79	87	82	89
Environmental clone MC17	83	91		
Environmental clone EA25	82	91	84	93
Environmental clone RB24			82	88
Environmental clone RB35			83	91
Environmental clone P19			83	92
Environmental clone P71			80	86
Environmental clone VeCb1	78	79	79	81
Environmental clone LD19		82		84

Values are given for two regions of the gene because sequence information is limited for most of the environmental clones. The origin of the environmental clones is described in the text.

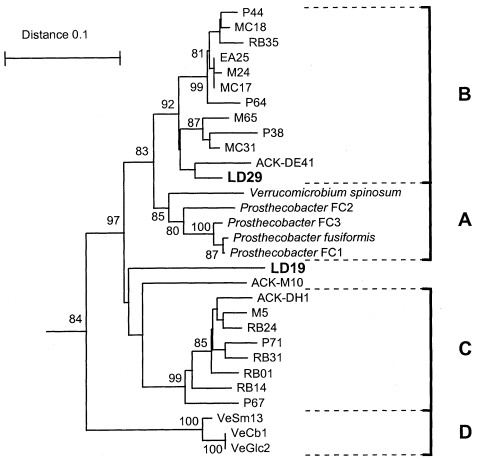

Fig. 2. Phylogenetic tree, constructed through neighbor-joining analysis, showing the placement of 16S rDNA sequences of Lake Loosdrecht environmental clones LD19 and LD29 (boldface) relative to those of other environmental clones and cultivated bacteria of the division *Verrucomicrobiales*. This tree was extracted from a larger neighbor-joining tree (of 85 taxa) containing representatives of all Bacterial phyla in which the *Verrucomicrobiales* division was coherent in 84% of 500 bootstrapped trees. 16S rRNA nucleotide positions correspond to *E. coli* positions 607–875. The scale bar represents 0.1 fixed point mutations per nucleotide sequence position. Values at the nodes indicate bootstrap percentages (only the values above 80 are shown). The origin of the environmental clones is described in the text.

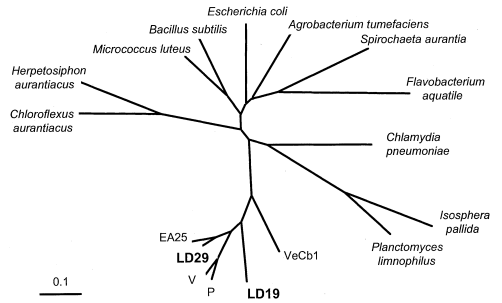

Fig. 3. Maximum likelihood tree, showing the placement of 16S rDNA sequences of Lake Loosdrecht environmental clones LD19 and LD29 (boldface) relative to those of representatives of the major bacterial lines of descent and members of the division *Verrucomicrobiales*. This tree was constructed using nucleotides 49–1406 (*E. coli* numbering). P and V denote *Prosthecobacter fusiformis* and *Verrucomicrobium spinosum*, respectively. Scale bar as in the legend to Fig. 2.

than 95% in analyses of larger regions of the gene (i.e. nucleotides 49–1041 and 49–1406, using all of those sequences that include these positions). For group C, only limited sequence information is available in these regions. The divergent sequence LD19 does not group within any of the groups A–D. However, its grouping within the 'supergroup' ABC is supported by a bootstrap value of 97%, consistent with the even more divergent nature of the rice paddy field isolates of group D. Taken together, the members of the division *Verrucomicrobiales*, including group D, form a monophyletic group. The separation of this group from other bacterial divisions is supported by bootstrap values of 100% if regions larger than 500 nucleotides are included.

The results from maximum likelihood analysis of nearly complete 16S rRNA gene sequences are displayed in Fig. 3. The tree supports the monophyletic clustering of the division *Verrucomicrobiales* with group D (VeCb1) on its outskirts and the separation of EA25 and LD29 (group B) from the *Verrucomicrobium* and *Prosthecobacter* sequences (group A). In addition, this analysis confirms the clear separation of the division *Verrucomicrobiales* from the closest bacterial divisions: the planctomycetes and members of the genus *Chlamydia* [20].

Table 2
Signature nucleotides in the 16S rRNA for the MC III clones

Position (*E. coli*)	Signature of MC III clones[a]	Planctomycetes	*Chlamydiae*	Group A	Group B	LD19	Group C	Group D
768	G	A	A	+	+	+	A	A
811	A	U/C	C	+	+	+	U/C	C
819	G	A	A	+	+	+	+	+
826, 874	UA	CG	CG	+/CG[b]	+	+	+	CG

Plus signs indicate accordance with signature. Groups A–D are radiations within the *Verrucomicrobiales* division as defined in Fig. 2. All members of these groups shown in Fig. 2 are included (A, $n = 5$; B, $n = 16$; C, $n = 10$; D, $n = 3$).

[a]According to Liesack et al. [18].

[b]*Prosthecobacter* FC2 and *Verrucomicrobium spinosum* have UA, others have CG.

Table 3
Signature nucleotides in the 16S rRNA for the planctomycetes

Position (*E. coli*)	Planctomycete signature[a]	Planctomycetes	*Chlamydiae*	Group A	MC cluster III clones	LD19 and LD29	EA25	Group D
47	G	+	+	+	+	+	unknown	+
48	A	+	+	+	+	+	unknown	U
50	U	+	G	A	A	A	G	A
52	G	+	+	+	+	+	A	+
53	G	+	+	A	A	A	A	A
353	U	+	+	A	A	A	A	A
570	U	+	G	G	G	G	G	G
933	A	+	G	G	G	G	G	G
955	C	+	U	+	+	+	+	+
983:1[b]	U	+	−	−	−	−	−	−
1109	Ac[c]	+	C	C	unknown	C	C	C
1384	U	+	C	C	unknown	C	C	C
1410	G	+	A	A	unknown	A	unknown	+

Plus signs indicate accordance with signature. Minus signs indicate no insertion. Groups A ($n = 5$) and D ($n = 3$) are radiations within the *Verrucomicrobiales* division as defined in Fig. 2. Less than half of the signature positions are known for most members of group B and all of group C.

[a]According to Woese et al. [6].

[b]Insertion.

[c]A for most species, C for less than 10% of species.

Further support for coherence of the groupings in the division *Verrucomicrobiales* comes from the presence of signature nucleotides unique to the MC cluster III clones (group B), as identified by Liesack et al. [18]. Table 2 shows that these signatures are shared by all 16 members of group B, by LD19 and by the members of group A (with the exception of the canonical RNA base pair at positions 826 and 874, where *Prosthecobacter* species FC1, FC3 and *P. fusiformis* have the more common 'CG' instead of 'UA'). Groups C and D share only two and one of these signatures, respectively, consistent with their phylogenetic distance to groups A and B.

Furthermore, Liesack et al. [18] reported that the MC cluster III clones shared four out of the 13 signature nucleotides of the planctomycetes (defined by Woese [6]) underscoring their relationship to this bacterial division. Table 3 shows that, in agreement with their relationship to the MC cluster III clones, all members of group A and LD19 and LD29 share the same four planctomycetes signatures. The rice paddy soil isolates in group D also display four of the planctomycetes signatures. However, one is at another position.

4. Discussion

In a study of the genetic diversity of the bacterial community in a freshwater lake we recovered two unique 16S rDNA sequences (clones LD19 and LD29) which appear to be related to the division *Verrucomicrobiales* [19]. The occurrence of the sequences LD19 and LD29 is noteworthy since the *Verrucomicrobiales* division is new and although the amount of environmental sequences in this division is growing, at present relatively few (nearly) complete gene sequences are available. Caution should however be taken with sequences extracted and amplified directly from the environment. Several sources of errors have been identified, such as polymerase misincorporations during the PCR, sequencing errors and, most deleterious, the formation of chimeric sequences [42]. Although we cannot exclude any of these errors, the number of errors of the first two categories can be limited by employing a low number of amplification cycles in the formation of clones and by obtaining sequence data from both strands. Limiting the number of amplification cycles also reduces the chance of chimeric sequence formation according to a study by Wang and Wang [43]. Furthermore, the sequences of LD19 and LD29 assumed the same

phylogenetic positions irrespective of the portion of the gene analyzed (not shown). In addition, all helix regions in which the complementary regions are separated more than 100 bases were fully compatible with known secondary structures for the 16S rRNA. These helix regions are variable between different phyla of the bacterial domain. Therefore, these sequences are very unlikely to be the product of recombination between organisms of different phylogenetic grouping. However, recombination between more closely related organisms remains a possibility, since the helix regions are highly conserved within the *Verrucomicrobiales* division.

The sequences of clones LD19 and LD29, together with other new sequences belonging to the *Verrucomicrobiales*, underscore the great diversity in this division. However, the amount of data now available allows the observation of phylogenetic structure within this diversity. At least four monophyletic groups (here designated A–D) can be discerned, which are clearly separated and coherent as apparent from phylogenetic analyses as well as the presence of signature nucleotides which are shared to different extents by the different groups. Cultivated bacteria are available from two of the groups (A and D), which may help in understanding the physiology and ecology of other members of the division. The morphology and habitat of the cultivated organisms from groups A and D is widely different; while members of the first group are prosthecate bacteria isolated from freshwater lakes, members of group D are coccoid ultramicrobacteria from soil. Nevertheless, there are metabolic similarities between cultivated strains from these groups. Both *Verrucomicrobium spinosum* from group A and the rice paddy soil isolates from group D can perform aerobic respiration as well as ferment various sugars under anaerobic conditions [41,44]. However, the genetic distance separating clones LD19 and LD29 from these cultivated strains (more than 13% 16S rDNA nucleotide divergence) allows a high degree of phenotypic variation. Therefore, deduction of physiological properties of these clones from those of the known cultivated strains is still speculative.

DGGE was utilized to relate the recovered clones to bands in the direct amplification pattern. The template DNA used for cloning of sequences was also used to generate the January DGGE pattern. Therefore, it is probable that bands from the January pattern and clones migrating to the same position as these bands contained identical sequences. For the bands corresponding to clones LD19 and LD29 we tried to confirm this through the determination of sequences obtained from the bands. Although two rounds of gel excision and reamplification were necessary to obtain relatively pure amplification products, indeed both directly determined and cloned sequences of these amplification products matched (with few mismatches) to the sequences from the corresponding clones. This shows that these sequence types were indeed represented in the targeted bands, although the possibility remains that other sequences were also present.

Both sequence types LD19 and LD29 showed low intensity signals in the direct amplification pattern relative to the total intensity of the sample. However, several factors, such as the number of 16S rRNA gene copies per cell, the DNA isolation efficiency and the amplification efficiency in PCR, may influence the intensity of the signal in an uncertain manner. For one, the sequence of the primers used for DGGE analysis mismatched at three positions with clone LD19 (positions 7 and 9 from the 3′ end of the forward primer and position 15 of the reverse primer) and at one position with clone LD29 (position 7 of the forward primer). This degree of mismatch may have adversely influenced the efficiency of amplification. Therefore, further experiments such as in situ hybridization are necessary for a more definite estimation of the relative abundance of these bacteria.

The presence of bands at the exact position of LD19 and LD29 in the samples from April and August 1995, as well as January 1996, suggests that the bacteria are present in the lake throughout the year. This observation makes it more likely that the lake water is their natural habitat. Remarkably, ACK-DE41, one of the closest sequences to LD29, was also recovered from the water column of a freshwater lake. However, presently, all other members of group B have been obtained from soils. Considering the genetic distance between LD19 and LD29, the corresponding bacteria may have marked physiological differences, enabling them to occupy different niches within the freshwater habitat. What their precise role is, and their relation to the domi-

nating cyanobacteria in the lake, remains to be established.

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References

[1] Rheinheimer, G. (1992) Aquatic Microbiology, 4th edn. John Wiley and Sons, Chichester.
[2] Kogure, K., Simidu, U. and Taga, N. (1979) A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. 25, 415–420.
[3] Brock, T.D. (1987) The study of microorganisms in situ: progress and problems. In: Symp. Soc. Gen. Microbiol. 41 (Fletcher, M., Gray, T.R. and Jones, J.G., Eds.), pp. 1–17. Cambridge University Press, Cambridge.
[4] Ward, D.M., Weller, R. and Bateson, M.M. (1990) 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature 345, 63–65.
[5] Suzuki, M.T., Rappe, M.S., Haimberger, Z.W., Winfield, H., Adair, N., Strobel, J. and Giovannoni, S.J. (1997) Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. 63, 983–989.
[6] Woese, C.R. (1987) Bacterial evolution. Microbiol. Rev. 51, 221–271.
[7] Stahl, D.A., Lane, D.J., Olsen, G.J. and Pace, N.R. (1985) Characterization of a Yellowstone hot spring microbial community by 5S rRNA sequences. Appl. Environ. Microbiol. 49, 1379–1384.
[8] Pace, N.R. (1997) A molecular view of microbial diversity and the biosphere. Science 276, 734–740.
[9] Reysenbach, A.-L., Giver, L.J., Wickham, G.S. and Pace, N.R. (1992) Differential amplification of rRNA genes by polymerase chain reaction. Appl. Environ. Microbiol. 58, 3417–3418.
[10] Suzuki, M.T. and Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. 62, 625–630.
[11] Giovannoni, S.J., Britschgi, T.B., Moyer, C.L. and Field, K.G. (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345, 60–63.
[12] Delong, E.F., Franks, D.G. and Alldredge, A.L.. (1993) Phylogenetic diversity of aggregate-attached vs free-living marine bacterial assemblages. Limnol. Oceanogr. 38, 924–934.
[13] Fuhrman, J.A. and Davis, A.A. (1997) Widespread archaea and novel bacteria from the deep sea as shown by 16S rRNA gene sequences. Mar. Ecol. Progr. Ser. 150, 275–285.
[14] Field, K.G., Gordon, D., Wright, T., Rappe, M., Urbach, E., Vergin, K. and Giovannoni, S.J. (1997) Diversity and depth-specific distribution of SAR11 cluster rRNA genes from marine planktonic bacteria. Appl. Environ. Microbiol. 63, 63–70.
[15] van Liere, L., Gulati, R.D., Wortelboer, F.G. and Lammens, E.H.R.R. (1990) Phosphorus dynamics following restoration measures in the Loosdrecht Lakes (The Netherlands). Hydrobiologia 191, 87–95.
[16] Gons, H.J., Otten, J.H. and Rijkeboer, M. (1991) The significance of wind resuspension for the predominance of filamentous cyanobacteria in a shallow, eutrophic lake. Mem. Ist. Ital. Idrobiol. 48, 233–249.
[17] Fisher, S.G. and Lerman, L.S. (1979) Length-independent separation of DNA restriction fragments in two dimensional gel electrophoresis. Cell 16, 191–200.
[18] Liesack, W. and Stackebrandt, E. (1992) Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. J. Bacteriol. 174, 5072–5078.
[19] Ward-Rainey, N., Rainey, F.A., Schlesner, H. and Stackebrandt, E. (1995) Assignment of hitherto unidentified 16S rDNA species to a main line of descent within the domain Bacteria. Microbiology 141, 3247–3250.
[20] Hedlund, B.P., Gosink, J.J. and Staley, J.T. (1996) Phylogeny of *Prosthecobacter*, the fusiform Caulobacters: members of a recently discovered division of the Bacteria. Int. J. Syst. Bacteriol. 46, 960–966.
[21] Lane, D.J. (1991) 16S/23S rRNA sequencing. In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 115–175. John Wiley and Sons, Chichester.
[22] Muyzer, G., de Waal, E.C. and Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.
[23] Don, R.H., Cox, P.T., Wainwright, B.J., Baker, K. and Mattick, J.S. (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. 19, 4008.
[24] Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J. and Woese, C.R. (1997) The RDP (Ribosomal Database Project). Nucleic Acids Res. 25, 109–111.
[25] Lipman, D.J. and Pearson, W.R. (1985) Rapid and sensitive protein similarity searches. Science 227, 1435–1441.
[26] Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence analysis. Proc. Natl. Acad. Sci. USA 85, 2444–2448.
[27] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–10.
[28] Van de Peer, Y., Jansen, J., De Rijk, P. and De Wachter, R. (1997) Database on the structure of small ribosomal subunit RNA. Nucleic Acids Res. 24, 111–116.
[29] De Rijk, P. and De Wachter, R. (1993) DCSE v2.54, an interactive tool for sequence alignment and secondary structure research. Comput. Appl. Biosci. 9, 735–740.
[30] Van de Peer, Y. and De Wachter, R. (1994) TREECON for

Windows: a software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Appl. Biosci. 10, 569–570.
[31] Jukes, T.H. and Cantor, C.R. (1969) Evolution of protein molecules. In: Mammalian Protein Metabolism, Vol. 3 (Munro H.N., Ed.), pp. 21–132. Academic Press, New York.
[32] Van de Peer, Y., Neefs, J.M. and De Wachter, R. (1990) Small ribosomal subunit RNA sequences, evolutionary relationships among different life forms, and mitochondrial origins. J. Mol. Evol. 30, 463–476.
[33] Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.
[34] Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.
[35] Hasegawa, M., Kishino, H. and Yano, T. (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J. Mol. Evol. 21, 160–174.
[36] Woese, C.R. (1992) Prokaryote systematics: The evolution of a science. In: The Prokaryotes, 2nd edn. (Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H., Eds.), pp. 3–18. Springer-Verlag, New York.
[37] Lee, S.-Y., Bollinger, J., Bezdicek, D. and Ogram, A. (1996) Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. Appl. Environ. Microbiol. 62, 3787–3793.
[38] Borneman, J. and Triplett, E.W. (1997) Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Environ. Microbiol. 63, 2647–2653.
[39] Wise, M.G., Mcarthur, J.V. and Shimkets, L.J. (1997) Bacterial diversity of a Carolina bay as determined by 16S rRNA gene analysis: confirmation of novel taxa. Appl. Environ. Microbiol. 63, 1505–1514.
[40] Hiorns, W.D., Methe, B.A., Nierzwickibauer, S.A. and Zehr, J.P. (1997) Bacterial diversity in Adirondack mountain lakes as revealed by 16S rRNA gene sequences. Appl. Environ. Microbiol. 63, 2957–2960.
[41] Janssen, P.H., Schuhmann, A., Morschel, E. and Rainey, F.A. (1997) Novel anaerobic ultramicrobacteria belonging to the *Verrucomicrobiales* lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. Appl. Environ. Microbiol. 63, 1382–1388.
[42] Liesack, W., Weyland, H. and Stackebrandt, E. (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed-culture of strict barophilic bacteria. Microb. Ecol. 21, 191–198.
[43] Wang, G.C.Y. and Wang, Y. (1996) The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology UK 142, 1107–1114.
[44] Schlesner, H. (1992) The genus Verrucomicrobium. In: The Prokaryotes, 2nd edn. (Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.-H., Eds.), pp. 3806–3808. Springer-Verlag, New York.