The impact of grassland management on archaeal community structure in upland pasture rhizosphere soil

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Summary
The community structure of rhizosphere soil *Archaea* from three grassland types, associated with different management practices, was examined at a site in the Borders region of Scotland, by analysis of 16S rRNA gene fragments amplified from 16S rDNA and from rRNA. Denaturing gradient gel electrophoresis (DGGE) and sequence analysis of amplified products indicated high relative abundance within the archaeal community of two distinct lineages of non-thermophilic (group 1) *Crenarchaeota*. Grassland management practices influenced archaeal community structure, as characterized by both 16S rRNA- and 16S rDNA-derived DGGE profiles. One band dominated DGGE profiles in all three grassland types examined, and reproducible differences in the presence and intensity of bands were observed between profiles from managed and natural grassland sites. Analysis of 16S rRNA-derived amplicons from managed and natural grasslands at sites in the north of England and the north of Wales also indicated high relative abundance of non-thermophilic crenarchaeotes within the archaeal community. The band dominating the Scottish grassland site also dominated DGGE profiles from the English and Welsh sites, and similar differences were seen between profiles derived from soils subjected to different management regimes. The study indicates that grassland archaeal communities are dominated by *Crenarchaeota*, with closely related members of this lineage ubiquitous in distribution in UK upland pasture, and indicate that management practices influence the nature of the crenarchaeotal community.

Introduction
The use of molecular techniques has greatly enhanced our understanding of microbial diversity in soil (Rondon *et al.*, 1999). In particular, the retrieval of 16S rRNA gene sequences has revealed that diverse microbial communities contain not only organisms from lineages with many cultured representatives, but also large abundances of phylogenetically diverse lineages with few or no cultured representatives (Hugenholtz *et al.*, 1998). This is exemplified by the ubiquity of organisms belonging to the domain *Archaea* in ‘non-extreme’ habitats. Cultivated *Archaea* have been isolated from environments considered to be extreme in temperature, salinity or anoxia (Woese *et al.*, 1990). However, molecular analyses indicate that uncultivated lineages of *Archaea* are distributed globally and are active in a wide variety of psychrophilic and mesophilic environments, with a lineage associated with the hyperthermophilic *Crenarchaeota* kingdom appearing to be the most ecologically diverse (DeLong, 1998). Non-thermophilic *Crenarchaeota* have been found in fresh water (Jurgens *et al.*, 2000), marine waters (DeLong, 1992; Fuhrman *et al.*, 1992; Karner *et al.*, 2001), palaeosol (Chandler *et al.*, 1998), sediments (Hershberger *et al.*, 1996; MacGregor *et al.*, 1997; Vetriani *et al.*, 1998) and the digestive tracts of marine and terrestrial animals (McInerney *et al.*, 1995; Preston *et al.*, 1996; van der Maarel *et al.*, 1998; Friedrich *et al.*, 2001). 16S rDNA sequences belonging to a non-thermophilic *Crenarchaeota* lineage have also been recovered from a wide variety of soil types, including forest (Bormann and Tripplett, 1997; Jurgens *et al.*, 1997) and agricultural soils (Ueda *et al.*, 1995; Bintrim *et al.*, 1997; Buckley *et al.*, 1998; Größkopf *et al.*, 1998; Chelius and Tripplett, 2001). Quantitative analysis by 16S rRNA probing has indicated that *Archaea* can contribute ~1% to total prokaryotic activity in soil (Buckley *et al.*, 1998; Sandaa *et al.*, 1999), suggesting that they could make a significant contribution to microbiologically mediated processes in soil.

Pastures represent over 25% of the earth’s land surface (Harrison and Pearce, 2001), and the majority of UK grazing pastures are in upland regions that are subject to a range of management regimes. Alteration of plant community structure and fertilizer applications can increase the productivity of natural grazing pastures (Haynes and Williams, 1993), and grassland management may affect
microbial community structure. For example, microbial activity is typically greater in unmanaged grasslands, where fungi dominate, whereas prokaryotes contribute more to the biomass of soils of managed pastures, which have greater fertility and nutrient availability (Grayston et al., 2001). This greater abundance is also reflected in the higher numbers of cultivable bacteria in fertilized soils (Lovell et al., 1995). The contribution of Archaea to soil microbial communities associated with grassland pasture, however, remains unknown.

The aim of this study was to determine the influence of grassland management practices on archaeal community structure by comparison of UK upland pasture rhizosphere soils subjected to different management regimes for prolonged periods. Archaeal community structure and activity were profiled using cloning and denaturing gradient gel electrophoresis (DGGE) analysis of both 16S rRNA and rDNA polymerase chain reaction (PCR) products, potentially differentiating between numerically abundant and active organisms (Felske and Akkermans, 1998). Molecular analysis avoided limitations imposed by the lack of availability of cultured Archaea from these soils. As the differences between different grassland types can easily be characterized, observable shifts in archaeal community structure may indicate those factors that influence soil Archaea, informing further study of the functional role of archaeal communities in grassland soil.

Results

**Characteristics of grassland rhizosphere soil at Sourhope**

Distinct differences were observed between different grassland soil types (Table 1). As reported previously (Webster et al., 2002), soil pH, carbon and nitrogen contents were all greatest in improved soil.

**Modification of primer PARCH340f**

The sequences of four previously published archaeal primers (Ar3F, Ar9R, PARCH340f and PARCH519r; Table 2) to be used in a nested PCR strategy were aligned

### Table 1. Characteristics of grassland rhizosphere soil samples.

<table>
<thead>
<tr>
<th>Grassland type</th>
<th>Water content (%)</th>
<th>pH</th>
<th>Organic matter by loss on ignition (%)</th>
<th>Nitrogen content (%)</th>
<th>Carbon content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Improved</td>
<td>51.6 (1.4)</td>
<td>6.6</td>
<td>22.5 (0.2)</td>
<td>0.9 (0.3)</td>
<td>13.2 (1.1)</td>
</tr>
<tr>
<td>Semi-improved</td>
<td>29.7 (0.6)</td>
<td>5.1</td>
<td>17.0 (0.1)</td>
<td>0.7 (0.2)</td>
<td>8.3 (0.8)</td>
</tr>
<tr>
<td>Unimproved</td>
<td>47.2 (2.0)</td>
<td>4.1</td>
<td>17.0 (0.2)</td>
<td>0.7 (0.2)</td>
<td>9.3 (0.9)</td>
</tr>
</tbody>
</table>

Data represent means of triplicate samples of soil bulked and homogenized from three individual subplots. Values in brackets represent the standard error of the mean. The standard errors of pH values were all <0.1.

### Table 2. Summary of primers and PCR conditions used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Positions*</th>
<th>Sequence 5¢-3¢</th>
<th>Reference</th>
<th>PCR round</th>
<th>PCR thermal cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ar3F</td>
<td>1–20</td>
<td>TTCCGGTTTGATCCCTGCCGGA</td>
<td>Giovannoni et al. (1988)</td>
<td>First</td>
<td>95°C for 5 min; followed by five cycles of 94°C at 30 s, 55°C at 30 s, 72°C at 1 min; followed by 30 cycles of 92°C at 30 s, 55°C at 30 s, 72°C at 1 min; followed by 72°C at 10 min</td>
</tr>
<tr>
<td>Ar9R</td>
<td>905–927</td>
<td>CCCCACAAATCTTCTTAAATGGTTTC</td>
<td>Jurgens et al. (1997)</td>
<td>Second</td>
<td>95°C for 5 min; followed by five cycles of 94°C at 30 s, 53.5°C at 30 s, 72°C at 1 min; followed by 30 cycles of 92°C at 30 s, 53.5°C at 30 s, 72°C at 1 min; followed by 72°C at 10 min</td>
</tr>
<tr>
<td>PARCH340f</td>
<td>340–357</td>
<td>CGCCCGCCGCGCGCCCGCCCGC</td>
<td>Øvreås et al. (1997)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PARCH519r</td>
<td>519–533</td>
<td>TTACCGCGGGGCACTACGGGGCGC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. E. coli numbering.

b. Primer SAF is a mixture of primers SAF(i) and SAF(ii) at a molar ratio of 2:1.

c. Sequence underlined corresponds to GC clamp (Muyzer et al., 1993) and is not homologous to 16S rRNA sequence.

K = G or T, S = G or C, Y = C or T.
with representative sequences from cultured isolates and environmental clones from both soil and non-soil environments of non-thermophilic *Crenarchaeota* and *Euryarchaeota* lineages. These alignments revealed that they exhibited broad coverage of archaeal sequences except for primer PARCH340f, which exhibited complete identity with only one of the 41 non-thermophilic crenarchaeotal sequences compared (from soil, marine and freshwater environments). This primer was designed when fewer archaeal 16S rDNA sequences were available, in particular those from the non-thermophilic *Crenarchaeota* lineage. Greater coverage of potential soil archaeal sequences (which were presumed to be methanogens and non-thermophilic crenarchaeotes) was achieved by modification of PARCH340f to generate primers SAf1 and SAf2 (Table 2). As SAf1 has one degeneracy, a 2:1 mixture of SAf1:2 was used leading to a final 1:1:1 ratio of the three different sequences represented as SAf. However, DGGE profiles obtained using either PARCH340f or SAf in nested amplification of the same template (first-round PCR product) showed no detectable difference.

**Comparison of 16S rRNA- and rDNA-derived DGGE profiles of archaeal communities**

To examine whether profiles generated using reverse transcription (RT)-PCR and PCR of 16S rRNA and rDNA, respectively, produced different profiles, DNA and rRNA extractions from 1 g soil samples were performed in duplicate from homogenized samples of both improved and unimproved soil. DGGE analysis was performed on nested PCR products obtained from both DNA and cDNA with Ar3F/Ar9R and SAf/PARCH519r primer sets (Fig. 1). Reproducible profiles were obtained from both templates for both soil treatments, with differences observed between DNA- and cDNA-derived profiles.

**Cloning archaeal PCR products for a DGGE clone ladder and phylogenetic analysis**

Using a semi-nested PCR approach, DNA- and RNA-derived PCR products from nucleic acid extractions of both improved and unimproved soil were pooled before purification and ligation into pGEM-T vector. After blue-white screening, 192 inserts were screened by PCR amplification using primers SAf and PARCH519r, followed by DGGE analysis, and clones representing 11 different migration patterns were selected for sequencing. Cloned sequences were prefixed with SUPA (Scottish Upland Pasture Archaea) and numbered 1–11 in relation to the migration of each band, with SUPA1 and SUPA11 migrating the shortest and greatest distances respectively. SUPA5 represented the most common migration position of all the clones screened. Eight of the 11 clones were used as markers for further DGGE analysis. Cloned sequences SUPA1 to SUPA4 migrated to similar positions on the DGGE gel, and only SUPA2 was included in the ladder.

BLAST similarity searches were performed on all 11 sequences to identify the closest related sequences retrieved in other studies. All 11 sequences showed high sequence similarity to other cloned 16S rDNA sequences (≥95%), although no sequence was found with 100% similarity. Phylogenetic analysis placed all 11 sequences within the non-thermophilic *Crenarchaeota* lineage, with each sequence falling in one of two previously described phylogenetic clusters of sequences retrieved from soil (Fig. 2). The basic tree topology was similar to that of previously published phylogenetic analyses of non-thermophilic *Crenarchaeota* (Buckley et al., 1998). Group 1 *Crenarchaeota* sequences used in Fig. 2 are described in Table 3. Sequences SUPA1–6, migrating in the upper sections of DGGE gels, could be placed within the terrestrial or 1.1b cluster (Buckley et al., 1998; DeLong, 1998 respectively), whereas sequences SUPA7–11 migrated to the lower section and fell within the FFSB or 1.1c cluster (Buckley et al., 1998; DeLong, 1998 respectively). Although primers SAf and PARCH519r produced a 117 bp DNA fragment (excluding primers) for all clones belonging.
Fig. 2. Phylogenetic tree showing the placement of cloned 16S rDNA sequences (SUPA1–11) within the Archaea. Korarchaeotal sequences pJP27 and pJP78 were used as outgroup references. Phylogenetic groups 1.1a and 1.3 are as described by DeLong (1998). Nodes marked A, B and C highlight three clusters of sequences in which SUPA clones are placed (see text). Environmental 16S rDNA clone sequences are presented as ‘name, accession number’ with environment of retrieval detailed in Table 3. Values at nodes represent the percentage of 1000 bootstrap replicates. Bootstrap values <50% are not shown.

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to both clusters, sequences SUPA1–6 and SUPA7–11 were 429 bp and 460 bp in length between primer sites Ar3F and PARCH519r respectively.

The influence of grassland management on archaeal community structure

Archaeal communities were characterized in the rhizosphere soil of three grassland types. At Sourhope Research Station, total DNA and 16S rRNA were extracted from homogenized soil for each of the three replicate soil samples from improved, semi-improved and unimproved grassland types. Nested PCR was performed in triplicate for each DNA extraction before pooling equal amounts of product for DGGE analysis to minimise the effect of PCR drift. Single nested PCR amplifications were performed on each RT reaction, as the increased amount of available template appeared to decrease any potential drift (unpublished data). A nested PCR strategy was necessary for generation of sufficient PCR product for analysis. DGGE analysis (Fig. 3) indicates that grassland type influenced archaeal community structure in the rhizosphere. Profiles of both DNA and RNA for all three samples of improved soil revealed two bands migrating at the top of the gel (positions marked ‘a’ and ‘b’) at intensities greater than those in unimproved and semi-improved soils (Table 4A). Bands at position ‘b’ co-migrated with SUPA2 in the marker lane. A dominant band co-migrating with SUPA5 (marked position ‘c’) was present in all lanes except for improved subplot 1, suggesting a widespread distribution of the organism possessing this sequence in grassland rhizosphere soil. Other band positions and, in particular, those migrating to positions lower than SUPA6 showed no apparent reproducible difference in intensity in soils from different management treatments. Comparison of DNA- and RNA-derived profiles from the same sample, as shown previously in Fig. 1, demonstrated clear differences at some band positions, with an intense band being present in the DNA lane but absent or clearly less intense in the RNA lane, and vice versa.

To assess the influence of grassland management on archaeal communities at other sites, 16S rRNA was extracted from rhizosphere soil samples from triplicate subplots of improved and unimproved grassland at Garrigill in the north of England and Aber in north Wales. The ladder produced from SUPA cloned sequences was run alongside the RT-PCR products to enable comparison with Sourhope grassland profiles (Fig. 4). Grassland-specific differences were clearly apparent in the soil samples from both sites. Bands co-migrating with SUPA2 (marked as position ‘a’) were observed, as in Sourhope soils, and the relative intensities of these bands were again greater in improved soil samples (Table 4B), although less markedly than in Sourhope soils. Bands marked at position ‘b’ co-migrated with SUPA5, the band position that dominated eight of nine subplot samples at Sourhope. SUPA5-like bands had greater relative intensity within lanes in unimproved soil samples from both sites. Although this was also observed for the rRNA-defined profiles at Sourhope, variability estimated as the standard error of values for triplicate subplots was much greater.
One improved subplot (number 1) at Sourhope had no visible band co-migrating to this position, and grassland management may affect the contribution of SUPA5-like organisms within the archaeal community. Profiles derived from improved subplots at both sites contained a band that was significantly more intense than in the equivalent unimproved subplots (marked at positions 'c' and 'd' in the Garrigill and Aber profiles respectively), again demonstrating a clear difference between improved and unimproved grassland types. In comparison with Sourhope rRNA-defined profiles, bands co-migrating to positions representative of group 1.1c were less diverse.

To determine whether the dominant bands in archaeal DGGE profiles from the three sites were of crenarchaeotal origin, as inferred by the analysis of cloned 16S PCR products, bands highlighted and numbered in Figs 3 and 4 were excised and sequenced. Sequences of bands from Sourhope-, Garrigill- and Aber-derived profiles were prefixed with bS, bG and bA respectively. Table 5 displays the sequences with closest similarity present in the GenBank database.

**Table 4.** Percentage values of band volumes relative to the total band volume in DGGE lanes profiling archaeal communities in grassland soils.

<table>
<thead>
<tr>
<th>Band position</th>
<th>Improved</th>
<th>Semi-improved</th>
<th>Unimproved</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>13.9 (0.7)</td>
<td>16.0 (3.5)</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>34.8 (17.3)</td>
<td>33.9 (8.5)</td>
<td>0</td>
</tr>
<tr>
<td>c</td>
<td>22.0 (11.1)</td>
<td>18.2 (10.2)</td>
<td>29.1 (4.7)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Band position</th>
<th>Improved</th>
<th>Unimproved</th>
<th>Improved</th>
<th>Unimproved</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>5.6 (1.8)</td>
<td>0.5 (0.5)</td>
<td>11.0 (3.6)</td>
<td>0.7 (0.7)</td>
</tr>
<tr>
<td>b</td>
<td>19.9 (2.3)</td>
<td>28.7 (1.2)</td>
<td>5.2 (1.7)</td>
<td>27.9 (1.4)</td>
</tr>
<tr>
<td>c</td>
<td>21.6 (7.4)</td>
<td>2.0 (1.3)</td>
<td>3.8 (0.2)</td>
<td>0</td>
</tr>
<tr>
<td>d</td>
<td>1.8 (0.8)</td>
<td>0.8 (0.8)</td>
<td>29.9 (0.6)</td>
<td>0.3 (0.3)</td>
</tr>
</tbody>
</table>

Values are the mean percentage of band volume from triplicate subplots. Values in brackets are standard errors of the mean.

(A) Sourhope (Fig. 3) band positions 'b' and 'c' co-migrate with the SUPA2 and SUPA5 marker sequences respectively.

(B) Garrigill and Aber (Fig. 4) band positions 'a' and 'b' co-migrate with the SUPA2 and SUPA5 marker sequences respectively.

Fig. 4. 16S rRNA-defined DGGE profiles of the dominating Archaea in triplicate improved and unimproved grassland plots at Garrigill and Aber. Volume data of bands migrating to positions 'a'–'d' are presented in Table 4B. Bands highlighted with numbered boxes were excised, sequenced and prefixed with bG or bA for Garrigill and Aber respectively. M indicates the SUPA ladder.
database after BLAST searches and the subsequent inference of cluster affiliation. All sequences showed high sequence similarity with other cloned crenarchaeotal 16S rDNA sequences. Bands bS-1a and bS-1b from DGGE of the Sourhope samples co-migrated with and were of the same sequence as SUPA2. Excised bands that co-migrated with SUPA5 (bS-3a, bS-3b and bS-3c) shared sequence identity with SUPA5. The excised band bS-2, however, shared the same sequence but migrated to a different position from the SUPA5-like sequences. This could be due to differences in the nucleotide sequence of these PCR fragments within the incorporated oligonucleotide only, as a result of primer degeneracies. Although the use of redundant positions in PCR primers may be necessary for optimal coverage of a particular phylogenetic group, they can result in more than one band being present on a DGGE profile for a single sequence being amplified. Alternatively, the 15 bp of sequence beside the incorporated PARCH519r oligonucleotide, which was not determined because of sequencing in one direction only, may have a different sequence in comparison with the SUPA5-like sequences. As suggested by migration towards the lower section of the DGGE gel, band bS-4 shared high sequence similarity with 1.1c group sequences, recovered from both Finnish and Colorado forest soils.

All five bands excised from Aber or Garrigill profiles migrated above 1.1b group positions in the marker lane, and each sequence displayed high sequence similarity to 1.1b sequences. Again, sequences co-migrating with SUPA5 (bG-1 and bA-2) from both sites were identical, indicating a ubiquitous distribution of closely related crenarchaeotes in UK upland pasture.

Discussion

Archaeal populations in grassland soils were characterized by analysis of 16S rRNA gene fragments amplified from extracted DNA and rRNA. Reproducible profiles were generated from replicate extractions of both DNA and rRNA. Although some bands were common to all profiles, differences were observed between DNA- and rRNA-defined profiles from the same sample, as observed in studies of bacterial communities (Felske and Akkermans, 1998; Kowalchuk et al., 1999; Duineveld et al., 2001). Comparison of DNA- and RNA-derived profiles may provide an indication of which members of the community have greater growth rate or metabolic activity, based on evidence that faster growing cells contain more ribosomes (Kerkhof and Kemp, 1999). However, it also assumes rapid degradation of rRNA after cessation of growth or activity. In this respect, microorganisms isolated from the environment appear to adopt different strategies. In some organisms, rRNA decay is thought to be rapid after the onset of starvation conditions (Kerkhof and Kemp, 1999), but maintenance of ribosome contents in excess of those required for protein synthesis has also been observed (Flardh et al., 1992). A large ribosome content in cells with low metabolic rates may allow a rapid response to changing nutrient availability (Alfreider et al., 1996), and individual strains may adopt different rRNA turnover strategies depending on previous physiological histories, e.g. during starvation after different rates of growth (Oda et al., 2000). The lack of availability of laboratory cultures representative of natural microbial populations, and of detailed physiological studies, therefore limits our ability to interpret differences between 16S rDNA- and rRNA-derived PCR fragments. The lack of non-thermophilic crenarchaeote cultures accentuates this problem, and the observed differences in profiles therefore provide only tentative indications of differences in relative activity. Nevertheless, 16S rRNA-based analysis also provides greater sensitivity, arising from the higher copy number of 16S rRNA per cell compared with rrn operon copy number, and growth and activity may be detected earlier through increases in ribosome content before cell division.

Previous phylogenetic analysis (Buckley et al., 1998) suggested that there were four major lineages or clusters of non-thermophilic Crenarchaeota, each of which could be associated with a particular environment: ‘freshwater’
and ‘marine’ clusters and two, termed ‘FFSB’ and ‘terrestrial’, from soil. Delong (1998) described these clusters according to the original ‘group 1’ designation with marine, terrestrial, FFSB and freshwater clusters termed groups 1.1a, 1.1b, 1.1c and 1.3 respectively. The retrieval of further archaeal 16S rDNA sequences from soil has shown, however, that soil-derived clones do not fall exclusively within groups 1.1b and 1.1c (Großkopf et al., 1998; Chelius and Tripllett, 2001) but are distributed throughout the major clusters of non-thermophilic Crenarchaeota (Fig. 2 and Table 3). All 11 SUPA sequences from the grasslands studied here fell within one of the two previously identified ‘soil’ clusters. DGGE analysis indicated that the basic phylogeny of a sequence (in terms of group 1.1b or 1.1c affiliation) could be putatively inferred by the migration pattern of a particular DGGE band using primer set SAF and PARCH519r. Sequences SUPA1–4 exhibited highest similarity (highlighted at node B in Fig. 2) to sequences retrieved either directly from plant roots or from soils in which crenarchaeotes colonized plant roots. Closest similarity (99% over 429 bases) was to sequences prefixed TRC, derived from DNA extracted from tomato root surfaces (Simon et al., 2000). As soil Crenarchaeota appear to be associated with roots, the plant communities associated with different management regimes at these sites may have led to the observed differences in relative sequence abundance, although differences in soil characteristics, such as pH, may also be important. The cluster containing sequences SUPAS and 6 (highlighted at node C) was affiliated with sequences retrieved from forest soil (USA) (Oline, Schmidt and Grant, 2000; GenBank submission) and a sequence from coastal marine water (CRO-7a) (Crum and Baross, 2000). Sequences SUPA7–11 were distributed throughout group 1.1c (highlighted at node A), and affiliated soil sequences were all retrieved from forest soil in geographically distant areas (Finland and USA) (Jurgens et al., 1997; Jurgens and Saano, 1999; Oline, Schmidt and Grant, 2000; GenBank submission). Clones belonging to group 1.1c have been retrieved in fewer studies than those in group 1.1b, and from less diverse soil types (forest soils only), possibly reflecting a different ecological distribution. The considerable sequence divergence between groups 1.1b and 1.1c may also indicate substantial differences in physiology.

Sequences of bands excised from DGGE gels of gene fragments amplified from extracted DNA were identical to those of co-migrating cloned sequences. Comparison with GenBank database entries inferred that those sequences migrating to 1.1b- and 1.1c-like positions belonged to those phylogenetic clusters. Although phylogenetic analysis was not performed on those short stretches of sequence from excised bands, the high level of similarity to previously described sequences and the substantial dissimilarity between 1.1b and 1.1c sequences support their affiliation with groups 1.1b or 1.1c. Interestingly, certain bands co-migrated to identical positions in profiles derived from soil samples from each of the three sites surveyed. Sequence analysis revealed that they were identical over these short stretches of 16S rDNA, indicating ubiquitous distribution of a closely related group of non-thermophilic crenarchaeotes. DGGE analysis revealed that grassland management resulted in reproducible shifts in archaeal community at these different locations. All differences in community profiles, inferred either by comparison with the migration patterns of cloned sequences or by excision and sequencing of DGGE bands, indicated that observable shifts in community structure were particularly associated with group 1.1b crenarchaeotes. No Euryarchaeota sequence was found, and no representative of methanogenic Archaea was detected. The findings therefore suggest that Crenarchaeota may be the dominant soil Archaea in upland pastures, which represent major grazing systems within the UK. Furthermore, differences in communities associated with grassland management regimes provide a basis for studying their role in the environment. Future work may provide an understanding of which characteristics of grassland management lead to selection for particular archaeal community structures, informing methods for enrichment and isolation of soil crenarchaeotes.

Experimental procedures

Soil sampling and storage

Soil samples were collected from Sourhope Research Station in the Scottish Borders (map reference NT 850 205) in October 1999 from triplicate plots of characteristic grassland designated improved, semi-improved and unimproved. Improved grassland vegetation was dominated by Lolium perenne (perennial ryegrass), Trifolium repens (white clover) and Cynosurus cristatus (crested dog’s-tail) vegetation. Plots received three fertilizer additions annually [50 kg N ha⁻¹ in March and August and 40 kg N ha⁻¹ N:P:K (40:20:20) in May] and were grazed intensively by sheep during spring, summer and autumn. Unimproved grassland represents natural grassland, with plant communities dominated by Festuca ovina (t cousc), Agrostis capillaries (common bent) and Galium saxatile (heath bedstraw). Plots received no fertilizer and were grazed by sheep in summer only. Semi-improved grassland was the same as unimproved but with a Holcus lanatus (common velvetgrass) and T. repens subcommunity.

A 5 × 5 m quadrat was randomly positioned in each plot, from which 10 soil cores were sampled randomly (80 mm diameter × 80 mm depth). Cores from each quadrat were pooled and sieved (3.35 mm mesh size). Dense root material was present in the cores and assumed to be in contact with the soil throughout. All soil was therefore considered to be rhizosphere (McCaig et al., 1999). Soil pH, moisture, organic matter and C and N contents were determined immediately after sampling, as described previously (Webster et al., 2003).
Rhizosphere soil samples were also collected from 5 × 5 m quadrats at Aber in north Wales (map reference SH 23 651 723) and Garrigill in northern England (map reference NY 35 761 381) as described previously (Clegg et al., 2000). At these two sites, 50 cores were sampled randomly within the 5 m × 5 m quadrat (30 mm diameter × 50 mm depth) before pooling, sieving and freezing at −20°C.

Nucleic acid extraction and purification

Nucleic acids were extracted from soil using a modification of the method described by Kowalchuk et al. (1997). Homogenized soil (1.0 g wet weight) was placed in a sterile 50 ml centrifuge tube with 1.0 g of sterile acid-washed glass beads (150–212 μm diameter; Sigma), 1 ml of 0.1 M phosphate buffer (pH 7.8) and 1 ml of Tris-buffered phenol (Fisher Scientific). Cells were lysed by vortexing for 3 × 30 s at full speed with chilling on ice between vortexing. After centrifugation at 5500 g at 4°C for 10 min, the aqueous phase was removed and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by further centrifugation at 16 000 g at 4°C for 10 min. The aqueous phase was removed and extracted with an equal volume of chloroform:isoamyl alcohol (24:1) before further centrifugation. Crude aqueous extract (100 μl) was electrophoresed at 75 V for 1.5–2 h through a 0.8% (w/v) agarose gel stained with ethidium bromide (0.1 μg ml−1) to separate genomic DNA, rRNAs and humic acids. Under UV illumination, DNA of ≈ 20 kb in size and 16S rRNA bands were excised. DNA and 16S rRNA were purified from agarose using a Hybaid purification kit II or Bio101 RNAid kit (Anachem), respectively, according to the manufacturer’s instructions. Nucleic acid concentrations were determined by comparison against known concentrations of DNA and RNA in Hyperladder I DNA marker (Bioline) or 0.17–1.77 kb RNA marker (Life Technologies), respectively, after agarose gel electrophoresis.

To minimize RNase activity, all solutions were pretreated with 0.1% (v/v) diethyl pyrocarbonate (DEPC), with the exception of solutions containing Tris, for which DEPC-treated water was used. After incubation at 37°C for 12 h, DEPC was removed by autoclaving. All glassware was baked at 180°C overnight, and non-disposable plasticware was rinsed thoroughly with 0.1 M NaOH, 1 mM EDTA and RNase-free water. Electrophoresis tanks were cleaned with detergent, ethanol, 3% H₂O₂ and, finally, RNase-free water.

Reverse transcription and PCR amplification of 16S rRNA and tRNA

Reverse transcription was used to produce cDNA of 16S rRNA for PCR amplification. Ribosomal RNA (> 2 ng) was treated with DNase to remove residual DNA after agarose purification using RQ1 DNase (Promega) in a 10 μl reaction according to the manufacturer’s instructions. Reverse transcription was then performed using Superscript RNase H− reverse transcriptase (Life Technologies) in a 20 μl reaction according to the manufacturer’s instructions, using primer Ar9R (Jurgens et al., 1997) to select for archaeal rRNA (Table 2).

For analysis by DGGE, DNA and cDNA were amplified by a nested PCR approach using primers Ar3F and Ar9R (Jurgens et al., 1997) in the first round, and primers SAI (this study) and PARCH519r (Övreås et al., 1997) in the second round (Table 2). Halobacterium salinarum DNA (≈ 0.1 ng per reaction) was used as a positive control. To obtain sufficient PCR product for cloning, a semi-nested approach was used, in which first-round PCR products, amplified using primer set Ar3F/Ar9R, were amplified using primers Ar3F/Ar9R-H (using the cycling conditions used for SAI/PARCH519r). Primer sequences and thermal cycling conditions are described in Table 2. All PCR amplifications were performed in 50 μl reactions on a Hybaid Omn-E thermal cycler with hot-lid using Thermus flavus Biopro DNA polymerase (Bioline) at 1 unit per reaction, MgCl₂ at 2 mM, dNTPs at 1 mM and each primer at 0.4 μM. One μl of template was added to each reaction, containing either 10 ng of genomic DNA or cDNA equivalent to 0.1 ng of total 16S rRNA.

DGGE analysis

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad) according to the manufacturer’s instructions. Gels were prepared as described previously (McCaig et al., 2001) and contained a linear gradient of 35–60% denaturant [with 100% denaturing solution defined as 40% formamide (v/v) and 7 M urea (42% w/v)]. Gels were electrophoresed in 7 l of 1× TAE buffer at a constant temperature of 60°C for 5.5 h at 200 V. Gels were silver stained, scanned and analysed using Phoretix 1D gel analysis software as described previously (McCaig et al., 2001).

Cloning PCR products and recovery of DGGE bands for sequence analysis

PCR products were cloned using pGEM-T Easy vector (Promega) according to the manufacturer’s instructions before transformation into Escherichia coli DH5α cells made competent by the method of Chung et al. (1989). Transformed cells containing vector with inserts were selected by blue–white screening. White colonies were streaked onto fresh plates, and colony PCR was performed with primers SAI/PARCH519r using the conditions described previously. PCR products were screened by DGGE to select for clones of different sequence with different migration patterns. For sequencing, plasmids were isolated from broth cultures of E. coli using the QiAprep Miniprep kit (Qiagen) according to the manufacturer’s instructions.

To recover DGGE bands for sequence analysis, DGGE gels were stained in 300 ml of 1× TAE containing ethidium bromide (0.1 μg ml−1) for 30 min and destained in 1× TAE for a further 30 min. Under UV illumination, DNA bands were excised and placed in a sterile microcentrifuge tube. The polyacrylamide slice was then finely ground using a sterile pipette tip, and 25 μl of dH₂O was added. The sample was left for 12 h at 4°C, and 1 μl of the gel suspension was used for PCR amplification using the primers used in the original PCR amplification. A sample of the subsequent PCR product

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was electrophoresed through a DGGE gel to ensure that only one band was present before sequence analysis.

Sequence reactions of PCR products or cloned inserts were performed using the BigDye terminator sequencing kit (PE Biosystems) with PARCH519r or M13f/M13r sequencing primers respectively. Sequencing reactions were analysed using an ABI 377 automated sequencer (PE Biosystems). Sequences were aligned with representative archaeal 16S rRNA sequences retrieved from the GenBank database (National Centre for Biotechnology Information) using the GENETIC DATA ENVIRONMENT (GDE) program running in ARB (Strunk and Ludwig, 1996). Phylogenetic relationships were calculated using LogDet/Paralinear distance analysis (Lake, 1994) of variable sites (Lockhart et al., 1996) using PAUP (version 4.0; Swofford, 1998). The proportion of variable sites was estimated by maximum likelihood analysis of the most parsimonious trees constructed from a subset of the sequence data representing the major phylogenetic groups. Bootstrap support for alignments was calculated 1000 times.

Nucleotide accession numbers

SUPA clone sequences have been deposited in the GenBank database with the accession numbers AF512957 to AF512967. Sequences ba1-1 and bs-4 of DGGE bands, which did not exhibit 100% identity to other sequences, have been deposited with accession numbers AF512968 and AF512969 respectively.

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References


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