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Identification of bacterial cultures from archaeological wood using molecular biological techniques

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Abstract

Anaerobic bacteria were isolated from a 1700-year-old wooden spear shaft, excavated from an archaeological site that dates from the iron age, in the southern part of Jutland, Denmark. The bacteria were cultivated in glucose- and xylose-supplemented media at 14°C and 20°C. A gene library with 21 clones was constructed by extracting and amplifying 16S rDNA sequences from the individual cultures. One clone was phylogenetically affiliated to the *Spirochaeta*. Eleven clones affiliated to an unidentified member of the *α-Proteobacteria* were present in all culture samples. Three clones were affiliated to the *β-Proteobacteria*. Four clones were clustered among the *Geobacteriaceae*, in the *δ-Proteobacteria*. A single clone was clustered with gram-positives. All the identified bacterial families are commonly found in soil or bog environments and many are able to utilize cellulose as their carbon or energy source.

Keywords: rDNA; Phylogeny; DNA

1. Introduction

Archaeological wood may survive for thousands of years despite the numerous threats of both human and natural origin (Florian, 1990). The degree of preservation of buried wooden artefacts depends upon the nature of the environment at the archaeological site. Cold, dark and near anaerobic conditions result in slower degradation (Blanchette, 2000; Kim et al., 1996; Powell et al., 2001). With the ever-increasing discovery of archaeological sites, but lack of funds for excavation and conservation, it is becoming common to consider either in situ preservation or reburial of the archaeological objects (Bergstrand, 2002; Brunning et al., 2000; Corfield, 2001; Gregory et al., 2002; Mouzouras et al., 1990; Olsson, 2001; Pournou et al., 1998). In such cases, it is necessary to ensure that further degradation is minimized, in order to preserve our cultural heritage for future generations. Active monitoring of environmental parameters is being undertaken world wide to gain further insight into factors which influence wood biodegradation (Gregory, 1998; Gregory et al., 2002; Hogan et al., 2001; Jordan, 2001; Powell et al., 2001).

One archaeological site that has received significant attention in Denmark is Nydam Mose, which is an iron age offering bog in the southern part of Jutland. It is a peat bog, where ground water has major influence on the conditions in the bog. The peat is weakly acidic, i.e. pH 6–7 (Aaby et al., 1999).

At least five or six different depositions of war offerings were made during the iron age. Analyses of the bog have shown that the first of these offerings, which was around 250 AD, was made in a fresh water lake. The youngest finds are dated at 475 AD. At that time, the lake had grown into a swampy bog in which the weapons were plunged (Rieck et al., 1999).

Excavations have been carried out in Nydam Mose since 1859. From 1992 to 1997, more than 15 000 mainly wooden objects were uncovered. There seems to be at least an equal number of artefacts still left in the bog. Since 1996, a preservation project referred to as the in situ project, around Nydam Mose has given a wealth of information. Today we know the facts about oxygen content at different ground

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levels, the peat acidity, the groundwater level and its temperature, the dissolved ions and the redox and corrosion potentials (Gregory et al., 2002). Archaeological objects, both wooden and metallic, from this site have been examined extensively Gregory, 2001). The present study concerns a piece of a spear shaft, which was one of the 400 wooden objects excavated (Aaby et al., 1999). A subset of the collected objects, which consisted mainly of spear shafts and arrows, was specifically stored for research purposes, and the rest were conserved.

The wooden objects from Nydam Mose appear visually to be intact immediately after excavation. However, when the objects were allowed to dry, they collapsed. It has been shown that the cellulose content in most of the artefacts is close to zero and that the density is only around 10% of its original value (Jensen, personal communication). The remaining material is composed almost exclusively of lignin. In the case of ash wood, the density of the archaeological objects was around 0.1 g/cm^3.

It is widely accepted, that bacteria are the main degraders of archaeological wood under near-anaerobic conditions (Björdal et al., 1999, 2000; Blanchette and Hoffmann, 1994; Blanchette, 2000; Daniel and Nilsson, 1997; Gregory et al., 2002; Kim et al., 1996; Kim and Singh, 2000; Powell et al., 2001). As it has not been possible to isolate these bacteria by conventional methods (Björdal and Nilsson, 2002; Kim and Singh, 2000; Nilsson and Daniel, 1983; Schmidt and Liese, 1994), the identification of bacteria that degrade wood under such conditions has been based mainly on the micromorphology of the decay pattern (Daniel and Nilsson, 1986; Daniel and Nilsson, 1997; Eaton and Hale, 1993; Schmidt and Liese, 1994; Singh et al., 1990; Singh and Butcher, 1991). The scope of the present work is to identify the microorganisms inhabiting archaeological wood in order to better understand the process of its degradation. Knowledge of nutritional requirements of these microorganisms and the effect of environmental factors on degradation could be used to model the degradation process as well as to identify optimal sediment or soil types for reburial sites.

To our knowledge, this paper describes the first successful cultivation of bacteria from the interior of archaeological wood. To allow cultivation of both anaerobic and facultative anaerobic bacteria, we have used strict anaerobic growth conditions and DNA-based identification of these bacteria. By extracting DNA from the cultures, followed by amplification of the 16S rDNA regions, and construction of clone libraries, six different bacteria have been identified and their full nucleotide sequences of the 16S rDNA regions have been determined and grouped phylogenetically.

2. Materials and methods

2.1. Material

The material for the present study was a piece of a spear shaft from an iron age war offering (around 300 AD) in Nydam Mose in Denmark (UTM co-ordinates are 546620/6090831 zone 32). This was one of many artefacts uncovered at a trial excavation in May 1999.

Immediately following excavation, all wooden artefacts were cleaned on site, packed in perforated polyolefin film and kept moist by covering with wet cotton textiles (Jensen et al., 1996). During the excavation period, the objects were kept in closed plastic boxes in tap water. After about 2 weeks, the boxes were transported to the conservation workshop and were stored together, still sealed, submerged in a 5000-l metal tank flushed with running water. The sample used for analysis was kept at the conservation workshop for about 14 months under these conditions.

2.2. Methods and sample preparation

2.2.1. Scanning electron microscopy of wooden objects

Photomicrographs of the physical appearance of the archaeological wooden sample was, together with those of a fresh ash wood sample, were taken on a JSM-5310 LV scanning electron microscope.

2.2.2. Growth conditions

The wooden sample (app. 5 × 1.4 cm^2) was equilibrated in an anaerobic glove-box for about 14 days. After anaerobic equilibration the sample was trimmed and wood taken from the inner part was transferred to four 20-ml anaerobic culture flasks containing minimal BA-medium (DSMZ medium no. 671, http://www.dsmz.de/media/med671.htm) without contaminating it with material from the surface of the wooden piece. Two of the anaerobic flasks were supplemented with 0.2% (w/v) glucose and two with 0.2% (w/v) xylose. The oxygen indicator dye resazurin was added to provide a visual indication of anaerobicity. The flasks were incubated for 14 days at 14°C and 20°C. Controls without wood were incubated under the same conditions to exclude false positive results.

After incubation, the cells were re-inoculated by transferring 100 μl cells to 10 ml of fresh medium solution with the same carbon sources as mentioned above, and were incubated under the same conditions.

2.2.3. Microscopy

A drop of each culture was transferred to a microscope slide and examined by phase-contrast microscopy using a Carl Zeiss Axioplan epifluorescence microscope (×630). Digital images were captured with a 12-bit cooled slow-scan charge-coupled device camera.

2.2.4. DNA extraction and purification

Aliquots (500 μl) of each of the four cell suspensions diluted 10 fold were mixed with Proteinase K and cells were disrupted in a Fast Prep FP120 Savant Bead Beater. Chloroform extraction of genomic DNA was performed according to Sambrook (Sambrook et al., 1989). The extracted DNA

was purified twice with a Wizard DNA-clean-up system kit (Wizard® DNA-clean-up system, Promega) following the manufacturer's protocol and dissolved in 250 µl TE-buffer (10 mM Tris · Cl; 1 µM EDTA).

2.2.5. Polymerase chain reaction (PCR) amplification

The bacterial small subunit (SSU) rDNA (=16S rDNA) was PCR-amplified from the extracted total DNA using oligonucleotide primers, which target all members of the domain Bacteria. The forward and reverse primers used were: 9F (5′-GAG TTT GAT CCT GGC GGC TCAG-3′) and 1512R (5′-ACG GCT ACC TTG TTA CGA CTT-3′) respectively (Snaidr et al., 1997; Lane, 1991). Primer pairs with a *Not*I sequence were used to facilitate later cloning steps: 9F-*Not*I (5′-CCA AAA ACC AAA AA**G CGG CCG C**GA GTT TGA TCC TGG CTC AG-3′) and 1512R-*Not*I (5′-CCA AAA ACC AAA AA**G CGG CCG C**AC GGC TAC CTT GTT ACG ACT T-3′).

All primers were supplied by TAG Copenhagen A/S, Denmark. The reaction cocktail for the PCR contained 0.5 µl template DNA prepared as described above, 2.5 µl 10 × PCR buffer (containing 100 mM Tris-HCl, pH 9.0, 15 mM $MgCl_2$ and 500 mM KCl), 1 µl each primer (10 µM), 1 µl dNTP mix containing 5 µm of each of dATP, dCTP, dGTP, dTTP), (Roche), 0.25 U of *Taq*I DNA Polymerase (Amersham Pharmacia Biotech) and sterile Milli-Q water to a final volume of 25 µl. The PCR was performed on a Biometra Trio-Thermoblock. The following PCR procedure was used: initial denaturation for 5 min at 95°C and 30 cycles consisting of denaturation at 95°C for 1 min, annealing at 52°C for 1 min and synthesis at 72°C for 2 min. Post-synthesis was carried out at 72°C for 5 min followed by cooling at 4°C. All PCR products were purified before further use by using GFX kit (Amersham Life Sciences) according to the manufacturer's protocol.

2.2.6. Construction of gene libraries

To identify the wood inhabiting bacteria, we wanted to obtain the nucleotide sequence of the 16S rDNA region from each of the species. As most of the cultures contained a variety of bacterial species, we had to construct a 16S rDNA library from each culture.

Aliquots (5 µl) of each PCR product was digested with *Not*I (GibcoBRL®) and ligated to *Not*I-digested plasmid pBluescript® II KS+ (Stratagene). To avoid self-ligation, the 5′-phosphate was removed by adding shrimp alkaline phosphatase (Roche). The dephosphorylated plasmid was purified with the use of the GFX kit. After ligation of the *Not*I-digested PCR products and plasmid, all the ligation mixes were used to transform competent *Escherichia coli* JM83 cells (Yanisch-Perron et al., 1985).

White transformants were picked after selection on LB plates containing ampicillin (100 µg/ml) and Xgal (50 µg/ml). The size of the inserts in pBluescript screened by colony PCR amplification using the vector specific primers T3 (5′-AAT TTA CCC TCA CTA AAAG G-3′) and T7 (5′-GTA ATA CGA CTC ACT ATA GG-3′).

2.2.7. Nucleotide sequence determination of 16S rDNA inserts

Plasmid DNA was extracted from each of the correct clones using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's protocol. The plasmid inserts were sequenced by the biotechnology company MWG-BIOTECH AG either with primer T7, and/or with primer 9F and 1512R, 357F and 530F.

2.2.8. Identification of bacteria by comparative sequence analyses of 16S rDNA

Initial identification of the bacteria was made using Blast (Altschul et al., 2001). Secondly, 16S rDNA sequences were aligned with the ARB software package (Strunk et al., 2001) and manually corrected for errors. Each new sequence was analysed against a phylogenetic tree containing all sequences in the ARB database using the maximum parsimony "quick add" tool to get a first estimate of the affiliation of new bacterial specimen. The family of each strain was identified and a new tree was reconstructed using sequences from all species within the corresponding family as described in Bergey's Manual (Garrity et al., 2001). Strains were identified using a consensus based on the neighbour joining, maximum parsimony and fastdna maximum likelihood algorithms and the bacterial nomenclature described in the latest edition of Bergey's Manual (Garrity et al., 2001). A base frequency filter was generated based on the selected sequences excluding all positions different in more than 70% of the strains to enable a comparison of homologous positions.

3. Results and discussion

3.1. SEM observations

Comparison of scanning electron micrographs of the piece of the spear (Fig. 1A) with fresh ash wood (Fig. 1B) suggested that much of the cellulosic material had been lost. The wood was heavily degraded, and only its waterlogged condition together with the preserved intercellular layer made the object retain its form over the past 1700 years.

3.2. Cultivation of bacteria from the interior of a wooden spear shaft under strict anaerobic conditions

Because of the limited success with microorganisms cultured from degraded wood under aerobic conditions (Nilsson and Daniel, 1983; Daniel and Nilsson, 1997; Schmidt and Liese, 1994) we performed the experiments under anaerobic conditions, using glucose and xylose, the dominant degradation products from cellulose and hemicellulose, as carbon sources. To increase selection for prototrophic bacteria, sources of vitamins and amino acids were omitted. Both equilibration of the wooden sample, inoculation and

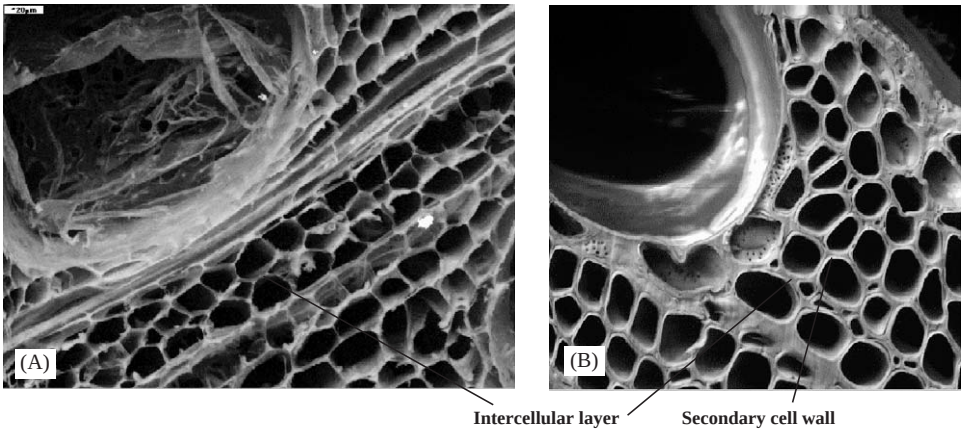

Fig. 1. Scanning electron micrographs of fresh and degraded ash wood (*Fraxinus excelcior*). (A) A 1700 year old piece of spear shaft from the Nydam offering bog. Only the compound middle lamellar system remains undegraded in the archaeological wood. (B) A piece of fresh ash wood. Both the secondary cell wall and the compound middle lamellae are clearly visible (photography: Ulrich Schnell).

the subsequent culturing were performed under strict anaerobic conditions. The inoculated flasks were incubated anaerobically at 14°C and 20°C. After 2 weeks, all four flasks were slightly turbid. The control flasks, which contained no wooden sample, showed no sign of growth. After dilution and re-inoculation of the cultures, these likewise showed turbidity after 14 days.

3.3. Microscopic examination of the wood-inoculated bacterial cultures

The resulting four bacterial cultures were observed by phase contrast microscopy. Fig. 2A shows a culture grown in glucose-supplemented medium at 14°C. At least five different types of bacteria were present in the suspension. The most frequent bacterium (marked 1) was a slender motile rod. Smaller spirillum-shaped (2), ellipsoid to coccoid (3) and fat rod-shaped (4) bacteria were also observed in high numbers. Tiny spirochaete-like bacteria (5) were present in the culture in lower numbers.

The bacterial selection shown in Fig. 2B had been cultured in glucose-supplemented medium at 20°C. At least three different morphologies were observed. Long rods were detected (marked 1), resembling the large rods in Fig. 2A (also marked 1). Spirillum-shaped bacteria (2) and smaller rods (3) with a corynebacterial appearance had no counterparts in Fig. 2A.

Fig. 3A shows a culture grown in xylose-supplemented medium at 14°C. This carbon source supports a different mixture of bacterial. The familiar class of large rod-shaped bacteria (marked 1) can be recognized, but the majority of the cells were smaller non-motile rods (2).

In Fig. 3B, a culture grown in xylose-supplemented medium at 20°C is shown. The population here seems to be very homogenous, although the differences in size of the single cells may indicate more than one species. The cells are long motile rods.

3.4. Identification of bacteria from cultures of wood inhabiting bacteria

Bulk DNA was extracted from each culture, and after amplification of the bacterial 16S rDNA regions four libraries were constructed (see materials and methods). A total of 21 clones were selected for sequencing. The full nucleotide sequence was determined for 15 clones, while six only provided sequence information between 555 and 883 bp. Table 1 shows the sequencing results for all clones.

3.5. Phylogenetic affiliations of the wood inhabiting bacteria

The phylogenetic analysis revealed a large diversity in affiliation (Figs. 4–9). Overall, there was a good agreement between the different algorithms (distance matrix, maximum parsimony and maximum likelihood) used for the phylogenetic analysis. The wood inhabiting bacteria clustered with bacteria as phylogenetically apart as spirochaetes, α-, β- and δ-*Proteobacteria*, *Verrumicrobiae* and gram-positives. More detailed information concerning these environmental sequences is given in Table 1.

Spirochaetes: The clone pACH87 forms a distinct branch in the *Treponema* cluster together with the *Spirochaeta aurantia* (Fig. 4). According to Lilburn et al. (2001), *Spirochaeta aurantia* is very common in aquatic habitats and able to fix nitrogen. It is facultative anaerobe, and ferments sugars such as glucose and cellobiose via the glycolytic pathway under anaerobic conditions (Canale-Parola, 1992).

α-*Proteobacteria*: Five clones clustered in the α-*Proteobacteria*. Together, with a previously described bacterial strain, KCB90 (Chin et al., 1999), isolated from anoxic bulk soil, they form a distinct branch (Fig. 5). Strain KCB90 is reported to grow anaerobically by fermenting a

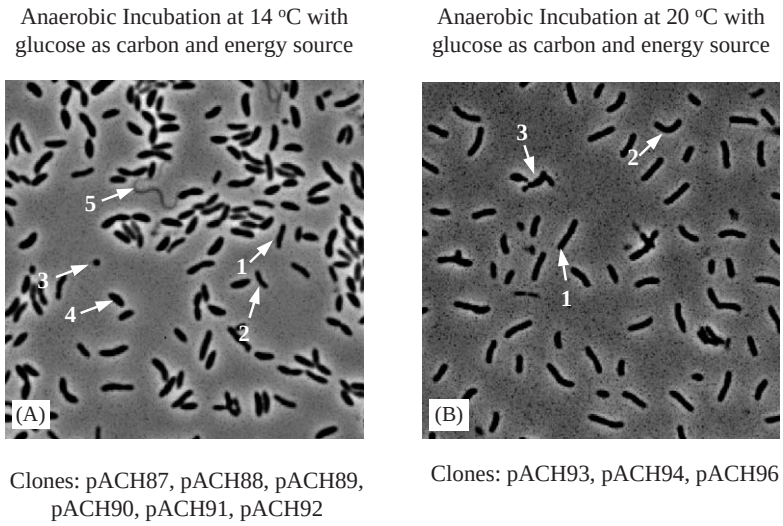

Fig. 2. Phase-contrast micrographs of secondary cultures are shown after grown at 14°C (A) or 20°C (B), with at least five and three different types of bacterium visible, respectively. 16S rDNA libraries were constructed from each of the cultures. The clones obtained from the particular culture are given below the micrographs.

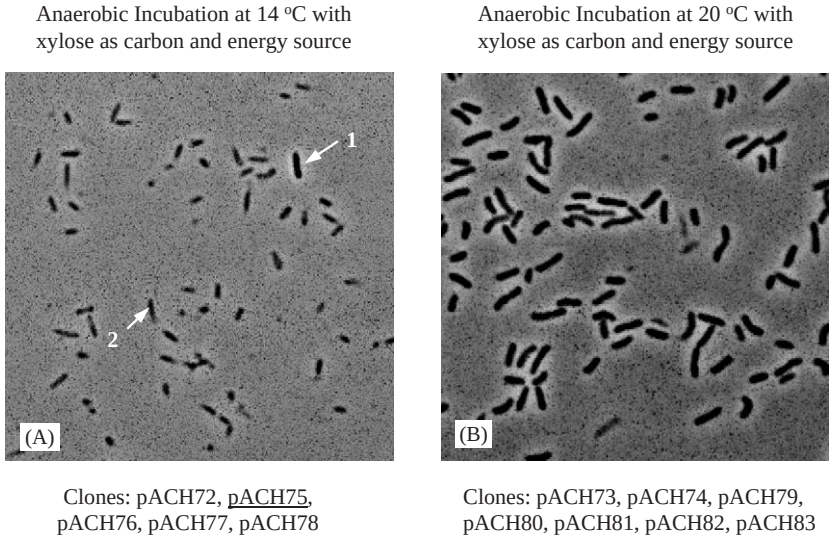

Fig. 3. Phase-contrast micrographs of secondary cultures are shown after grown at 14°C (A) or 20°C (B). In (A) two different species can be recognized. In (B) there appears to be only one species present. 16S rDNA libraries were constructed from each of the cultures. The clones obtained from the particular culture are given below the micrographs.

range of sugars and sugar polymers, including cellulose. The KCB90 cells are rod shaped and motile. The clones most likely originated from the large motile rod-shaped bacteria detected in all samples (marked 1 in Figs. 2A, B and 3A and seen in Fig. 3B).

β-Proteobacteria: Three clones formed a distinct branch in the *Rhodocyclaceae* family. They grouped with *Propionivibrio dicarboxylicus*, *Propionibacter pelophilus* and *Rhodocyclus tenuis* (Fig. 6). *P. dicarboxylicus* is a curved rod, with a cashew nut or kidney-shaped appearance, found in anaerobic mud or freshwater sediments (Tanaka et al., 2003). It may be motile by means of a single polar flagellum. *P. dicarboxylicus* is a strictly anaerobic organism with a fermentative metabolism, known to decarboxylate succinate to propionate ((Brune et al., 2002). *P. pelophilus* is a rod-shaped bacterium, which has been found to be motile by means of one polar flagellum. It ferments several substrates (sugars and cellulose among others) by propionic fermentation. *P. pelophilus* grows best under anoxic or near-anoxic conditions. Normally, it is found in anoxic freshwater and estuarine sediments (Meijer et al., 1999).

R. tenuis is curved in a spiral of one or two complete turns and is motile by means of polar flagella. It prefers growing under anaerobic conditions with numerous carbon sources and electron donors. Growth is possible under microaerobic to aerobic conditions in the dark (Imhoff and Trüper, 1989).

Table 1
Similarity in partial 16S rDNA sequences of clones to sequences of their closest bacterial relatives available in the Genbank nucleotide sequence database

Clone name	Sequencing primers	Number of sequenced bases	Phylogenetic affiliation (GenBank accession number)	Sequence identity (%)	Accession number
14°C, glucose					
Spirochaeta					
pACH87	T7,357F,1512R	1467	*Spirochaeta aurantia* (M57740)	98	AY297804
Proteobacteria					
pACH88	T7	687	α-*Proteobacterium* (AJ229247)	100	
pACH92	9F	555	α-*Proteobacterium* (AJ229247)	99	
pACH89	9F,1512R	1436	*Rhodocyclus tenuis* (D16209)	95	AY297805
pACH91	9F,530F,1512R	1378	*Rhodocyclus tenuis* (D16209)	96	AY297807
Verrumicrobia					
pACH90	9F,530F,T7	1497	*Opitutus terrae* (AJ229246)	96	AY297806
14°C, xylose					
Proteobacteria					
pACH72	T7,357F,1512R	1405	*Geobacter chapelleii* (U41561)	96	AY297795
pACH75	9F,1512R	1447	*Geobacter chapelleii* (U41561)	96	AY297797
pACH76	9F,530F,1512R	1399	*Geobacter chapelleii* (U41561)	96	AY297798
pACH78	9F,530F,1512R	1439	*Geobacter chapelleii* (U41561)	96	AY297799
pACH77	T7	600	α-*Proteobacterium* (AJ229247)	99	
20°C, glucose					
Erysipelotrichaceae					
pACH93	9F,357F,1512R	1400	Uncultured low G + C gram-positive (AY133091)	97	AY297808
Proteobacteria					
pACH94	9F,530F,T7	1478	*Rhodocyclus tenuis* (D16209)	96	AY297809
pACH96	357F	883	α-*Proteobacterium* (AJ229247)	99	
20°C, xylose					
Proteobacteria					
pACH73	9F	720	α-*Proteobacterium* (AJ229247)	99	
pACH74	T7,530F	1383	α-*Proteobacterium* (AJ229247)	99	AY297796
pACH79	T7,357F,1512R	1381	α-*Proteobacterium* (AJ229247)	99	AY297800
pACH80	9F,530F	854	α-*Proteobacterium* (AJ229247)	99	
pACH81	9F,530F	1357	α-*Proteobacterium* (AJ229247)	99	AY297801
pACH82	9F,530F,1512R	1357	α-*Proteobacterium* (AJ229247)	99	AY297802
PACH83	9F,530F,T7	1417	α-*Proteobacterium* (AJ229247)	98	AY297803

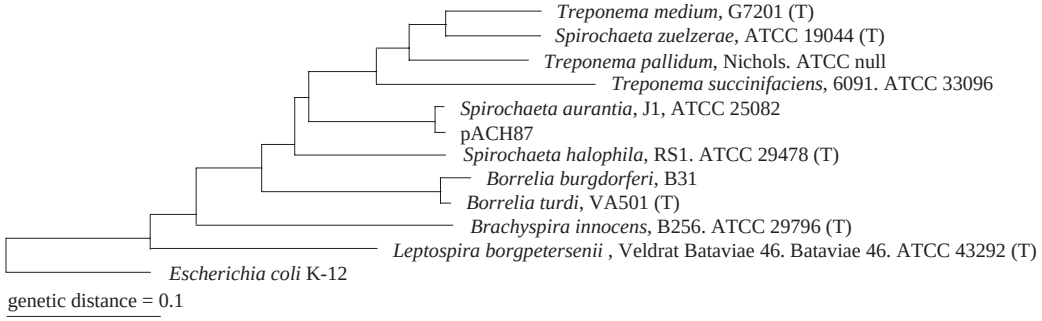

Fig. 4. Dendrogram showing the phylogenetic relationships of clone pACH87 with close relatives. The sequence of *E. coli* was included as an out-group. The scale bar represents a 10% difference in nucleotide sequences.

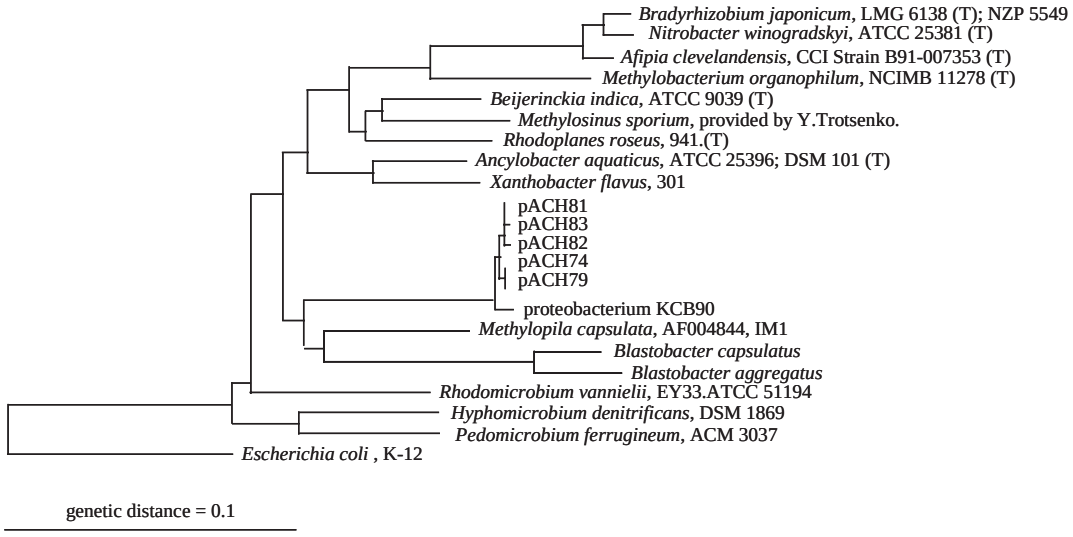

Fig. 5. Dendrogram showing the phylogenetic relationships of clones pACH74, pACH79, pACH81, pACH82 and pACH83 with close relatives. The sequence of *E. coli* was included as an out-group. The scale bar represents a 10% difference in nucleotide sequences.

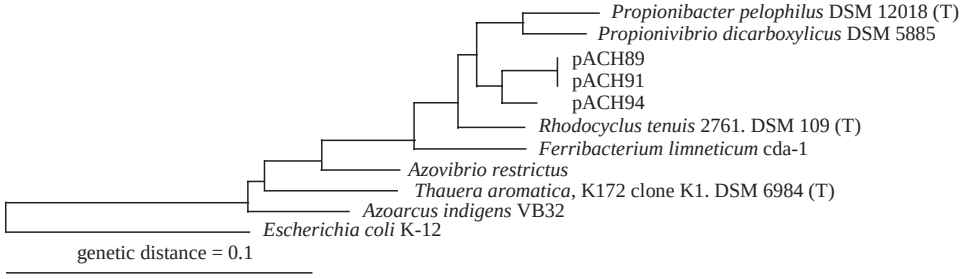

Fig. 6. Dendrogram showing the phylogenetic relationships of clones pACH89, pACH91 and pACH94 with some close relatives. The sequence of *E. coli* was included as an out-group. The scale bar represents a 10% difference in nucleotide sequences.

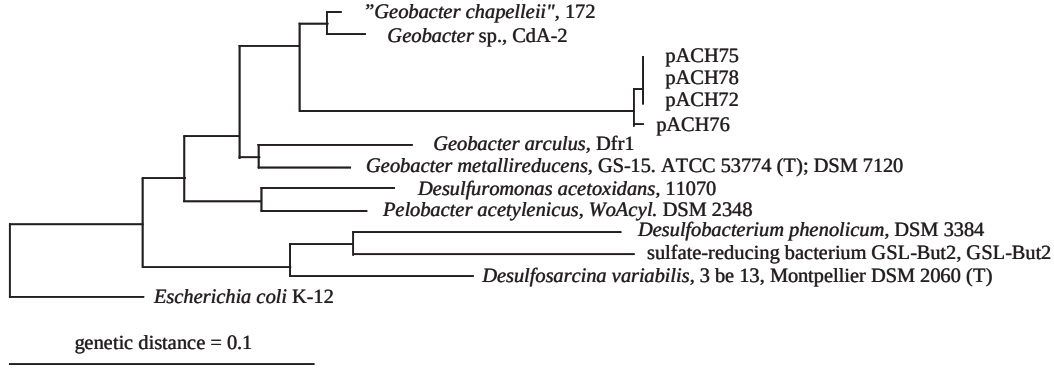

Fig. 7. Dendrogram showing the phylogenetic relationships of clones pACH72, pACH75, pACH76 and pACH78 with some close relatives. The sequence of *E. coli* was included as an out-group. The scale bar represents a 10% difference in nucleotide sequences.

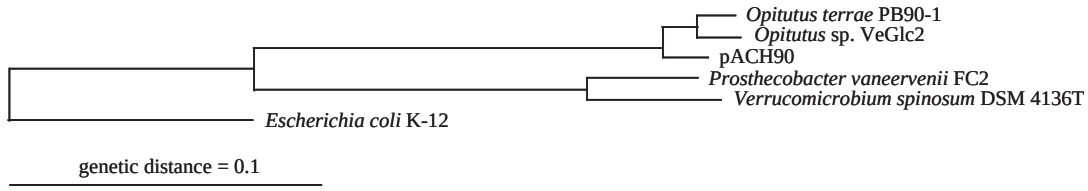

Fig. 8. Dendrogram showing the phylogenetic relationships of clone pACH90 with some close relatives. The sequence of *E. coli* was included as an out-group. The scale bar represents a 10% difference in nucleotide sequences.

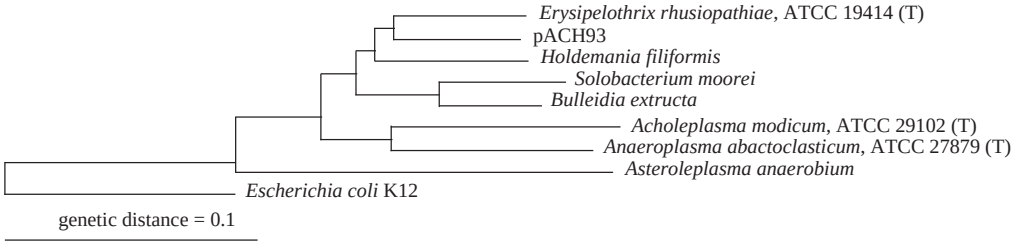

Fig. 9. Dendrogram showing the phylogenetic relationships of clone pACH93 with some close relatives. The sequence of *E. coli* was included as an out-group. The scale bar represents a 10% difference in nucleotide sequences.

The clones pACH89, pACH91 and pACH94 could have originated from the spirillum-shaped bacteria observed in the glucose-supplemented medium (Fig. 2A and B) cultured at both 14°C and 20°C.

δ-Proteobacteria: Four clones formed a very distinct branch, in the *δ-Proteobacteria* (Fig. 7). They are grouped in the *Geobacteraceae* family with *Geobacter chapelleii* as the closest relative. However, the four clones form a deep branch in the Geobacter family, which indicates that they might constitute a new genus. When the nucleotide sequences of the four clones are compared to the 16S rDNA sequence of *G. chapelleii*, the differences are uniformly distributed along the DNA, arguing that the large evolutionary distance is not due to chimera formation or faulty DNA amplification (data not shown). *G. chapellei* is strictly anaerobic, rod-shaped, non-motile, and chemo-organotrophic (Coates et al., 2001). It oxidizes acetate, ethanol, lactate and formate with the concomitant reduction of Fe(III). Generally, Geobacter species are known as the predominant Fe(III)-reducing bacteria, using iron as terminal electron acceptor in anaerobic respiration (Coates et al., 2001). Interestingly members of *Geobacteraceae* are also known to be responsible for microbial reduction of humic substances in sedimentary environments (Coates et al., 1998). These clones could have originated from the non-motile rod-shaped cells observed in the xylose-supplemented medium (Fig. 3A) grown at 14°C.

Division Verrumicrobia: One clone was found to be closely affiliated with *Opitutus terrae* (Fig. 8), which belongs to the subdivision 4 of the *Verrumicrobia*. According to Hengstmann et al. (1999) subdivision 4 environmental 16S rDNA clones have been detected only in peat bogs and rice paddy soils. *O. terrae* forms rod-shaped to oval cells that are motile by means of a flagellum and are non-spore-forming. *O. terrae* is strictly anaerobic, cellulolytic and nitrate reducing. The closest relative of pACH90, *O. terrae* PB90-1, was detected in the same anoxic soil bulk as the bacterial strain KCB90, described under *α-subdivison* of the *Proteobacteria* (Chin et al., 1999). This clone pACH90 could have originated from the small ellipsoid or coccoid cells detected in the glucose-supplemented medium (Fig. 2A) grown at 14°C.

Class Mollicutes: One clone is the sole gram-positive clone in the study. It forms its own distinct branch in the phylogenetic tree (Fig. 9), *Erysipelothrix rhusiopathiae* being the most closely related relative cultured. It belongs to the genus *Eubacterium*, which contains, anaerobic, non-spore-forming, rod-shaped bacteria (Willems et al., 1997). This clone pACH93 could have originated from the smaller rods detected in the 20°C glucose-supplemented medium (Fig. 2B).

Even though the cultured bacteria might represent the bacterial biota, it is highly probable that they constitute a smaller physiologically homogenous group, selected by the particular growth conditions. In ongoing work, the bacterial biota have recently been analysed through bulk extraction of DNA from the interior of the wooden sample and amplification of bacterial 16S rDNA species. It will be interesting to identify the sequences of the cultured bacteria within this 16S rDNA library. In the case of identical sequences, this will confirm that the anaerobic cultured species are part of the total bacterial species present.

The successful cultivation and phylogenetic identification of these bacteria has provided us with indications of their

physiology and growth requirements. This knowledge will be used in future attempts to obtain pure cultures of each bacterium and in assessments of their wood degradation capacity.

4. Conclusion

The present work has demonstrated what had been previously postulated by Nilsson and co-workers (Kim et al., 1996) and Blanchette and co-workers (Blanchette, 2000), that archaeological woods contain living bacteria. The 16S rDNA identification showed that a wide range of bacteria inhabited the degraded archaeological wood examined. It is noteworthy that the closest relatives to most identified bacteria are strictly anaerobic, or strongly prefer anoxic environments. We therefore ascribe the success in cultivation of these bacteria to the extensive anaerobic equilibration of the wooden sample before inoculation and the anaerobic growth conditions. Many close relatives of the sequenced bacteria were found to be known cellulose degraders. The species of *Geobacter*, which was cultured at 20°C with xylose as carbon source, might be a humic acid degrader, like its closest relative *Geobacter chapelleii*. We therefore believe that a number of the cultivated and sequenced bacteria are, or have been, involved in the degradation process in and around waterlogged archaeological wood.

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