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Wide bacterial diversity associated with tubes of the vent worm *Riftia pachyptila*

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Summary

We carried out a 16S rDNA-based molecular survey of the prokaryotic diversity associated with the chitin tubes of the giant vent tubeworm *Riftia pachyptila* (collected at the East Pacific Rise, 9°N and 13°N). Scanning electron microscopy showed dense microbial populations, particularly on the external surface of the middle and upper tube regions, which included very diverse prokaryotic morphotypes. We used archaeal- and bacterial-specific primers for polymerase chain reaction (PCR) amplification, but only bacterial amplicons were obtained. We analysed a total of 87 clones. Most belonged to the ε-Proteobacteria, but also to the δ-, α- and γ-Proteobacteria. A broad diversity of phylotypes belonging to other bacterial divisions was detected, including Verrucomicrobia, the *Cytophaga–Flavobacterium–Bacteroides* group and the candidate division OP8. We also retrieved a sequence, R76-B150, of uncertain phylogenetic affiliation, which could represent a novel candidate division. The sequence of the *R. pachyptila* γ-proteobacterial endosymbiont was not detected. The bacterial diversity found suggests that complex metabolic interactions, particularly based on sulphur chemistry, may be occurring in different microniches of the *R. pachyptila* tubes.

Introduction

Deep-sea hydrothermal vents are among the most studied extreme environments on earth. In addition to the high pressure inherent to the deep ocean, hydrothermal fluids are hot (up to ≈ 350°C in black smokers) and enriched in reduced chemical species. Steep thermal and chemical gradients are established with the surrounding cold (≈ 2°C on average) and highly oxygenated deep-sea waters. These environments host the most hyperthermophilic prokaryotes known to date, which have been studied extensively by both classical (isolation and cultivation) and molecular (16S rRNA-based) methods (for a review, see Stetter, 1996; Jeanthon, 2000; Reysenbach and Cady, 2001). Microbial communities associated with hydrothermal vents are largely composed of chemoheterotrophs or chemoautotrophs that exploit the reduced fluid components as an energy source (Stetter, 1996). They are at the base of a light-independent ecosystem and sustain flourishing animal communities. Diverse endemic animal species are directly dependent on bacterial chemoautotrophic endosymbionts for growth (Rau and Hedges, 1979; Cavanaugh *et al*., 1981; Felbeck, 1981; Felbeck and Jarchow, 1998). Several metazoans are known to carry bacterial epibionts. Among these are the tubeworm *Alvinella pompejana* (Haddad *et al*., 1995; Campbell and Cary, 2001) and the shrimp *Rimicaris exoculata* (Polz and Cavanaugh, 1995). Tubes from vent tubeworms also offer suitable colonization surfaces for microorganisms (Gaill and Hunt, 1991). Thus, recent enrichment cultures showed the presence of iron and sulphur reducers in the tubes of *A. pompejana* (Campbell *et al*., 2001; Slobodkin *et al*., 2001). Many of these epibionts belong to the ε-Proteobacteria, which appear to be very abundant and diverse at deep vent sites (Longnecker and Reysenbach, 2001).

Riftia pachyptila is a giant vestimentiferan tubeworm endemic to deep-sea hydrothermal vent areas in the Pacific. It is a gutless species hosting a specific endosymbiotic chemolithoautotrophic γ-Proteobacterium (Cavanaugh *et al*., 1981; Felbeck, 1981; Distel *et al*., 1988; Cary *et al*., 1993) in a specialized organ, the trophosome. The *R. pachyptila* endosymbiont has raised considerable attention. In contrast to other symbionts coevolving with their hosts (Peek *et al*., 1998), there is compelling evidence that the *R. pachyptila* endosymbiont is newly acquired by each generation (Cary *et al*., 1993; Laue and Nelson, 1997). The existence of a motile free-living stage is further supported by the presence of flagellin genes, although it has never been detected in the hydrothermal vent environment (Millikan *et al*., 1999). One intriguing possibility is that the symbiont also exists in the *R. pachyptila* chitin tube, as a chitinase gene has been identified in the genome sequence of

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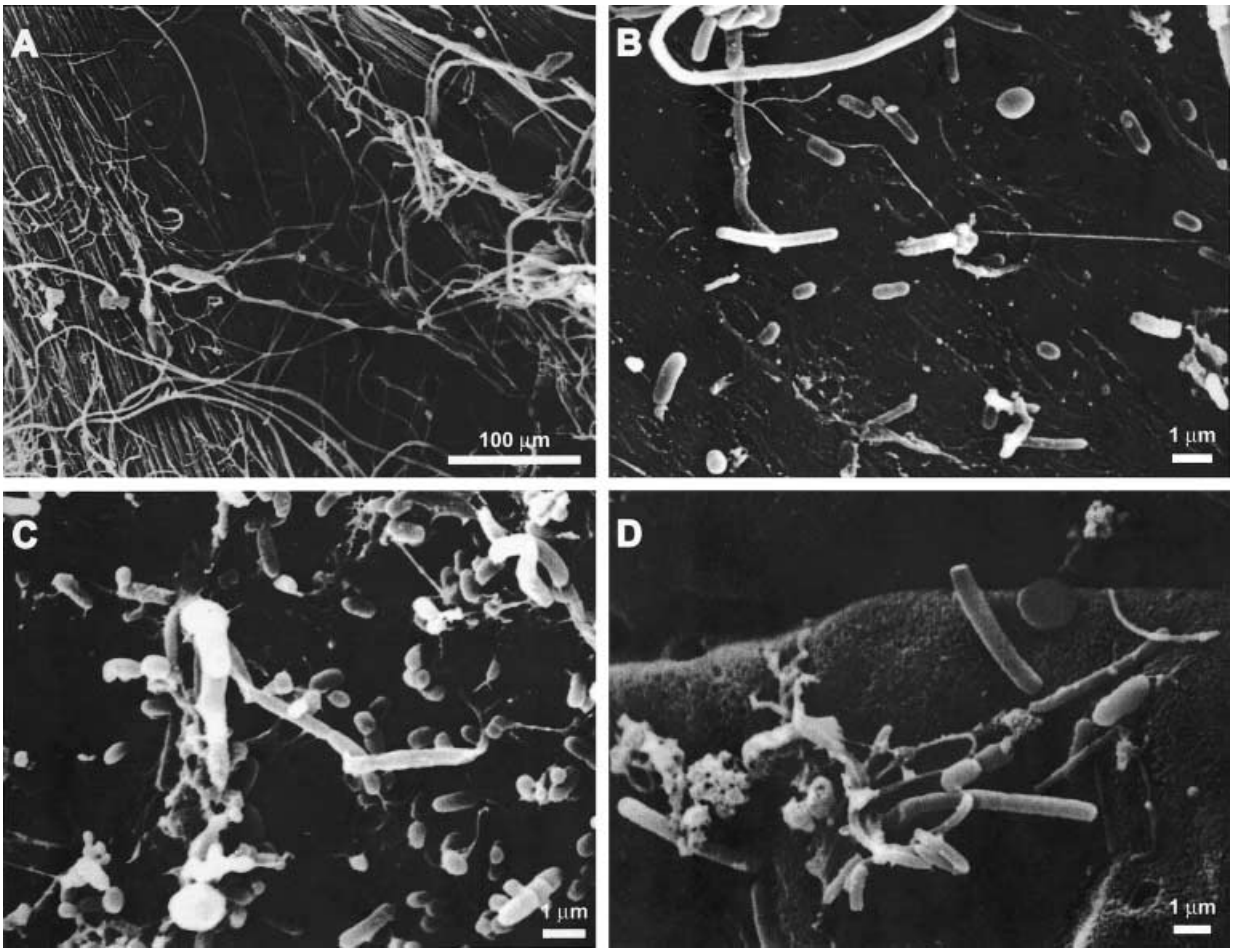

Fig. 1. Scanning electron micrographs of the outer surface of adult *Riftia pachyptila* tubes.
A. Basal, fibrous, part of the tube.
B–D. Middle part of the tube showing a variety of prokaryotic morphotypes.

the endosymbiont (H. Felbeck, personal communication). As stated above, *R. pachyptila* tubes were a priori appropriate colonization substrates. However, the microbial diversity potentially associated with *R. pachyptila* tubes has not been assessed. Consequently, the aim of our work was to carry out a 16S rRNA-based molecular survey of the bacterial diversity inhabiting these chitin tubes, which could also lead, eventually, to the detection of the endosymbiont there. In our study, we found a large diversity of bacterial sequences, but were not able to detect the endosymbiont sequence. Most of the phylotypes identified corresponded to ε-Proteobacteria, but also to α-, δ- and γ-Proteobacteria, the *Cytophaga–Flavobacterium–Bacteroides* (CFB) group, Verrucomicrobia and the candidate division OP8. One sequence did not branch close to any of the known bacterial divisions and might represent a novel candidate division.

Results

Scanning electron microscopy of the R. pachyptila *tube*

We observed by scanning electron microscopy (SEM) three different parts of the tubes of two adult *R. pachyptila* specimens corresponding to the basal, middle and upper regions. These tubes were ≈ 40 cm high. Additionally, we observed the equivalent regions of a juvenile tube ≈ 13 cm high and ≈ 1 cm in diameter. All tubes were composed of several concentric chitin layers. The basal parts showed a fibrous appearance at the external surface, whereas the middle and upper regions appeared squamous with detached areas of the most external chitin layers and numerous fissures and interstices (Fig. 1). This irregular external surface seems to be favourable for the colonization of microorganisms. Indeed, we observed a variety of prokaryotic morpho-

Table 1. Phylogenetic affiliations of bacterial clones from *R. pachyptila* tubes as deduced from BLAST searches.

Clone	Sequence length (bp)	No. of similar sequences[a]	Closest 16S rDNA match in database (accession number)	Percentage identity	Phylogenetic ascription
R76-B150	1442	1	*Bacillus* sp. BD-85 (AF169519)	80	Uncertain[b]
R76-B58	1439	1	*Cytophaga fermentans* (M58766)	90	CFB group
R103-B20	1451	3	*Polaribacter filamentus* (U73726)	88	CFB group
R76-B13	808	1	*Cytophaga* sp. strain BD1-16 (AB015525)	90	CFB group
R76-B102	1475	2	Clone WFeA1-35 (AF050554)	95	OP8 candidate division
R76-B18	1492	2	Clone Sva0107a (AJ241016)	94	Verrucomicrobia[b]
R103-B35	1489	7	Clone Sva0107a (AJ241016)	95	Verrucomicrobia[b]
R76-B23	1462	1	*Thiomicrospira* sp. Milos-T2 (AJ217758)	97	Gamma Proteobacteria
R103-B53	1476	1	*Desulfobulbus rhabdoformis* (U12253)	93	Delta Proteobacteria
R103-B27	1477	6	*Desulfobulbus rhabdoformis* (U12253)	93	Delta Proteobacteria
R103-B61	1385	1	Uncultured *Roseobacter* NAC11-6 (AF245634)	94	Alpha Proteobacteria
R103-B9	1388	2	Slope strain DI4[a] (AF254104)	95	Alpha Proteobacteria
R103-B63	1432	2	*Desulphuromusa kysingii* (X79414)	94	Epsilon Proteobacteria[b]
R103-B70	1432	2	'Endosymbiotic eubacterium' (D83061)[b]	95	Epsilon Proteobacteria
R76-B30	1428	2	Uncultured hydrocarbon seep bacterium BPC056 (AF154091)	94	Epsilon Proteobacteria
R76-B151	1430	1	Uncultured hydrocarbon seep bacterium BPC056 (AF154091)	96	Epsilon Proteobacteria
R103-B37	1433	4	Uncultured hydrocarbon seep bacterium BPC056 (AF154091)	93	Epsilon Proteobacteria
R103-B18	1431	1	Benzene-mineralizing consortium clone SB-17 (AF029044)	94	Epsilon Proteobacteria
R103-B22	1431	5	Benzene-mineralizing consortium clone SB-17 (AF029044)	95	Epsilon Proteobacteria
R76-B78	1429	1	Uncultured hydrocarbon seep bacterium BPC056 (AF154091)	97	Epsilon Proteobacteria
R103-B15	1429	2	Clone BD2-1 (AB015531)	96	Epsilon Proteobacteria
R76-B47	1428	32	Clone BD2-1 (AB015531)	96	Epsilon Proteobacteria
R103-B76	1432	3	Uncultured hydrocarbon seep bacterium BPC056 (AF154091)	96	Epsilon Proteobacteria
R76-B61	1429	1	Clone BD7-6 (AB015582)	94	Epsilon Proteobacteria
R76-B12	797	2	Clone NKB-10 (AB013262)	96	Epsilon Proteobacteria

R76 and R103 indicate clones from two worm tubes collected at 13°N and 9°N in the East Pacific Rise respectively.
a. Sequences 98–100% identical, including partial sequences.
b. See text for details.

types colonizing it. The highest microbial density was observed at the middle and, to a lesser extent, at the apical tube outer surfaces (Fig. 1). The basal outer surface was also colonized, but microbial density appeared to be much lower. In contrast, the inner part of the tube wall was much more uniform and smooth in all cases and was completely devoid of microorganisms (not shown). The juvenile tube was thinner. It showed similar trends to the adult tubes regarding structural appearance and microbial distribution, except for a better preservation of the external chitin layers and the fact that microbial colonization was less important. Among the prokaryotic morphologies observed in *R. pachyptila* tubes, we identified cocci of very different diameters, small and large rods and multicellular filamentous bacteria (Fig. 1). Some cells appeared to display filamentous structures for attachment.

Initial bacterial 16S rRNA-based diversity and rarefaction analyses

We extracted total DNA from the middle part of the tubes of two different *R pachyptila* adult specimens coming from 13°N and 9°N at the East Pacific Rise, samples R76 and R103 respectively. DNA was extracted from the external chitin layers (≈ 0.3–0.5 mm thick). Our choice was influenced by previous SEM observations showing that the middle part of the tube was richer in epibionts and that the internal surface lacked microorganisms.

We carried out polymerase chain reaction (PCR) amplification assays using 16S rDNA primers specific for bacteria and archaea (see *Experimental procedures*). We failed to obtain archaeal amplification products under various PCR conditions. Bacterial amplification products were obtained from the two *R. pachyptila* tubes. These were cloned, generating two different libraries. A selection of 87 clone inserts from these libraries (48 clones from R76 and 39 clones from R103) was partially sequenced. Partial sequences (≥750 bp) were compared by BLAST with sequences existing in the databases. Most of the sequences from the two libraries matched to ε-Proteobacteria (68%), several matched to other Proteobacteria of the α (3%), γ (1%) and δ (8%) subdivisions, to the CFB group (7%) and to the OP8 candidate division (2%) (see Table 1 and Fig. 2). A considerable proportion (11%) of clones first matched to the 'uncultured *Planctomyces'* clone Sva017a (Ravenschlag *et al.*, 1999) (Table 1). However, subsequent phylogenetic analyses showed a clear affiliation of this group of sequences with the Verrucomicrobia (see below). Finally, one sequence

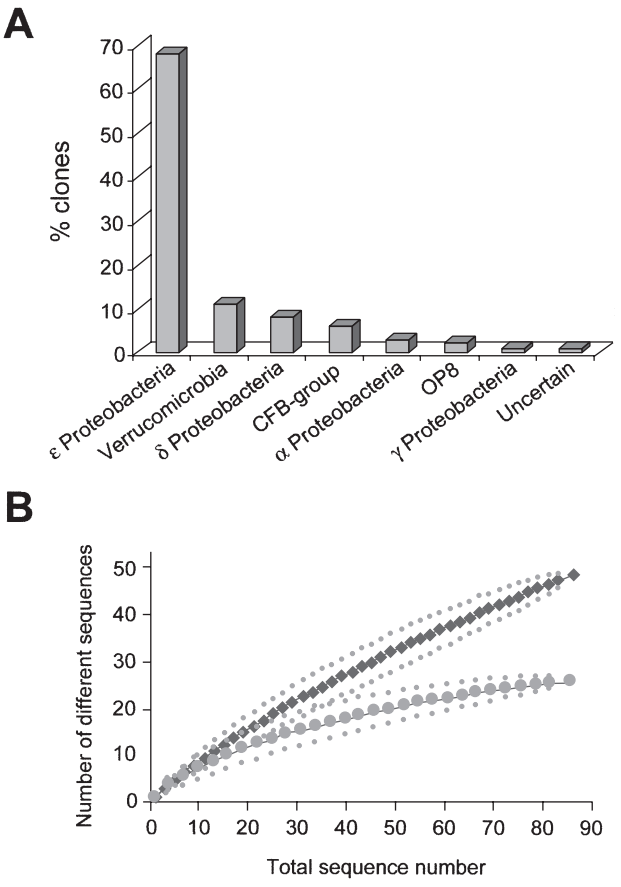

Fig. 2. Quantification of the diversity found in two 16S rDNA bacterial libraries from *R. pachyptila* tubes.
A. Histogram showing the proportions of clones belonging to different bacterial phylogenetic groups.
B. Rarefaction curves for the bacterial phylotypes analysed at the 'strain level' (all sequences with more than one substitution were considered different, closed diamonds) and at 'species level' (only those sequences diverging by more than 2% were considered different, closed circles). 95% confidence intervals are indicated for both curves with smaller diamonds and circles respectively.

showed no clear close match, BLAST being able to align only 80% of the sequence to a group of environmental *Bacillus* sp. clones (Table 1).

We applied rarefaction analysis to our sequences to see if we were far from describing the bacterial diversity present in our clone libraries. We used two different criteria to construct rarefaction curves. When we considered as different all sequences with more than one substitution ('strain level' criterion), the curve did not reach a plateau (Fig. 2). However, when we considered as different only those sequences diverging by more than 2% ('species level' criterion), the curve was significantly closer to the saturation plateau (Fig. 2). This indicated that an important fraction of the diversity found corresponded to groups of phylotypes with small sequence variability. In fact, a preliminary phylogenetic analysis of the 87 sequences carried out by distance (neighbour-joining, NJ) methods allowed us to identify several groups of identical or very closely related sequences (not shown). This preliminary analysis was also used to select 40 clones for complete sequencing, including representatives of different bacterial phylogenetic groups and also moderately close sequences within the same phylogenetic group.

Phylogenetic analyses and novel bacterial groups

As our libraries contained very broad bacterial diversity, we carried out phylogenetic analyses of two complementary data sets in order to include an adequate taxonomic sampling compatible with the most time-consuming phylogenetic methods [in particular, the maximum likelihood (ML) analyses]. The first contained the complete sequences of our proteobacterial clones, as well as their closest relatives and sequences representative of the different proteobacterial subdivisions (Fig. 3). As already deduced from BLAST searches, the vast majority of our proteobacterial phylotypes belonged to the ε subdivision. Across this group, a large diversity was observed, although none of our sequences was closely related to other vent animal epibionts, including those from *R. exoculata* (Polz and Cavanaugh, 1995) and the tube worm *A. pompejana* (Haddad *et al*., 1995). Remarkably, the ε-proteobacterial strains isolated from *A. pompejana* tubes ('Am-H' and '18.2') (Campbell *et al*., 2001) branch very distantly from our sequences (Fig. 3). We found a group with a strong statistical support [bootstrap proportion (BP) of 96%] that included 11 of our phylotypes and the environmental clones NKB8 and BD2-1, both retrieved from deep-sea sediments close to Japan (Li *et al*., 1999a,b). Most of our partial sequences also belong to this group (Table 1). Our remaining complete ε-proteobacterial sequences were mostly interspersed with other environmental phylotypes retrieved from deep-sea sediments in the Nankai Trough (NKB10), an *in situ* growth chamber deployed at a mid-Atlantic ridge hydrothermal vent (VC2.1), and a benzene-mineralizing bacterial consortium (SB-17) (Phelps *et al*., 1998; Li *et al*., 1999a; Reysenbach *et al*., 2000). One sequence (R103-B70) belonged to the *Arcobacter* group, and was closely related to a sequence referred as to 'endosymbiotic eubacterium' by Naganuma *et al*. (1997), who thought it to be the trophosome endosymbiont of *R. pachyptila*. However, it has been extensively shown that the *R. pachyptila* endosymbiont is a single organism belonging to the γ-Proteobacteria (Cary *et al*., 1993). This suggests that these authors had a contamination, probably from the worm tube. We found an additional ε-proteobacterial sequence (R103-B63) that branched close to the base of this subdivision, after the emergence of *Desulphurella propionica* and the *A. pompejana* tube strains. Interestingly, the BLAST search

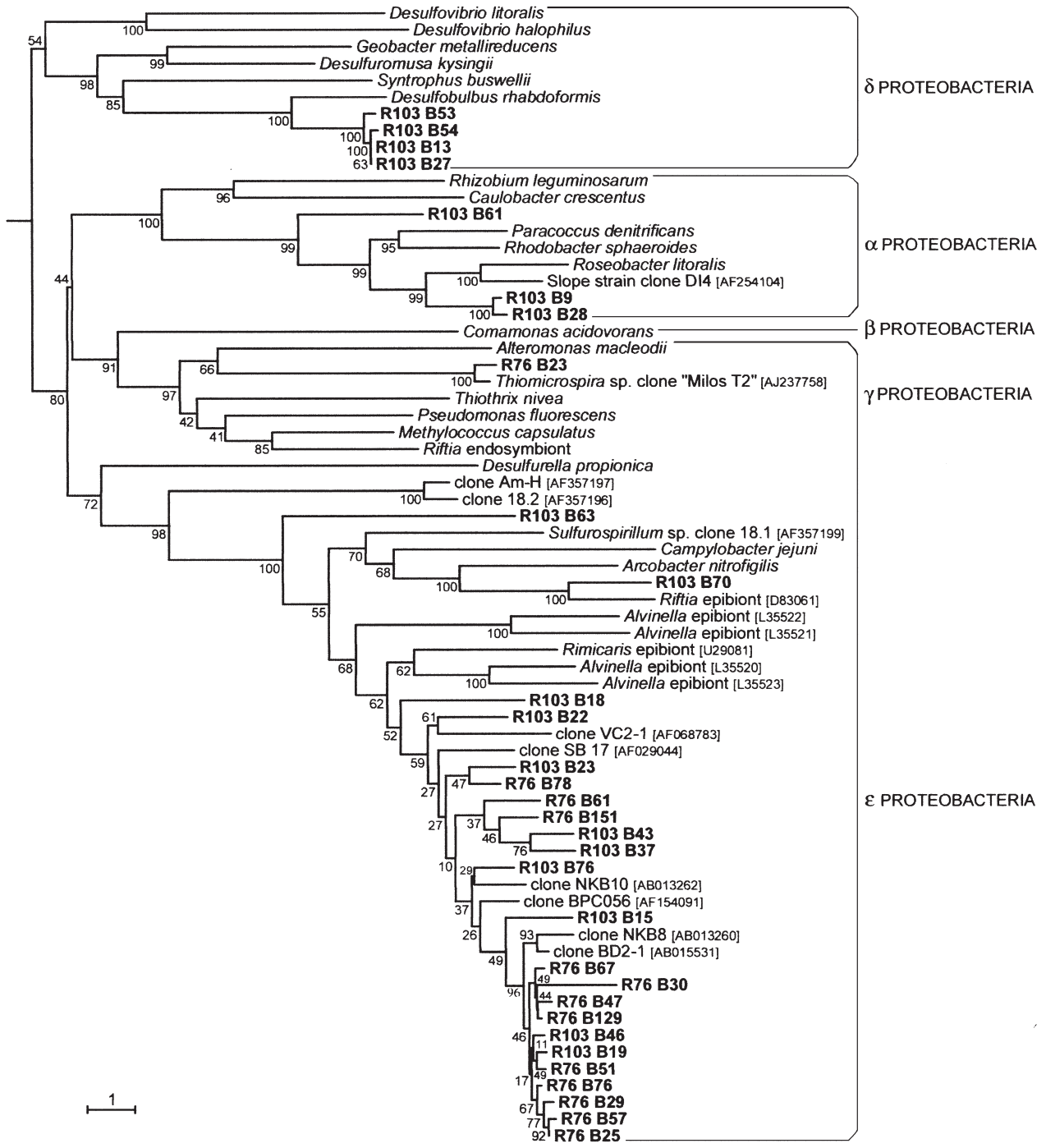

Fig. 3. Maximum likelihood (ML) phylogenetic tree of the proteobacterial lineages detected in *R. pachyptila* tubes. A total of 1210 unambiguously aligned positions were used. Numbers at nodes are ML bootstrap values. Accession numbers for environmental sequences retrieved from databases are indicated in brackets. The tree is rooted with the sequences of the Gram-positives *Acidimicrobium ferrooxidans*, *Clostridium litorale* and *Micrococcus luteus*, and the cyanobacteria *Nostoc muscorum* and *Synechococcus elongatus* as the outgroup (not shown). The scale bar represents one substitution per 100 positions for a unit branch length.

retrieved several δ-Proteobacteria as the best matches for this phylotype (Table 1), although our phylogenetic analysis consistently clustered it with the ε-Proteobacteria (Fig. 3).

We did find true members of the δ subdivision, which represented the next most abundant group of Proteobacteria in our libraries (Fig. 2). All our δ-proteobacterial phylotypes form a compact cluster (BP of 100%) related to

the sulphate reducer *Desulfobulbus rhabdoformis* (Fig. 3). Although only 3% of our library clones were affiliated to the α-Proteobacteria, these were comparatively more diverse. Two of these sequences, R103-B9 and R103-B28, were closely related. They shared as δ closest relative the strain DI4. It is a thiosulphate-oxidizing bacterium that was isolated from a continental slope sediment collected off the coast of New England (Teske *et al*., 2000). The other α-proteobacterial phylotype, R103-B61, did not have very close relatives, showing 94% BLAST identity with several *Roseobacter* strains (Table 1 and Fig. 3). Only one γ-proteobacterial phylotype was identified, clone R76-B23. It was closely related to the thiosulphate oxidizer strain *Thiomicrospira* sp. Milos, isolated from shallow hydrothermal vent fields (Brinkhoff *et al*., 1999), and far from the γ-proteobacterial *R. pachyptila* endosymbiont (Fig. 3). β-Proteobacterial phylotypes were not detected in our libraries.

The second data set contained all our complete sequences that did not belong to the Proteobacteria, their closest matches as identified by BLAST searches and representatives of various bacterial divisions (Fig. 4). The diversity of these non-proteobacterial phylotypes was scattered across a broad spectrum of bacterial groups. The most abundant clade corresponded to a group of sequences closely related to the '*Planctomyces*-like' environmental clone Sva0170a (Ravenschlag *et al*., 1999). However, our ML phylogenetic analysis strongly supported that Sva0170a and our clones are closer to the Verrucomicrobia (BP of 100%) instead of the Planctomycetales. Thus, in addition to clone Sva0170a, the closest relative to our sequences in the bacterial alignment (see *Experimental procedures*) was the environmental clone WCHB1-41, retrieved from a hydrocarbon and chlorinated solvent-contaminated aquifer (Dojka *et al*., 1998), and other Verrucomicrobia (Fig. 4). In addition to these Verrucomicrobia-related phylotypes, we identified a member of the Flavobacteria, R103-B20 (BP of 100%) and two members of the Cytophagales, R76-B58 and R76-B13 (BP of 100%, R76-B13 was only partially sequenced and was not included in the tree in Fig. 4). We identified a putative member of the OP8 candidate division (BP of 92%), the closest relative of which was the environmental clone WFeA1-35 (not included in the tree because it is partial), retrieved from an iron/sulphate-reducing zone in a contaminated aquifer (Dojka *et al*., 1998). One clone, R76-B150, had no close relatives in the databases. Its best BLAST match, *Bacillus* sp. BD-85, displayed only 80% sequence identity (Table 1). Our phylogenetic analysis confirmed this preliminary observation, as the clone R76-B150 appeared to be distant from all other bacterial groups, and its position in the tree was only marginally supported (BP of 52% for its relationship with a clade encompassing the green sulphur bacteria and the CFB group).

Discussion

Chitin tubes from the giant vent tubeworm *R. pachyptila* offer *a priori* favourable surfaces for diverse microbial colonization, as they are exposed to energy-rich complex chemical-mixing media, where reduced chemical species from the hydrothermal fluids mix with highly oxygenated cold deep-sea water. In fact, *R. pachyptila*, like several other vent animals, rely directly on sulphide-oxidizing endosymbionts for growth, which make use of both reducing sulphur species and dissolved oxygen (Cavanaugh *et al*., 1981; Felbeck, 1981; Distel *et al*., 1988). In our work, we have confirmed this surmise by direct scanning electron microscopy (SEM) observation and molecular 16S rRNA-based surveys of the bacterial diversity associated with *R. pachyptila* tubes. Only the external surface appeared to be colonized by microorganisms according to SEM analyses, and these were mostly distributed in the less fibrous middle and upper regions of the tube (Fig. 1). Several microbial morphotypes were observed, which already suggested the presence of different lineages. This was subsequently shown by the analysis of two bacterial 16S rDNA libraries constructed from DNA extracted from the outer tube layers of two *R. pachyptila* specimens from the East Pacific Rise at 9°N and 13°N respectively.

Phylogenetic bacterial diversity

The most abundant clones in both libraries corresponded to the ε-Proteobacteria (Table 1, Figs 2 and 3). This is in accordance with previous findings suggesting the predominance of ε-Proteobacteria in moderately hot to warm areas of vent regions. They appear to colonize all available surfaces around hydrothermal vents, having been identified in mats (Moyer *et al*., 1995), artificial colonizing substrates (Reysenbach *et al*., 2000), the *A. pompejana* tube (Campbell *et al*., 2001) and as epibionts of *A. pompejana* and *R. exoculata* (Haddad *et al*., 1995; Polz and Cavanaugh, 1995). Not only were our *R. pachyptila* tube phylotypes very abundant in the libraries, but they were also surprisingly diverse. They form at least eight groups at species or even genus difference level, widely scattered among the ε-Proteobacteria (Fig. 3). However, none was closely related to any of the described ε-proteobacterial epibionts in *Rimicaris* and *Alvinella* spp. or in the tube of the latter. On the contrary, most of our sequences were related to deep-sea sediment phylotypes. This may result in part from differences in the physico-chemical conditions at the *R. pachyptila* tubes, as the *A. pompejana* tubes are exposed to much higher temperatures and a reducing environment, living closer to the hydrothermal fluid. This also indicates that ε-Proteobacteria have been remarkably successful in colonizing different surfaces along the thermal and chemical

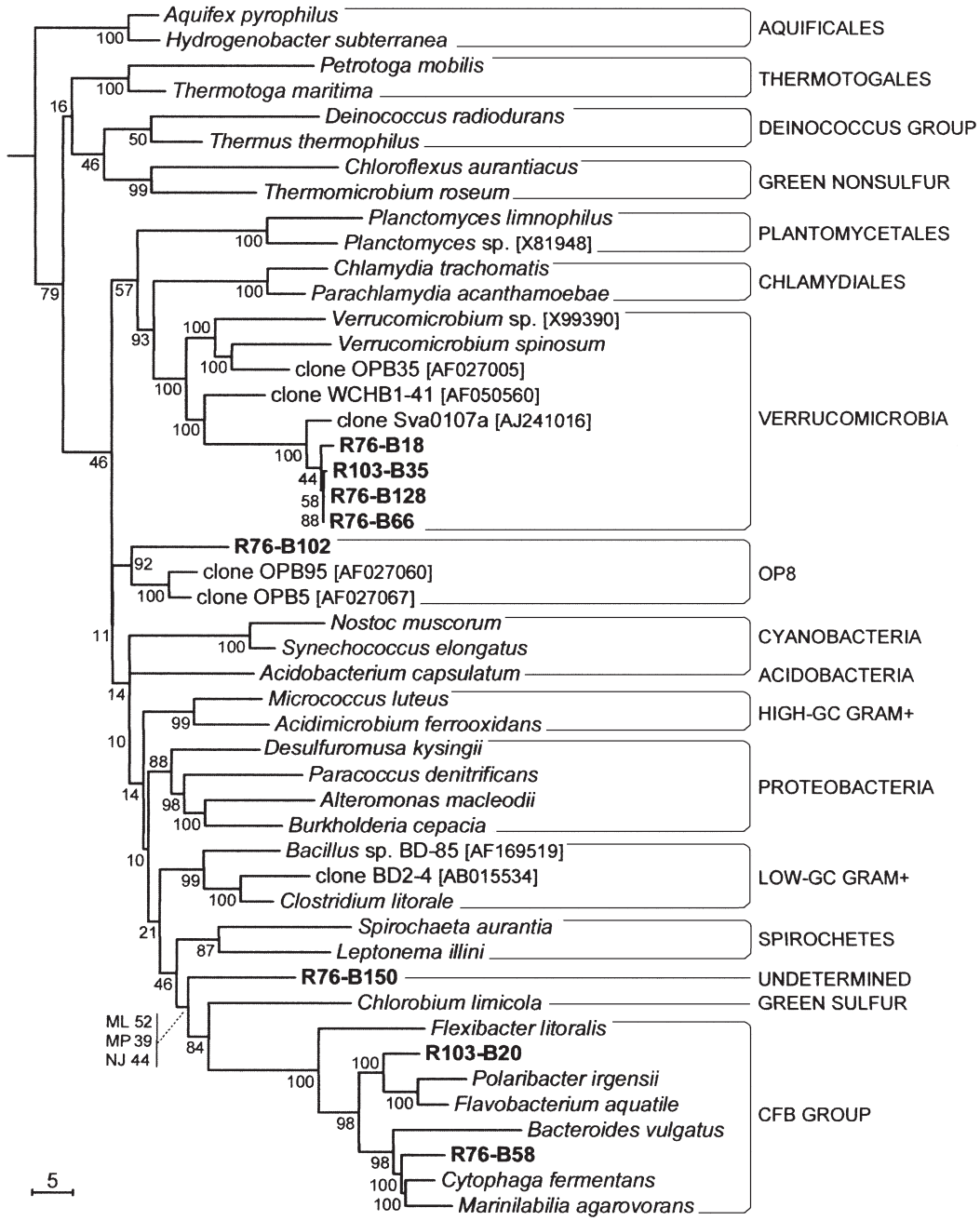

Fig. 4. Maximum likelihood (ML) phylogenetic tree of bacterial lineages other than Proteobacteria detected in *R. pachyptila* tubes. A total of 1130 unambiguously aligned positions were used. Numbers at nodes are ML bootstrap values. For the deeply divergent clone R76-B150, bootstrap values for neighbour-joining (NJ) and maximum parsimony (MP) analyses are also shown. Accession numbers for environmental sequences retrieved from databases are indicated in brackets. The tree is rooted with the sequences of the archaea *Methanothermus fervidus*, *Thermococcus hydrothermalis*, *Pyrodictium occultum* and *Thermophilum pendens* as the outgroup (not shown). The scale bar represents five substitutions per 100 positions for a unit branch length.

gradient, from the more thermophilic areas close to chimneys to cold, deep-sea sediments. Our δ-Proteobacteria, which formed a compact cluster, and the much more diverse α-Proteobacteria were not particularly related to hydrothermal vent sequences, but several of these were related to deep-sea and continental slope strains (Teske *et al.*, 2000). Despite the fact that γ-Proteobacteria are common in deep-sea plankton (Fuhrman and Davis, 1997), only one of our clones belongs to this subdivision. It is related to other phylotypes retrieved from hydrothermal environments, but it is very distant from the *R. pachyptila* γ-proteobacterial endosymbiont.

We also found a wide diversity of phylotypes ascribing to other bacterial divisions. An interesting group of sequences form a compact cluster related to the Verrucomicrobia, although quite far from any of its known members (Fig. 4). Verrucomicrobia are a recently recognized bacterial division (Liesak and Stackebrandt, 1992; Ward-Rainey *et al*., 1995). They include heterotrophic, aerobic and anaerobic members that have been found in forest and paddy soils, freshwater and sulphide-rich marine coastal sediments (Liesak and Stackebrandt, 1992; Jansen *et al*., 1997; O'Farrell and Janssen, 1999; Tanner *et al*., 2000). They have also been detected in the anoxic Cariaco basin, at 500 m depth (Madrid *et al*., 2001). This is the first time that members of this division have been detected in deep-sea hydrothermal areas. Our Verrucomicrobia sequences are very closely related to the environmental clone Sva0170a recovered from Arctic coastal sediments (Ravenschlag *et al*., 1999). We also detected several members of the CFB group, including one *Flavobacterium*-like and two *Cytophaga*-like phylotypes. Members of the CFB group are widespread and diverse. They are very abundant in marine waters (Glöckner *et al*., 1999) and sediments (Ravenschlag *et al*., 2001) and have been identified in many different environments, including deep-sea sediments (Teske *et al*., 2000). They have even been detected as part of the epibiotic community on a marine nematode (Polz *et al*., 1999). Our phylotypes, however, are distantly related to other CFB sequences. We also detected a distant member of the candidate division OP8, with no cultivated representatives. This group was first detected in a Yellowstone hot spring, the Obsidian Pool (Hugenholtz *et al*., 1998), but was later identified in non-thermophilic environments including contaminated aquifers and the anoxic waters of the Cariaco basin (Dojka *et al*., 1998; Madrid *et al*., 2001). One interesting finding corresponds to the clone R76-B150, which is not related to any bacterial lineage in the databases. Not only is the statistical support of this sequence in the tree very low, but it is also very unstable, changing its position depending on the kind of phylogenetic analysis performed and on the taxonomic sampling used. R76-B150 most probably represents the first member of a novel candidate division.

Metabolic hypotheses

Although we do not have direct evidence for the type of metabolism displayed by the microorganisms detected in the *R. pachyptila* chitin tube, the physiology of some of them can be reasonably hypothesized from their phylogenetic association with bacterial groups with consistent phenotypes. Most δ-Proteobacteria are sulphate reducers, reducing sulphate under strict anoxic conditions using H_2 or a variety of organic molecules as electron donors (Madigan *et al*., 1997). Thus, the group of δ-proteobacterial members identified in *R. pachyptila* tubes might be reducing sulphate in anoxic parts of the tube. Indeed, oxygen concentration must vary importantly in different tube microniches, evolving from the oxygenated external surface to microaerophilic and strictly anaerobic conditions towards the interior of fissures and crevices and, possibly, the internal layers of the tube. This would not be surprising, as anaerobic sulphate-reducing bacteria are present in anoxic parts of the *A. pompejana* dorsal integument, as evidenced by the presence of a variety of bisulphite reductase genes coding for the terminal redox enzyme that catalyses the reduction of sulphite to sulphide (Cottrell and Cary, 1999). The electron donors for sulphate reduction could be H_2 or small organic compounds (acetate, lactate, formate, etc.) resulting from the metabolic activity of other bacteria present in these tubes. Among these could be the Cytophagales and the Verrucomicrobia. *Cytophaga* spp. are known to degrade long polymers, being cellulolytic or even degrading chitin (Madigan *et al*., 1997). This is particularly interesting because transmission electron microscopy (TEM) has revealed the presence of colonial-like bacterial islands within the tube, which appear to be mostly located in the outer third of the tube thickness (J. P. Lechaire *et al*., personal communication). It could thus be hypothesized that the *Cytophaga*-like lineages live in the tubes degrading chitin, although they could also use long exopolymeric substances secreted by bacteria attached to the tube. This would result in the transformation of complex biopolymers to simpler organics that could then be used by fermentative bacteria. Candidate fermenters are the Verrucomicrobia lineages, as some members of this bacterial division are known to carry out fermentation (Jansen *et al*., 1997).

The ability to oxidize reduced sulphur species (sulphide, thiosulphate and elemental sulphur) seems to be a usual feature of ε-Proteobacteria. Many of the ε-Proteobacteria isolated from hydrothermal vent environments, including the *A. pompejana* tube, are chemoautotrophic or chemoheterotrophic sulphur oxidizers (Campbell *et al*., 2001). An aerobic autotrophic sulphide- oxidizing metabolism has been inferred for the epibiont of *R. exoculata* and *A. pompejana* (Polz and Cavanaugh, 1995). Hence, given the large diversity of ε-proteobacterial members identified in *R. pachyptila* tubes, it could be speculated that some of them are oxidizing sulphur and sulphide under aerobic and anaerobic conditions. Under anaerobic conditions, nitrate, manganese or iron oxides species could be used to oxidize reduced sulphur species. Fe(III)-reducing ε-Proteobacteria have been enriched from hydrothermal environments (Slobodkin *et al*., 2001). Interestingly, the *R. pachyptila* tubes are frequently covered by iron oxides. Additionally, putative thiosulphate-oxidizing bacteria could also be

present in the tubes. These could include, in addition to ε-Proteobacteria, the *Flavobacterium*-like, the α-proteobacterial and the γ-proteobacterial lineages. The last is closely related to the thiosulphate-oxidizing *Thiomicrospira* spp., and two of the α-proteobacterial phylotypes are close to a thiosulphate-oxidizing strain isolated from slope sediments (Teske *et al*., 2000). All these groups are frequent thiosulphate oxidizers in deep-sea and hydrothermal vent regions (Teske *et al*., 2000).

These observations suggest a prevalent role for sulphur in the metabolism of these bacteria. They also point to the existence of a complete or nearly complete sulphur cycle in the *R. pachyptila* tube with an important contribution from members of the Proteobacteria, although other bacterial divisions could also be involved. Further analyses will be required to ascribe the identified phylotypes to the different morphotypes observed by SEM. Fluorescent *in situ* hybridization (FISH) with specific probes should allow this, as well as making quantitative inferences. *R. pachyptila* tubes could constitute a good model to study the succession of bacteria involved in sulphur cycling in deep-sea vent-affected environments. They possibly represent an intermediate step in the gradient established between more thermophilic sulphur-dependent communities (e.g. *A. pompejana*-associated bacteria) and the cold, deep-sea sediments.

Experimental procedures

Sampling and scanning electron microscopy

The *R. pachyptila* specimens used in this study were collected in 1996 during the HOT cruise at the East Pacific Rise 13°N (sample R76) and 9°N (sample R103) at 2600 m depth. The tubes were cut into segments that were immediately frozen and stored in liquid nitrogen. Small sections of 5 mm × 5 mm were removed from the basal, middle and apical tube segments and dehydrated in increasing ethanol concentrations (50%, 70%, 90% and 100%). Samples were then critical point dried and gold coated. Observation was carried out with a Jeol (JSM 840A) scanning electron microscope (SEM) operating at 17 kV.

Nucleic acid extraction

Fragments from the middle part of the tube from two adult specimens from different geographic locations (samples R76 and R103) were used for DNA extraction. These were ≈ 2 cm long, with a lumen of ≈ 3 cm, and 2–3 mm thick. Tube fragments were thawed at room temperature. Each tube fragment was washed with 5 ml of phosphate saline buffer (130 mM NaCl, 10 mM phosphate buffer, pH 7.7, PBS) to remove possible accidentally attached deep planktonic bacteria, by repeatedly projecting (approximately 30 times) with a pipette 1 ml of this volume on the surface. Subsequently, the outer part of the tube fragments (≈ 0.3–0.5 mm thick) was peeled off on a sterile mortar, frozen in liquid nitrogen by partial immersion of the recipient and pounded. The resulting powder was recovered in Eppendorf tubes and subjected to four additional freezing–thawing cycles in liquid nitrogen. Samples were suspended in 500 μl of PBS and incubated at 37°C with 1 mg ml^{-1} lysozyme for 30 min. Then, 80 μg ml^{-1} proteinase K, 1% SDS, 1.4 M NaCl, 0.2% β-mercaptoethanol and 2% CTAB (final concentrations) were added sequentially. Lysis suspensions were incubated overnight at 55°C. Lysates were extracted twice with phenol–chloroform–isoamyl alcohol and once with chloroform–isoamyl alcohol. Nucleic acids were concentrated by ethanol precipitation.

16S ribosomal RNA gene libraries and sequencing

16S rRNA gene PCR amplification assays were carried out using specific forward primers for bacteria (B-27F, AGAGTTTGATCATGGCTCAG) and archaea (21F, TTCCG GTTGATCCTGCCGGA) and a reverse primer specific for prokaryotes (1492R, GGTTACCTTGTTACGACTT). PCRs were normally performed under the following conditions: 30 cycles (denaturation at 94°C for 15 s, annealing at 55°C for 30 s, extension at 72°C for 2 min) preceded by 2 min denaturation at 94°C and followed by 10 min extension at 72°C. Archaeal amplicons were not obtained with the primers tested, even when lower annealing temperature (50°C) and more cycles (35) were used. Two bacterial rDNA clone libraries (R76 and R103) were constructed using the Topo TA cloning system (Invitrogen) according to the manufacturer's instructions. After plating, positive transformants were screened by PCR amplification of inserts using flanking vector primers. A total of 87 expected size amplicons from both libraries was partially sequenced (Genome Express) with the primer 1492R. After preliminary phylogenetic analysis (see below), 40 clones representative of the phylogenetic diversity were chosen for complete sequencing using primer B-27F.

Phylogenetic analysis

Close relative sequences in the database were identified by BLAST (Altschul and Koonin, 1998). A preliminary phylogenetic analysis of partial sequences was done using CLUSTALX (Thompson *et al*., 1997), which allowed the identification of identical or nearly identical sequences. Complete sequences were aligned manually using the program ED from the MUST package (Philippe, 1993) with 16 407 prokaryotic 16S rDNA sequences. Most of these sequences were retrieved in an aligned format from the Ribosomal Database Project (Maidak *et al*., 2001). The rest corresponded to close relative sequences to ours, which were retrieved from GenBank and aligned manually. Clone sequences were checked by the Ribosomal Database Project CHECK_CHIMERA tool to avoid the inclusion of artifactual sequences in our analysis (Maidak *et al*., 2001). From our 40 complete sequences, one was discarded as chimerical. For our phylogenetic analyses, gaps and ambiguously aligned positions were excluded. Neighbour-joining (NJ) trees were generated using different sequence substitution models with the program NJ from the MUST package (Philippe, 1993). Maximum parsimony

(MP) trees were constructed with the program PAUP 3.1.1 (Swofford, 1993) using a heuristic search. Maximum likelihood (ML) trees were constructed with the program NUCML from the MOLPHY 2.3 package (Adachi and Hasegawa, 1996) using a heuristic quick-add OTUs search with a Tamura–Nei substitution model (Tamura and Nei, 1993). ML analyses accounting for among-site rate variation with a gamma law correction were carried out using PUZZLE (Strimmer and von Haeseler, 1996). NJ and MP bootstrap proportions were inferred using 1000 replicates. ML bootstrap proportions were estimated using the RELL method upon the 2000 top-ranking trees (Kishino and Hasegawa, 1989).

Rarefaction analysis

Rarefaction curves were produced using the program ECOSIM 6.0 (Gotelli and Entsminger, 2001) by comparison of the diversity patterns of 1000 Monte Carlo randomized pseudocommunities with those found in the real data matrix, allowing the calculation of 95% confidence intervals. Rarefaction analyses were done at the 'strain' level and at the 'species' level by considering as different all sequences with more than one substitution and sequences diverging by more than 2% respectively.

Nucleotide sequence accession numbers

The sequences reported in this study were submitted to GenBank with accession numbers AF449222 (R103-B61), AF449223 (R103-B28), AF449224 (R103-B9), AF449225 (R76-B23), AF449226 (R103-B53), AF449227 (R103-B54), AF449228 (R103-B27), AF449229 (R103-B13), AF449230 (R103-B63), AF449231 (R103-B15), AF449232 (R103-B18), AF449233 (R103-B19), AF449234 (R103-B22), AF449235 (R103-B23), AF449236 (R103-B37), AF449237 (R103-B43), AF449238 (R103-B46), AF449239 (R103-B70), AF449240 (R103-B76), AF449241 (R76-A19), AF449242 (R76-B29), AF449243 (R76-B30), AF449244 (R76-B25), AF449245 (R76-B47), AF449246 (R76-B51), AF449247 (R76-B57), AF449248 (R76-B61), AF449249 (R76-B67), AF449250 (R76-B76), AF449251 (R76-B78), AF449252 (R76-B129), AF449253 (R76-B151), AF449254 (R76-B12), AF449255 (R76-B128), AF449256 (R76-B66), AF449257 (R76-B18), AF449258 (R103-B35), AF449259 (R103-B20), AF449260 (R76-B58), AF449261 (R76-B13), AF449262 (R76-B150) and AF449263 (R76-B102).

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