Assignment of hitherto unidentified 16S rDNA species to a main line of descent within the domain *Bacteria*

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Phylogenetic analysis of the almost complete 16S rDNA sequence of the fimbriate prosthecate bacterium *Verrucomicrobium spinosum* confirms the unique phylogenetic position of this organism, as previously shown by oligonucleotide cataloguing and partial reverse transcriptase sequencing. Comparative 16S rDNA sequence analysis of *V. spinosum* with a group of environmental clones, considered to represent a novel phylum, reveals their relatedness, and allows assignment of these clones to a known main line of descent. This phylogenetic relationship is supported by the presence in the 16S rDNA sequence of *V. spinosum* of signature nucleotides previously considered unique for the environmental clone cluster.

Keywords: 16S rDNA, *Verrucomicrobium spinosum*, environmental clone sequences

INTRODUCTION

The results obtained from molecular microbial ecology studies (mostly based on 16S rRNA/rDNA analysis) have led to the suggestion that many more organisms exist in natural environments than have been isolated and characterized in pure culture. The molecular approach to microbial ecology has been applied to a wide range of environments. The first analyses of 16S rRNA sequences obtained directly from environmental biomass were performed on cyanobacterial mats from Octopus Spring in Yellowstone National Park (Weller *et al.*, 1991; Ward *et al.*, 1990). This study demonstrated the failure of cultivation techniques to describe the full extent of biodiversity in a natural sample. The authors recovered sequences of cyanobacteria unrelated to *Synechococcus lividans*, the organism believed to be the sole cyanobacterial component of the mat, and other novel taxa, some of which could not be assigned to any known phylum. The first reports of 16S-rRNA-based molecular ecology studies of the marine environment described the composition of marine plankton from the Sargasso Sea (Britschgi & Giovannoni, 1991; Giovannoni *et al.*, 1990) and the Central Pacific (Schmidt *et al.*, 1991). Both studies reported the recovery of 16S rRNA gene sequences from members of the cyanobacteria and the Proteobacteria.

While the cyanobacterial clones were closely related to cultured marine *Synechococcus* species (Giovannoni *et al.*, 1990; Schmidt *et al.*, 1991), 16S rRNA sequences were recovered that formed clusters within the alpha and gamma subdivisions of the Proteobacteria unrelated to cultured members of these subdivisions. The lack of close phylogenetic relationship between cloned environmental 16S rRNA/rDNA sequences, and those available from previously sequenced pure cultures, recurred in subsequent investigations of marine environments (Fuhrman *et al.*, 1993).

Novel archaeal groups have also been detected, including clones related to the methanogens in marine samples (De Long, 1992), numerous new lineages from thermal spring samples (Barns *et al.*, 1994) and the Antarctic marine environment (De Long *et al.*, 1994).

The 16S rRNA/rDNA techniques that were first applied successfully to aqueous samples have been subsequently employed in the investigation of other environments. The first molecular ecology study of the terrestrial environment was performed on a forest soil from Australia (Liesack & Stackebrandt, 1992a, b; Stackebrandt *et al.*, 1993). Among the findings of these investigations was the recovery of cloned sequences clustering within the radiation of the planctomycetes, and a novel clone group (Cluster III) sharing common ancestry with members of the planctomycetes and the chlamydiae. A third group were unexpectedly found to be deep-branching members

of the order Actinomycetales, some of which were related to the iron-oxidizing strain TH3.

The most striking outcome of this study, as with the aquatic environments described above, was the recovery of sequences with only distant phylogenetic relationships to cultured micro-organisms. These findings have stimulated debate as to whether the cloned sequences represent so-called 'unculturable' or 'uncultured' organisms, or whether their apparent novelty is due to the fact that the 16S rRNA/DNA sequences of many cultivated organisms are not available for comparison (Fuhrman *et al.*, 1994; Ward *et al.*, 1995). Given that the diversity of uncultured micro-organisms is considered to exceed that of cultured microbes by an order of magnitude, it is unlikely that sequences of cultured but as yet unsequenced strains will match closely those of environmental clones.

Here we demonstrate the relationship of the Cluster III 16S rDNA clones, previously thought to be members of a novel line of descent (Liesack & Stackebrandt, 1992b), to *Verrucomicrobium spinosum*, an organism for which full sequence data was not previously available.

METHODS

Verrucomicrobium spinosum strain 145^T (IFAM 1439, DSM 4136) was cultivated on agar-solidified medium M13 (Schlesner, 1986). The extraction of genomic DNA and the amplification of 16S rDNA were performed as described previously (Rainey *et al.*, 1992). The PCR product was purified using the Prep-A-Gene kit (Bio-Rad) according to the manufacturer's instructions. The Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) was used to directly sequence the PCR product, following the protocol provided by the manufacturer. The sequence reactions were run on the Applied Biosystems 373A DNA sequencer.

The 16S rDNA sequence of *V. spinosum* was aligned against a set of sequences of representatives of the main lines of descent within the domain Bacteria, and the clone sequences of Cluster III (Liesack & Stackebrandt, 1992b). Pairwise evolutionary distances were computed using the correction of Jukes & Cantor (1969). The least-squares distance method of De Soete (1983) was used in the construction of phylogenetic dendrograms from distance matrices. Phylogenetic analysis was also performed using the programs contained in the PHYLIP package (Felsenstein, 1993). Bootstrap values, which were based on the analysis of 1000 trees of 600 polymorphic sites, were calculated using the programs NJFIND and NJBOOT.

RESULTS AND DISCUSSION

The genus *Verrucomicrobium*, containing the species *V. spinosum*, was described for a group of strains isolated from the Vollstedter See, a small eutrophic lake in Holstein, Germany (Schlesner, 1987). The strains are prosthecate, but differ from other prosthecate organisms in being fimbriate (the fimbriae originating from the tips of the prosthecae), and in DNA base composition.

The almost complete 16S rDNA sequence (1492 nucleotides) of *V. spinosum* was determined in this study. In a previous phylogenetic study (Albrecht *et al.*, 1987), analysis was based on the 16S rRNA catalogue, and 515

Fig. 1. Phylogenetic dendrogram indicating the position of *Verrucomicrobium spinosum* as a distinct lineage within the domain Bacteria, and demonstrating its relatedness to environmental clone sequences. The root was determined by the inclusion of the sequence of *Thermotoga maritima*. The scale bar represents 10 inferred nucleotide substitutions per 100 nucleotides.

nucleotides obtained by 16S rRNA reverse transcriptase sequencing. The authors reported difficulties in determining sequence of the 5' and 3' regions of the molecule, due to sequence ambiguities at many positions, arising from fragmented rRNA or highly stabilized rRNA secondary structure. We did not encounter sequence ambiguities, probably because the direct sequencing of the PCR-amplified 16S rRNA gene circumvents the problem of secondary structure. The availability of the almost complete sequence allowed a more accurate determination of the phylogenetic position of *V. spinosum*. The phylogenetic position of *V. spinosum* as a distinct lineage within the domain Bacteria is shown in Fig. 1. The *V. spinosum* lineage would seem to share a common branching position with the planctomycetes and chlamydia lines of descent, but bootstrap values determined for these branching points were found to be insignificant.

Comparison of the *V. spinosum* 16S rDNA sequence with all available 16S rDNA sequences revealed a relationship to the Cluster III 16S rDNA clone sequences previously recovered from DNA that was isolated from an Australian

Table 1. 16S rDNA similarity values between Cluster III clones, *Verrucomicrobium spinosum*, and *Pirellula staley*

Strain	1.	2.	3.	4.	5.	6.
1. Clone MC15	—					
2. Clone MC18	98.2	—				
3. Clone MC17	95.2	93.9	—			
4. Clone MC31	91.8	91.3	91.4	—		
5. <i>Verrucomicrobium spinosum</i>	86.2	86.5	86.1	86.5	—	
6. <i>Pirellula staley</i>	73.0	73.2	73.7	72.7	72.0	—

terrestrial soil. The 16S rDNA sequences of the Cluster III clones were more highly related to *V. spinosum* than to any members of other lineages within the domain Bacteria.

16S rDNA sequence similarity values between the clones of Cluster III and *V. spinosum* ranged from 86.1 to 86.5% (Table 1). In the analysis described here, the Cluster III clones were shown to have similarities of 91.3–98.2% to each other, in a similar range (89.8–97.5%) to that found by Liesack & Stackebrandt (1992b). Previous phylogenetic analysis based on a very small data set found *V. spinosum* to have highest sequence similarity to *Escherichia coli* (77.1%) (Albrecht *et al.*, 1987).

The phylogenetic relationship between *V. spinosum* and the Cluster III clones is clearly demonstrated by the topology displayed in the phylogenetic dendrogram (Fig. 1). The distant relationship of the Cluster III clones to, and possible common ancestry with, the planctomycetes and chlamydiae reported by Liesack & Stackebrandt (1992b) is confirmed, as is the relationship of *V. spinosum* to these phyla (Albrecht *et al.*, 1987). The phylogenetic depth of the *V. spinosum* group could be interpreted in two ways. Firstly, the taxa represented by the clone sequences, showing 86.1–86.5% similarity to *V. spinosum*, could be considered as species of the genus *Verrucomicrobium*; such low similarity ranges between species have been reported for other genera, for example the genus *Spirochaeta* (83–90%) (Rainey *et al.*, 1992). Alternatively, the clone sequences could represent species of a separate genus, since numerous examples exist in the literature of individual genera showing greater than 87% sequence similarity. Bootstrap analysis indicated that the *V. spinosum*/Cluster III clones group was recovered in all 1000 trees generated, thus demonstrating the phylogenetic coherence of this group. Phylogenetic analysis using other methods, e.g. neighbour joining, maximum likelihood and maximum parsimony, clearly showed the phylogenetic relatedness of the Cluster III clones to *V. spinosum*.

The 16S rDNA sequence of *V. spinosum* was examined for the presence of the signature nucleotides reported to be present in the sequences of the Cluster III clones by Liesack & Stackebrandt (1992b). All of the signatures for the Cluster III 16S rDNA clones were found in the sequence of *V. spinosum*, as were those positions shared by the Cluster III clones and *Chlamydia psittaci*. These signature nucleotides support the phylogenetic relation-

ship of Cluster III and *V. spinosum*, and the possible common ancestry of the Cluster III clones–*V. spinosum* lineage and the chlamydiae. The only exceptions were at positions 88 and 955 (*E. coli* numbering, Brosius *et al.*, 1978), due to errors in the original report (Liesack & Stackebrandt, 1992b). The nucleotide at position 570 pairs with that at position 880, not 88. At position 955, the Cluster III clones and *C. psittaci* were reported to have a U residue. This residue is in fact a C in *C. psittaci*, the Cluster III clones and *V. spinosum*, in common with the planctomycetes, and in contrast to other members of the domain Bacteria, which have a U at position 955.

The discovery that cloned species from an acid soil are related to isolates from an alkaline lake (pH 9.5) is not necessarily surprising, as the study of Liesack & Stackebrandt (1992b) also demonstrated the presence in soil of planctomycete sequences, while all pure cultures of planctomycete species and all as-yet uncultured planctomycete species have been isolated from, or observed in, only aquatic samples. Liesack & Stackebrandt (1992b) pointed out that growth requirements within the planctomycetes vary widely, and that the four planctomycete genera have not been found together in the same habitat. Therefore, it is also possible that the phylogenetic group presently represented by *V. spinosum* and the Cluster III 16S rDNA clone sequences contains taxa of widely differing physiological types living in diverse environments.

The results of this study demonstrate the potentially useful information that resides in already cultured organisms for which either no 16S rRNA/rDNA sequence, or only partial sequence, has been determined. This information, if available, would be a valuable resource in comparative analysis of cloned environmental 16S rDNA sequences, and would probably allow a number of so-called 'unculturable/uncultured' bacterial species to be assigned to existing lineages. The generation of sequence data from cultured organisms is therefore a prerequisite for a more realistic interpretation of microbial diversity. In order to build up an overview of microbial diversity from all studied environments, it is paramount that previously determined clone sequences are included in phylogenetic analyses of new cultured micro-organisms.

DESCRIPTION OF NEW TAXA

Verrucomicrobiales Ward-Rainey, Rainey, Schlesner and Stackebrandt (ord. nov.)

ver.ru'co.mi.cro.bi.a'les. *Verrucomicrobiaceae* type family of the order, -ales ending to denote an order, *Verrucomicrobiales* the *Verrucomicrobiaceae* order. The description is the same as for the type family.

Verrucomicrobiaceae Ward-Rainey, Rainey, Schlesner and Stackebrandt (fam. nov.)

ver.ru'co.mi.cro.bi.a'ce.ae. *Verrucomicrobium* type genus of the family, -eae ending to denote a family, *Verrucomicrobiaceae* the *Verrucomicrobium* family. The descrip-

tion is based on that of the genus *Verrucomicrobium* (Schlesner, 1987).

Unicellular, Gram-negative bacteria with numerous fimbriate prosthecae extending in all directions from the cell surface. Isolated from aquatic environments and found as 16S rDNA clone sequences in soil. Phylogenetically, represents a phylum of the domain Bacteria. The presence of signature nucleotides in the 16S rDNA sequences of the type species of the type genus of the family, and of uncultured representatives of the family from soil, define this family phylogenetically. DNA base composition 57.9–59.3 mol% G + C (one species).

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CORRIGENDUM

Cholera toxin (CTX) genetic element in *Vibrio cholerae* O139

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Microbiology (1995), **141**, 1977–1983

p. 1978, Table 1:

MO1	Clinical, Madras, India	O139, CT ⁺
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should read

MO1	Clinical, Madras, India	O1, El Tor, CT⁺
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