Soil pH regulates the abundance and diversity of Group 1.1c Crenarchaeota

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Abstract
Archaeal communities in many acidic forest soil systems are dominated by a distinct crenarchaeal lineage Group 1.1c. In addition, they are found consistently in other acidic soils including grassland pasture, moorland and alpine soils. To determine whether soil pH is a major factor in determining their presence and abundance, Group 1.1c community size and composition were investigated across a pH gradient from 4.5 to 7.5 that has been maintained for > 40 years. The abundances of Group 1.1c Crenarchaeota, total Crenarchaeota and total bacteria were assessed by quantitative PCR (qPCR) targeting 16S rRNA genes and the diversity of Group 1.1c crenarchaeal community was investigated by denaturing gradient gel electrophoresis (DGGE) and phylogenetic analysis. The abundance of Group 1.1c Crenarchaeota declined as the pH increased, whereas total Crenarchaeota and Bacteria showed no clear trend. Community diversity of Group 1.1c Crenarchaeota was also influenced with different DGGE bands dominating at different pH. Group 1.1c Crenarchaeota were also quantified in 13 other soils representing a range of habitats, soil types and pH. These results exhibited the same trend as that shown across the pH gradient with Group 1.1c Crenarchaeota representing a greater proportion of total Crenarchaeota in the most acidic soils.

Introduction
Molecular techniques have revealed the abundance and ubiquity of Archaea in all terrestrial and marine ecosystems (Fuhrman et al., 1992; Buckley et al., 1998) despite earlier indications that these organisms were restricted to extreme environments. The presence of members of the two major established archaeal kingdoms, Crenarchaeota and Euryarchaeota in nonthermophilic environments has been demonstrated by 16S rRNA gene surveys, with Crenarchaeota often more common in soil environments (Nicol et al., 2003). In particular, nonthermophilic ‘Group I’ Crenarchaeota have been reported in numerous environments including marine (Delong, 1992; Fuhrman et al., 1992) and freshwater environments (Jurgens et al., 2000), sediments (Hershberger et al., 1996; MacGregor et al., 1997; Schleper et al., 1997), bulk soil (Bintrim et al., 1997; Jurgens et al., 1997; Buckley et al., 1998; Sandaa et al., 1999; Ochsenreiter et al., 2003) and the rhizosphere and mycorrhizosphere (Simon et al., 2000; Bomberg et al., 2003). Estimates based on 16S rRNA gene copy numbers suggest that Crenarchaeota constitute 1–5% of the total prokaryotic community in soils (Buckley et al., 1998; Sandaa et al., 1999; Ochsenreiter et al., 2003), with some estimates as high as 12–38% (Kemnitz et al., 2007).

Evidence from both molecular and cultivation-based studies suggests that nonthermophilic Crenarchaeota play a role in ammonia oxidation (Treusch et al., 2005; Francis et al., 2007; Prosser & Nicol, 2008). Much of this evidence is based on analysis of the functional amnA gene, encoding a subunit of ammonia monooxygenase. Comparison of amnA gene abundance and transcript abundance suggests that ammonia-oxidizing archaea may be more active in the soil than ammonia-oxidizing bacteria (Leininger et al., 2006). In addition, all Group I Crenarchaeota cultivated so far are ammonia oxidizers. The only nonthermophilic Group 1.1a crenarchaeon isolated, Nitrospumilus maritimus, grows autotrophically with ammonia as the sole energy source (Könneke et al., 2005). In addition, two highly enriched cultures obtained from thermophilic environments, Nitrosocaldus yellowstoneii (de la Torre et al., 2008) and Nitrososphaera gargensis (Hatzenpichler et al., 2008), placed within...
Materials and methods

Site description, soil sampling and nucleic acid extraction

Soil samples were obtained from pH-manipulated plots at the Scottish Agricultural College on Craibstone Estate, NE Scotland (Grid ref. NJ 867112) in August 2006. The soil plots have been maintained at 0.5 pH unit intervals in the range 4.5–7.5 for the past 40 years, by the addition of lime or aluminium sulphate. The soil, which is a sandy loam, is subject to an 8-year crop rotation that minimizes the potential effects of vegetation. The soil had supported a crop of potatoes in the previous year. Three individual soil samples were obtained from the surface 10 cm of each plot, sieved through a 3.35-mm diameter metal mesh and stored at −20 °C. Dry weight was determined following drying at 105 °C for 24 h and total N and total C were determined on oven-dried soil using an automated Fison NA 1500 NCS Analyser mass spectrophotometer (Elemental Microanalysis Ltd, Okehampton, UK). Soil pH was determined after shaking a soil suspension for 15 min in deionized water [1:2 soil:water (w/v)] and settling for 30 min, and ammonia concentration was determined colorimetrically by flow injection analysis (FIA star 5010 Analyzer, Tecator) (Allen, 1989).

With the exception of pH, measured soil physicochemical characteristics showed little variability between plots and no significant trends were observed, as determined by ANOVA. The average water, carbon and nitrogen contents across all plots on an oven dry weight basis were 23.6(±0.4)%, 0.4(±0.02)% and 7.0(±0.4)%, respectively, and the average ammonia concentration was 1.5(±0.1) μg NH4+-N g−1 soil.

To compare with data obtained from the pH-manipulated plots, soil samples from a further 13 sites were obtained and represented a diverse range of soil, habitat and pH ranges (Table 1). For all sites, soil was collected from the top 0–20 cm depth. DNA was extracted from 0.5 g triplicate samples for all sites using the method of Griffiths et al. (2000) with some modifications (Nicol et al., 2005).

Primer design

Primers were designed to target Group 1.1c Crenarchaeota specifically in quantitative PCR (qPCR) and DGGE assays and to discriminate against other major crenarchaeal or bacterial groups. To design primers, sequences representing major crenarchaeal lineages, including 1.1a, 1.1b, 1.1c and 1.1c-associated groups, were retrieved from GenBank and aligned using CLUSTALW (Thompson et al., 1994) implemented in BIOEDIT sequence alignment editor (Hall, 1999). Using comparison of consensus sequences within each individual group and also Primrose (Ashelford et al., 2002), suitable discriminatory primer sets were identified and tested with
Groups 1.1b, 1.1c and 1.1c-associated crenarchaeal templates to verify their specificity for Group 1.1c Crenarchaeota and their ability to exclude 1.1b and 1.1c-associated Crenarchaeota frequently found in soil (in silico and PCR of cloned templates from different lineages).

**PCR and denaturing gradient gel electrophoresis (DGGE)**

A nested PCR strategy was used to obtain Group 1.1c amplicons for DGGE analysis for all samples. First-round PCR was performed using Group 1.1c specific primer 200F and the universal primer 1492R (Table 2). These products were then diluted and used for nested PCR amplification with Group 1-specific primers 771F and 957R (with GC clamp) (Table 2). For all standard end-point PCR, amplification was performed in 50-μL reaction mixtures consisting of 1 μL 50-fold diluted DNA extract (approximately 10 ng DNA), 1 × PCR buffer (Bioline, London, UK), 20 mM MgCl₂, 200 nM each primer, 1 mM dNTPs and 1 U Taq polymerase (Bioline). Cycling conditions used were 95 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 or 1 min (for first and second round, respectively), followed by one cycle of 72 °C for 10 min. DGGE analysis was performed using a 30–70% gradient containing 8% polyacrylamide as described previously (Nicol et al., 2005). Electrophoresis was performed at 75 V for 960 min in 1 × TAE buffer (at 60 °C) and gels were visualized by silver staining (Nicol et al., 2005) before scanning with Epson GT9600 scanner (Epson, Hemel Hempstead, Hertfordshire, UK).

**Phylogenetic analysis**

Based on the trends of differences in Group 1.1c crenarchaeal communities identified by DGGE analysis, cloning, screening and sequencing were performed to obtain clones representative of band positions of interest. Group 1.1c sequences were amplified from environmental samples with 200F and 1492R primers as described above, generating amplicons approximately 1300 bp in length. A total of six clone libraries of 16S rRNA genes were constructed from soils of pH 4.5, 6.0 and 6.5 (two, one and three clone libraries, respectively). However, due to poor amplification from one sample of pH 6.0 soil, a semi-nested PCR was performed with primers 200F and 957R, generating amplicons approximately 800 bp in length. PCR products were purified using a NucleoSpin Extract II Kit (Clontech, Mountain View, CA), cloned into pGEM-T easy vector system (Promega, Southampton, UK) and transformed into...
XL1-Blue supercompetent *Escherichia coli* cells (Stratagene, Cambridge, UK). Clones were screened for inserts using M13F/M13R vector primers before performing nested PCR using 771F and 957R-GC primers. DGGE was performed to screen for clones representing the major environmental DGGE bands. Selected clones were sequenced and included in phylogenetic analysis, with LogDet/Paralinear distances and tree construction performed using *PAUP* v4.01 (Swofford, 1998) as described previously (Nicol et al., 2005). Bootstrap support was calculated using for distance (*PAUP*), parsimony (*PAUP*) and maximum likelihood (*PHYML*; Guindon & Gascuel, 2003) methods (1000, 1000 and 100 bootstrap replicates, respectively).

**qPCR**

The abundance of 16S rRNA genes from Group 1.1c, total Group 1 Crenarchaeota and total Bacteria was determined by qPCR amplification of 16S rRNA genes of extracted DNA. qPCR was performed using the BioRad MyIQ Single-Color Real-Time PCR Detection System (Biorad, Hertfordshire, UK) with Qiagen QuantiFast SYBR Green PCR Master Mix (Qiagen, West Sussex, UK). Reaction mixtures of 25 μL contained 12.5 μL Qiagen Master Mix, 400 nM of each primer for bacterial (1369F and 1492R) and general crenarchaeal (771F and 957R) assays and 1200 nM of each primer for the Group 1.1c assay (200F and 385R) (Table 2). qPCR standards were generated by PCR amplification of crenarchaeal 16S RNA genes from extracted DNA. Clones were screened for Group 1.1c sequences (using specific primers and confirmation by sequencing) before amplification of the insert and quantification. A bacterial qPCR standard was generated by amplifying and quantifying the 16S rRNA gene of *E. coli*, using primers 27F and 1492R (Table 2). For all runs, amplification of a serial dilution of standard template (in triplicate) from 10⁶ to 10⁴ copies was used to generate standard curves. Amplification efficiencies for all reactions ranged from 99.4% to 111.5% with an r² value of > 0.99 for standard curve regression. For all qPCR assays, the following cycling conditions were used: 95 °C for 15 min, followed by denaturation at 94 °C for 10 s, combined annealing and extension for 30 s and fluorescence reading at 72 °C for 6 s, followed by 72 °C for 10 min. To test the specificity of the qPCR, after each run a melting curve analysis was performed between 72 and 95 °C to determine the presence of specific products. The PCR products were also subject to standard agarose gel electrophoresis to ensure amplicons of the expected size only were produced. Ten nanograms of extracted environmental DNA was used as template.

**Statistical analysis**

The effect of pH on gene abundances was analysed by one-way ANOVA of the ln-transformed data for Group 1.1c Crenarchaeota and nontransformed data for total Crenarchaeota and Bacteria. A Student’s *t*-test was carried out to compare the abundances of Bacteria and Crenarchaeota. Levene statistics was used to test for the homogeneity of variances. All statistical analyses were performed using the SPSS statistics package (SPSS Inc., Chicago, IL).

**Accession numbers**

All sequences have been deposited in the GenBank database with accession numbers GQ141959–GQ141977.

**Results**

**Effects of pH on Group 1.1c crenarchaeal community abundance**

Abundances of Group 1.1c, total Crenarchaeota and total bacteria were determined by real-time PCR amplification of 16S rRNA genes using group-specific primers. Group 1.1c was detected only is soils at pH 4.5–6.0 (detection limit 2.5 × 10⁵ copies g⁻¹ dry soil). The abundance of Group 1.1c Crenarchaeota was greatest (3.8 × 10⁶ 16S rRNA gene copies g⁻¹ dry soil) at the lowest pH and gene abundance declined steadily as pH increased (Fig. 1). There was at least one order of magnitude difference in 16S rRNA gene abundance between soils at pH 4.5 and 6.0 and, given the lack of detection at pH values > 6.5, the difference in abundance between the two pH extremes studied, 4.5 and 7.5, was more than two orders of magnitude. Statistical analysis confirmed a significant effect of soil pH on Group 1.1c crenarchaeal abundance (*ANOVA*, *P* < 0.001). In contrast to Group 1.1c Crenarchaeota, the 16S rRNA gene abundances of total Crenarchaeota and total Bacteria did not vary significantly with soil pH (Fig. 1) (*P* = 0.536 and *P* = 0.215, respectively). Bacterial 16S rRNA gene abundance was significantly greater than that of Crenarchaeota (Student’s *t*-test, *P* < 0.001) with proportions of total Crenarchaeota : total Bacteria ranging from 1.1% to 2.2%. Group 1.1c Crenarchaeota constituted 0.1–1.8% of total crenarchaeal abundance in plots where quantification was possible.

**Effects of soil pH on crenarchaeal community structure**

pH-associated changes in Group 1.1c community structure were analysed by DGGE of amplified 16S rRNA gene fragments. A nested PCR approach was used for all samples, and the reproducibility of DGGE profiles between biological field triplicates was high at low pH but less so in the neutral pH (7.0 and 7.5) soils (Fig. 2). This may reflect lower abundances of Group 1.1c 16S rRNA gene numbers at neutral pH, resulting in increasingly stochastic amplification. The DGGE profiles from the lowest pH soils were

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Archaea in acid soil

dominated by three major bands, at positions I, II and IV (Fig. 2). The relative intensity of DGGE band position IV decreased with increasing pH and the band was not detectable in many neutral pH replicates. A similar impact of pH was seen for band positions I and II. While the relative intensity of these bands did not diminish greatly, they were not detectable in many replicates from soils of pH 6.5 or higher. Band positions III and V showed a reverse trend, increasing in intensity as pH increased. They were, however, absent from pH 7.5 soil, possibly due to low abundance of 1.1c Crenarchaeota, as for most other phylotypes detected by DGGE at lower pH. Several additional minor bands appeared sporadically in at least one of the triplicates from samples of pH 5.5 or above, but these showed no detectable trend with pH and probably account for a relatively small part of the community.

**Phylogenetic analysis**

Clone library analysis and sequencing were performed to determine the identity of major DGGE bands of interest and confirm affiliation within Group 1.1c. For five libraries, ~1300 bp products were cloned. For one library (pH 6.0), a nested PCR strategy was required to generate sufficient product for cloning. Individual clones were then amplified with the DGGE primer set for screening by DGGE and comparison with the environmental DGGE profiles. Between two and eight clones comigrating with each DGGE band of interest were chosen for sequencing. Where possible, clones with the same band position were selected from libraries obtained from soil samples of different pH. Phylogenetic analysis was performed on 619 unambiguous positions using variable sites only (65%) with reference crenarchaeal Group 1.1c sequences from various forest soils, moorland and alpine soils (Fig. 3). A marker composed of these clones was run alongside the environmental samples in DGGE analysis (Fig. 2) and most sequences showing identical migration patterns formed monophyletic groups in phylogenetic analyses (Fig. 3). Clones sequenced in this study were closely related to Group 1.1c 16S rRNA gene sequences from a variety of other acidic soils, including coniferous forest and mature alpine soils. Phylogenetic clusters corresponding to band positions I and V contained DNA sequences from both low and neutral pH soils, while the other clusters originated from several clone libraries of one pH. Sequences dominating at one pH range do not appear to be monophyletic.

**Abundance of Group 1.1c Crenarchaeota in other soil systems**

In order to verify findings on the influence of pH on Group 1.1c crenarchaeal abundance at Craibstone, 13 other soils with different characteristics and varying pH were investigated by qPCR of Group 1.1c, crenarchaeal and bacterial 16S rRNA genes. The highest abundance of Group 1.1c was $1.3 \times 10^7$ copies g$^{-1}$ dry soil in a Welsh podzol of pH 4.3 (Fig. 4; Table 1). High abundances of Group 1.1c were also observed in acidic moorland, coniferous and deciduous forest soils in the Cabrach area (north-east Scotland) (Fig. 4; Table 1, sites 1, 2, 3, 5 and 6). Group 1.1c at these sites accounted for between 36% and 69% of total Crenarchaeota and the 16S rRNA gene copy numbers ranged from $8.8 \times 10^5$ to $9.3 \times 10^6$ copies g$^{-1}$ dry soil. A grassland soil (site 9),...
which was located in the vicinity of these sites and possessed the same parent material, had a higher pH and low abundance of Group 1.1c, which comprised only 0.9% of the total crenarchaeal community at this site. Although Crenarchaeota were detected in all soils investigated, Group 1.1c were below detection at several sites of near-neutral pH (Fig. 3). Abundances of total Crenarchaeota and Bacteria varied between soils by up to two orders of magnitude.

Discussion

Effect of pH on the microbial community size and structure

Real-time PCR quantification of 16S rRNA genes demonstrated little variation and no apparent trends in total crenarchaeal abundance with soil pH but greater abundance of Group 1.1c Crenarchaeota in low pH soils. This is

![Fig. 4. Quantification of 16S rRNA gene abundance of Group 1.1c Crenarchaeota, total Group 1 Crenarchaeota and total Bacteria in a range of soils with contrasting habitat and soil properties. Error bars represent SEs of means of three biological replicates. Soil characteristics are described in Table 1.](image-url)
consistent with results of earlier ecological soil surveys, in which Group 1.1c Crenarchaeota have been found in the most acidic soil sites (Nicol et al., 2005; Oline et al., 2006; Bomberg & Timonen, 2007). Soil pH has previously been described as a major factor in determining microbial community structure and abundance (Fierer & Jackson, 2006) and Group 1.1c appear to support this view. Nicol et al. (2008) demonstrated a pH-related decrease in crenarchaeal amoA gene abundance, in the soil pH plots investigated in this study, which corresponded to an increase in soil pH and a simultaneous structural change in crenarchaeal community (Nicol et al., 2008). Whereas their observations could be explained in terms of established mechanisms for inhibition of ammonia oxidation at low pH, nothing is known of Group 1.1c function, and associated amoA genes have not been identified. Comparative abundances of archaeal amoA genes from the study by Nicol et al. (2008) and 16S rRNA genes presented here are similar, but differences do exist in the overall trends, with crenarchaeal amoA abundance decreasing with increasing pH while 16S rRNA gene copies remain relatively constant. This may indicate that other Crenarchaeota are present, particularly in high pH soils that do not possess ammonia monoxygenase genes or may indicate issues with primer specificity or bias. Our data agree with previous estimates of crenarchaeal 16S rRNA gene abundances (around 10^7 copies g^-1 dry soil) and ratios of Crenarchaeota to Bacteria in soil environments (around 1%) (Buckley et al., 1998; Ochsenreiter et al., 2003). The lower abundance of Group 1.1c Crenarchaeota at neutral pH was not associated with a general reduction in prokaryote abundance, but with changes in relative abundance.

Even at low pH, Group 1.1c Crenarchaeota accounted for only a modest proportion of total Crenarchaeota. This is perhaps unsurprising as this study was performed on an arable soil, and Group 1.1c populations have been shown to be a minor component of total archaeal communities in arable and pasture soils (Nicol et al., 2003, 2008). The field site was selected due to the long-term maintenance of a pH gradient with other factors (e.g. vegetation) remaining constant and previous detection of Group 1.1c (Nicol et al., 2008). Comparison of a range of soils from other sites revealed higher abundance of Group 1.1c at acidic forest sites and in moorland, where they constituted a significant component or even the majority of crenarchaeal community. They were also absent or below detection in soils near neutral pH (pH > 5.8), which agrees with the qPCR data from the pH-adjusted plots, where Group 1.1c was below detection at pH > 6.5.

At lower soil pH values in the Craibstone plots, the biological replicates were consistent and trends in relative band intensities in DGGE profiles suggest adaptation of different phylotypes within Group 1.1c to specific pH ranges. The decrease in the reproducibility between replicates from the higher pH soils is probably indicative of low template copy numbers, which leads to stochastic amplification (Ou et al., 2005). Group 1.1c 16S rRNA gene abundance was below the detection limit of 2.5 × 10^4 copies g^-1 dry soil by qPCR (which corresponds to 10 copies per qPCR reaction). It is likely that PCR products were only detected in DGGE analysis because all the samples were subjected to nested amplification (i.e. another 35 cycles of PCR).

**Phylogenetic analysis**

DGGE bands with different migration behaviour fell into distinct monophyletic groups, which were consistent for comigrating clones from different clone libraries, and relationships were therefore seen between phylogenetic groups and soil pH. Thus, clones corresponding to DGGE positions I and V (Figs 2 and 3) were derived from both pH 4.5 and 6.5 soils, while other DGGE band positions contained clones from one pH only. Clones associated with DGGE positions III and V may represent phylotypes adapted to soils of higher pH, although they are polyphyletic. The most closely related database sequences originate from a moorland-coniferous forest transect and developed alpine soils, which have pH lower than any studied here (Nicol et al., 2005, 2007), suggesting that these Crenarchaeota may be able to inhabit soils over a wide acidic pH range.

**Potential biases and methodological limitations**

The abundance of different microbial groups was estimated by the qPCR analysis of 16S rRNA genes. A recognized limitation of this approach is variable 16S rRNA gene copy number per genome, which in bacteria ranges from 1 to 13 depending on ecological strategy (Klappenbach et al., 2000). While Euryarchaeota may possess up to four copies of 16S rRNA genes per genome, thermophilic Crenarchaeota only have one copy (Lee et al., 2009). The genomes of _N. maritimus_ and _Cenarchaeum symbiosum_, which are currently the only available nonthermophilic crenarchaeal genomes, each have only one ribosomal operon. This suggests that crenarchaeal 16S rRNA gene copy numbers measured by qPCR may be representative of cell numbers in the studied soils. In addition, primer biases cannot be entirely ruled out. In a mixed template population (e.g. environmental DNA), the choice of primers can introduce a stronger bias than the template (Suzuki & Giovannoni, 1996). The efficiency of nucleic acid extraction and reproducibility of standard curve reconstruction may affect the outcome of qPCR. Because the same extraction method was used for all samples, and biological replicates were similar, it is unlikely that significant bias resulted from the nucleic acid extraction procedure. Also amplification efficiencies, linear regression coefficients, melting curves and reproducibility of all qPCR assays were optimal.
Potential function of Group 1.1c Crenarchaeota

Group 1.1c Crenarchaeota represent a greater proportion of all Crenarchaeota in acidic soils and may have an important ecological function under specific conditions. Although some organisms within the related Groups 1.1a and 1.1b lineages are confirmed ammonia-oxidizing organisms, there is no evidence to suggest that Group 1.1c are involved in nitrogen transformations. However, there is indirect evidence for the presence of the amoA gene in the marine 'ALOHA' group (Mincer et al., 2007), which forms a monophyletic lineage with both the 'PSL12' group and Group 1.1c Crenarchaeota (Prosser & Nicol, 2008). Together they also belong to a potentially monophyletic group of archaeal ammonia oxidizers, the deepest branching member of which is the thermophile N. yellowstonei (del la Torre et al., 2008).

Conversely, however, there is evidence that some Crenarchaeota within this group do not have the potential to oxidize ammonia. Agogué et al. (2008) demonstrated that a specific subcluster of marine Group 1.1a Crenarchaeota may lack the amoA gene and instead may have a heterotrophic lifestyle (Agogué et al., 2008). Ammonia availability is limited under acidic conditions and, while nitrifying activity has been demonstrated in many acid soils, it often requires specific adaptations, which Group 1.1c may or may not possess (De Boer & Kowalchuk, 2001).

Association with ectomycorrhizae may influence Group 1.1c composition and increase archaeal diversity (Bomberg & Timonen, 2007). However, the crop species grown at the Craibstone field site (turnip, potato, grasses, wheat and barley) are known to be either nonmycorrhizal or have arbuscular mycorrhizal associations. The field site was also subjected to an 8-year crop rotation cycle and each plot contained the same plant species at one time. The most recent crop supported was potato, which possesses arbuscular mycorrhiza. Biases introduced by vegetation or the presence of mycorrhiza are therefore unlikely. Although other biotic (e.g. arbuscular mycorrhizas, plant species) and abiotic (e.g. forms and amounts of carbon and nitrogen) factors will most likely drive crenarchaeal diversity and abundance in soils, these results indicate that soil pH is a major determinant of the abundance of Group 1.1c Crenarchaeota.

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