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Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms

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Summary

Ammonia oxidation, as the first step in the nitrification process, plays a central role in the global cycling of nitrogen. Although bacteria are traditionally considered to be responsible for ammonia oxidation, a role for archaea has been suggested by data from metagenomic studies and by the isolation of a marine, autotrophic, ammonia-oxidizing, non-thermophilic crenarchaeon. Evidence for ammonia oxidation by non-thermophilic crenarchaea in marine and terrestrial environments is largely based on abundance of bacterial and archaeal ammonia monooxygenase (*amo*) genes, rather than activity. In this study, we have determined the influence of temperature on the response of ammonia-oxidizing bacteria and archaea in nitrifying soil microcosms using two approaches, involving analysis of transcriptional activity of 16S rRNA genes and of a key functional gene, *amoA*, which encodes ammonia monooxygenase subunit A. There was little evidence of changes in relative abundance or transcriptional activity of ammonia-oxidizing bacteria during nitrification. In contrast, denaturing gradient gel electrophoresis analysis of crenarchaeal 16S rRNA and crenarchaeal *amoA* genes provided strong evidence of changes in community structure of active archaeal ammonia oxidizers. Community structure changes were similar during incubation at different temperatures and much of the activity was due to a group of non-thermophilic crenarchaea associated with subsurface and marine environments, rather than soil. The findings suggest a role for crenarchaea in soil nitrification and that further information is required on their biogeography.

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Introduction

Molecular techniques have demonstrated that *Archaea*, traditionally associated with extreme environments, constitute a significant proportion of prokaryotic cell abundance in non-thermophilic environments. In particular, 'Group 1' crenarchaea (DeLong, 1998) represent significant proportions of prokaryotes in marine plankton (e.g. Karner *et al*., 2001; Herndl *et al*., 2005) and ≥ 1% of soil prokaryotes (Sandaa *et al*., 1999; Ochsenreiter *et al*., 2003). The abundance of crenarchaea implies their importance in biogeochemical cycles and ecosystem processes, while the different physiological characteristics of archaea and bacteria provide the potential for significant differences in process characteristics and rates from those of already cultivated groups.

Recent metagenomic studies (Schleper *et al*., 2005; Treusch *et al*., 2005; Hallam *et al*., 2006) have demonstrated co-occurrence of crenarchaeal 16S rRNA genes and genes homologous to those encoding bacterial ammonia monooxygenase, AMO, a key functional enzyme in ammonia oxidation, the first step in nitrification. Crenarchaeal *amoA* genes appear to be ubiquitously distributed in soils, sediments and aquatic habitats (Francis *et al*., 2005) and quantification of bacterial and archaeal *amoA* genes in terrestrial environments (Leininger *et al*., 2006) indicates that ammonia-oxidizing archaea may be more abundant than ammonia-oxidizing bacteria. In marine environments, the abundance of *amo* genes appears to be correlated with ammonia oxidation (Wuchter *et al*., 2006). There is also evidence from the analysis of natural carbon abundances in crenarchaeal tetraether lipids and incorporation of inorganic ^{13}C into crenarchaeal lipids (such as crenarchaeol) for autotrophic metabolism by non-thermophilic crenarchaea (Pearson *et al*., 2001; Wuchter *et al*., 2003; Ingalls *et al*., 2006). The ability of non-thermophilic crenarchaea to oxidize ammonia was confirmed by cultivation of the first non-thermophilic crenarchaeon, *Nitrosopumilus maritimus*, a chemolithotrophic marine ammonia oxidizer (Könneke *et al*., 2005), which possesses *amoA*, *amoB* and *amoC* gene homologues with sequences similar to those found in metagenomic studies and in environmental surveys.

N. maritimus grows chemolithoautotrophically by aerobically oxidizing ammonia to nitrite and physiological characteristics (cell yield on ammonia, maximum specific growth rate) are similar to those of chemolithoautotrophic ammonia-oxidizing bacteria.

The microbial oxidation of ammonia is a key process in the global cycling of nitrogen. It determines the balance between oxidized and reduced forms of nitrogen in terrestrial and aquatic ecosystems, limits throughput of material in wastewater treatments processes and significantly reduces quality of groundwater, through production of nitrate, and of the atmosphere, through production of the greenhouse gas nitrous oxide. Knowledge of the key players in nitrification, and measurement and prediction of their activity, is therefore essential for environmental and economic reasons.

Most of the evidence for archaeal ammonia oxidation in the environment is based on the presence and relative abundance of 16S rRNA and functional marker genes, rather than assessment of activity. Discrimination of ammonia-oxidizing activity by bacteria and archaea is currently not possible at the process level. However, the presence and activity of the organisms can be discriminated through sequence differences in their respective 16S rRNA and *amoA* genes. These differences are exploited in this study to determine growth and activities of archaeal and bacterial ammonia oxidizers, as determined by taxonomic and functional genes, during soil nitrification and to assess potential differences in relative activities with temperature. In using molecular techniques, particularly those involving polymerase chain reaction (PCR) amplification, biases associated with nucleic acid extraction, choice of primers, amplification efficiency, gene copy number and other factors must be considered when interpreting results. Approaches employing functional genes also make assumptions about gene function in the environment and characterization of ammonia oxidizers through analysis of *amoA* genes is based on the assumption that *amoA* gene products are involved in ammonia oxidation. For this reason, both archaeal and bacterial ammonia oxidizers characterized using this approach should be considered as putative ammonia oxidizers.

Results and discussion

Process response to temperature

Nitrification was preceded by an apparent lag phase in all soil microcosms, with undetectable or small increases in nitrite + nitrate concentration within the first 12 days of incubation. This lag period may reflect the time necessary for cells to adjust to the conditions of the experiment, as soil was refrigerated prior to establishing the microcosms. Alternatively, nitrite + nitrate production may have been undetectable due to low ammonia oxidizer abundance. After 12 days, nitrite + nitrate production increased significantly ($P < 0.05$), correlating positively with temperature, up to concentrations of 14 and 61 μg N g^{-1} soil at 10°C and 30°C, respectively, after incubation for 55 days (Fig. 1). During this period, ammonia concentration decreased from 7 to less than 1 μg NH_4^+-N g^{-1}, through oxidation to nitrite and nitrate, and further product formation was supported by ammonia that was presumed to be generated by mineralization. The influence of temperature on ammonia oxidation is typical of other studies, with reported optimum temperatures for potential nitrification in the range 20–37°C (Stark, 1996).

Influence of temperature on ammonia oxidizer community structure

Community structure and composition of active archaeal and bacterial ammonia oxidizers were characterized by denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene transcripts amplified from RNA extracted from soil after incubation of microcosms for 40 days. Denaturing gradient gel electrophoresis profiles of bacterial ammonia oxidizers across all incubation temperatures were dominated by seven bands (Fig. 2A). Only two phylotypes, represented by bands 1 and 2, were influenced by temperature. Relative intensity of both bands decreased (ANOVA, $P < 0.05$), but only at 30°C, and these bands represented a relatively small proportion of the total community. In contrast, archaeal community DGGE profiles contained six bands (Fig. 2B) which were influenced by temperature. Relative intensities of bands 3 and 4 increased with temperature, while those of the remainder decreased (ANOVA, $P < 0.05$). Effects were

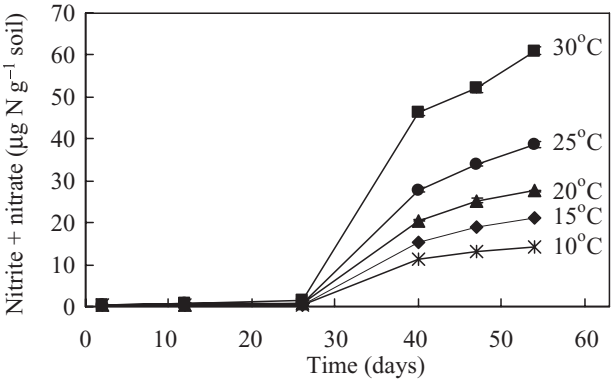

Fig. 1. The influence of temperature on soil nitrification kinetics, assessed as changes in the concentrations of nitrite and nitrate in KCl-extracted soil solution during incubation of soil microcosms at 10°C, 15°C, 20°C, 25°C and 30°C for 55 days. Results are means calculated from triplicate microcosms and all standard errors were less than the symbol size.

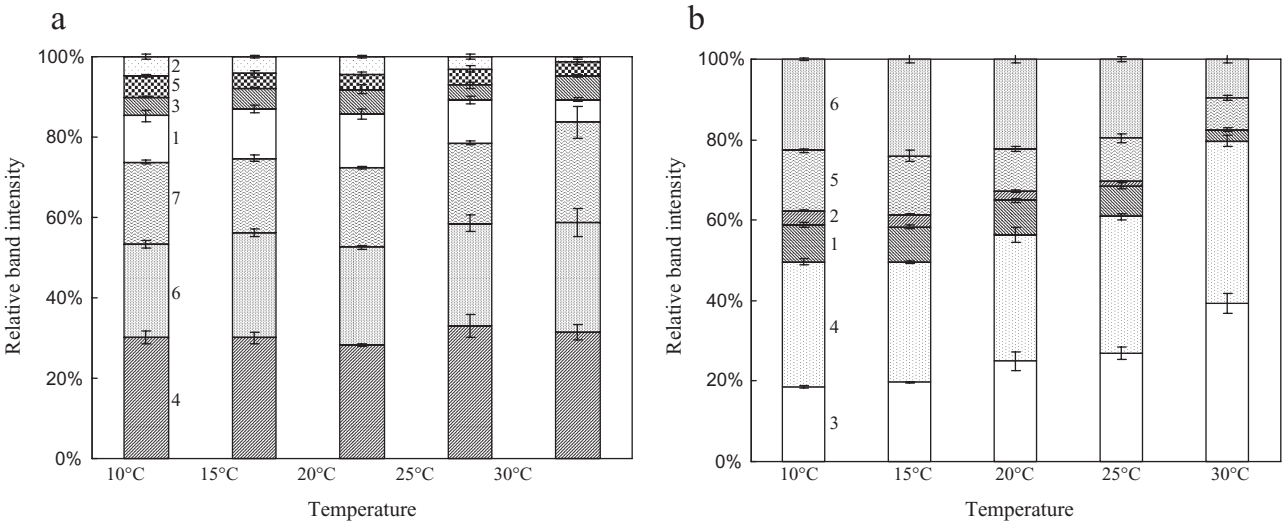

Fig. 2. The influence of temperature on changes in the relative abundance of 16S rRNA gene transcripts of major phylotypes of active ammonia-oxidizing bacteria and crenarchaea during incubation of soil microcosms for 40 days at temperatures in the range 10–30°C. Relative abundance was determined by DGGE analysis of amplicons following amplification, from extracted RNA, of 16S rRNA genes using primers specific for ammonia-oxidizing bacteria (a) and crenarchaea (b). Histograms represent relative intensity of bands within individual lanes in DGGE gels derived from triplicate microcosms and are presented as means of triplicate values and standard errors. Numbers relate to bands whose relative intensities are referred to in the text.

greatest at 30°C, where band 2 was not detectable, but were also significant at other temperatures. Soil nitrification was therefore associated with significant changes in the relative abundances of different phylotypes.

To determine which members of the ammonia oxidizer communities were active, DGGE analysis was also performed on *amoA* gene transcripts. Reproducible DGGE patterns between replicate microcosms were not obtained following amplification of bacterial *amoA* gene transcripts (data not shown), presumably due to stochastic effects arising from low levels of template concentration (Taberlet *et al.*, 1996). Indeed, a gene-specific reverse primer was required to increase sensitivity (Lekanne Deprez *et al.*, 2002) in order to detect bacterial *amoA* transcripts. In addition, amplification efficiency was reduced when using primers used prior to DGGE analysis, due to incorporation of a GC clamp. Reproducible DGGE profiles of archaeal *amoA* gene transcripts were obtained from all replicate microcosms (Fig. 3). Changes in community structure became evident earlier at higher temperatures and, interestingly, changes in relative intensities were detected after incubation for only 12 days, before detection of increases in nitrite and nitrate concentrations. Band 5 was undetectable at 2 days but relative intensity of this band and of band 6 increased with time and with temperature (ANOVA, $P < 0.05$). The relative intensities of bands 1, 3 and 4 generally decreased with time and with temperature. Band 1 was not detectable at day 2, but appeared at other sampling times at most temperatures, but not at 30°C.

Changes in patterns of *amoA* gene transcript relative abundance were most evident at 30°C and provide evidence of selective growth of archaeal, rather than bacterial ammonia oxidizers, and of selection for particular archaeal ammonia oxidizer phylotypes within soil microcosms undergoing active nitrification. Temporal changes in patterns were similar to those at different temperatures. It is likely therefore that changes in community were due to selective growth and increased activity of particular groups of archaeal ammonia oxidizers, and that these changes were more evident at higher temperature due to higher growth rate.

Phylogenetic analysis of crenarchaeal 16S rRNA and amoA *gene sequences*

In previous studies of this soil (e.g. Stephen *et al.*, 1998), all sequences amplified by the bacterial ammonia oxidizer primers used here fell within recognized bacterial ammonia oxidizer phylogenetic groups. However, to determine identity of crenarchaeal DGGE bands, clone sequences (CBS16S1–6) representing the six dominant band positions in 16S rRNA gene DGGE profiles (Fig. 2b, bands 1–6) were subjected to phylogenetic analysis (Fig. 4a). A minimum of three clones obtained from two independent clone libraries were included for each band position. As expected, clones that exhibited identical migration in DGGE gels formed monophyletic groups to the exclusion of sequences representing other migration positions. The PCR primers used for first-round

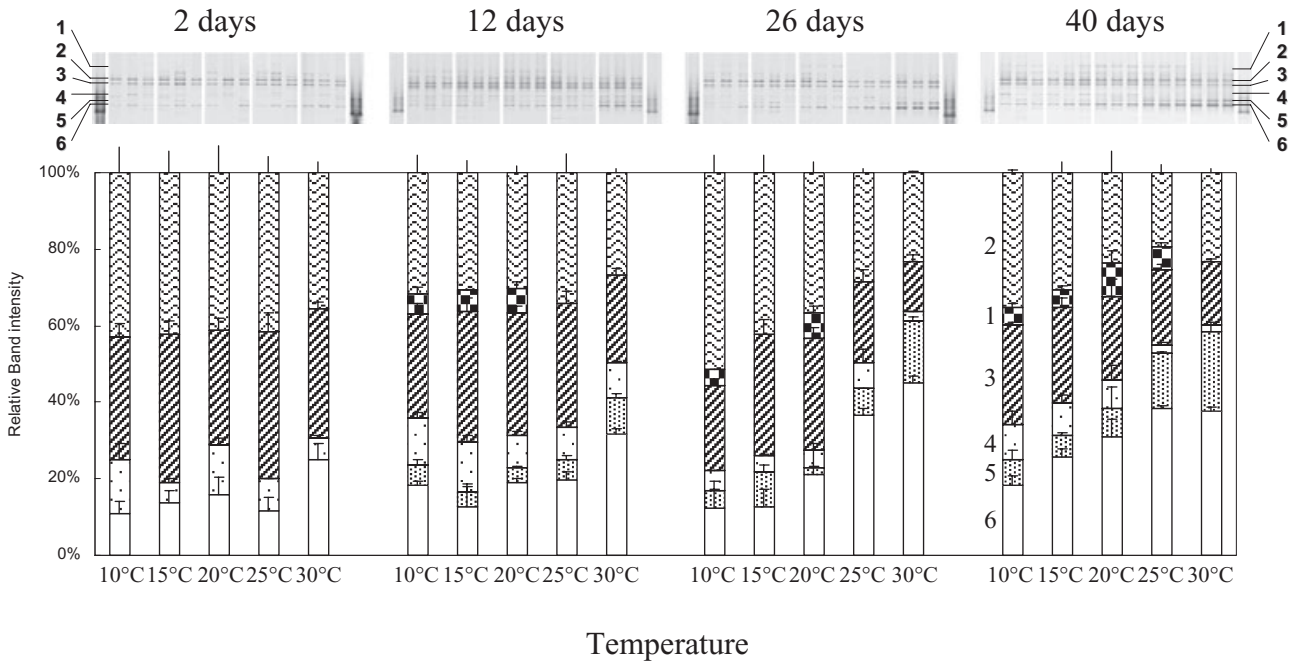

Fig. 3. Changes in transcriptional activity of different archaeal ammonia oxidizer phylotypes during incubation of soil at temperatures ranging from 10°C to 30°C. Activity was determined by DGGE analysis of archaeal *amoA* gene transcripts in soil microcosms incubated at temperatures in the range 10–30°C and sampled at 2, 12, 26 and 40 days. Histograms represent relative intensity of bands within individual lanes in DGGE gels derived from triplicate microcosms and are presented as means of triplicate values and standard errors. Numbers relate to bands that were sequenced and whose relative intensities are referred to in the text.

amplification of 16S rRNA genes were biased to non-thermophilic crenarchaeal groups and therefore it was presumed that these sequences would be placed within Group 1 'soil' lineages (e.g. 1.1b). In fact, sequences associated with band positions 3 and 4 (Fig. 2B), which increased in relative intensity with temperature, were placed within two quite distinct phylogenetic clusters. Band position 4 sequences (CBS16S4) were placed within the Group 1.1b lineage, which is generally considered to be associated with soil habitats. However, band position 3 (CBS16S3) sequences were surprisingly placed within the Group 1.1a lineage. Although this lineage is dominated by sequences retrieved from marine habitats, the three CBS16S3 sequences were associated with a clade containing sequences retrieved mainly from terrestrial subsurface.

Sequences representing six dominant band positions in crenarchaeal *amoA* gene transcript profiles (CBSamoA1–6) were also subjected to phylogenetic analysis (Fig. 4b). Previous analyses have revealed the presence of phylogenetic clades dominated by either soil or marine sequences, as for crenarchaeal 16S rRNA gene phylogenies. As observed for the 16S rRNA gene phylogeny, some *amoA* sequence groups (CBSamoA1, 5 and 6 were placed in the 'marine' lineage). Also, band positions that increased in relative intensity with increasing temperature (CBSamoA5 and 6) were associated exclusively within this sequence group, and again clustered with sequences retrieved from subsurface environments. Therefore, both 16S rRNA and *amoA* gene phylogenetic analyses provide evidence that crenarchaea associated with a lineage containing sequences from marine and subsurface habitats exhibited a temperature response.

The lack of an increase in the relative intensity of *amoA* DGGE bands associated with the 'soil' lineage contrasts with that of 16S rRNA gene sequences analysis. Although it is possible that crenarchaeal *amoA* gene phylogenies are not congruent with 16S rRNA phylogeny, the recovery of soil and marine sequences in various studies (e.g. Francis *et al*., 2005; Hallam *et al*., 2006; Leininger *et al*., 2006) that fall in two distinct clades (which include those of *N. pumilus*, *Cenarchaeum symbiosum* and soil fosmid 54d9) would suggest that this is not the case. It is therefore possible that the increase in the CBS16S4 sequence group in DGGE profiles reveals a temperature response of a crenarchaeal group that does not possess an associated *amoA* gene.

Conclusion

Two different molecular approaches failed to detect significant changes in bacterial ammonia oxidizer

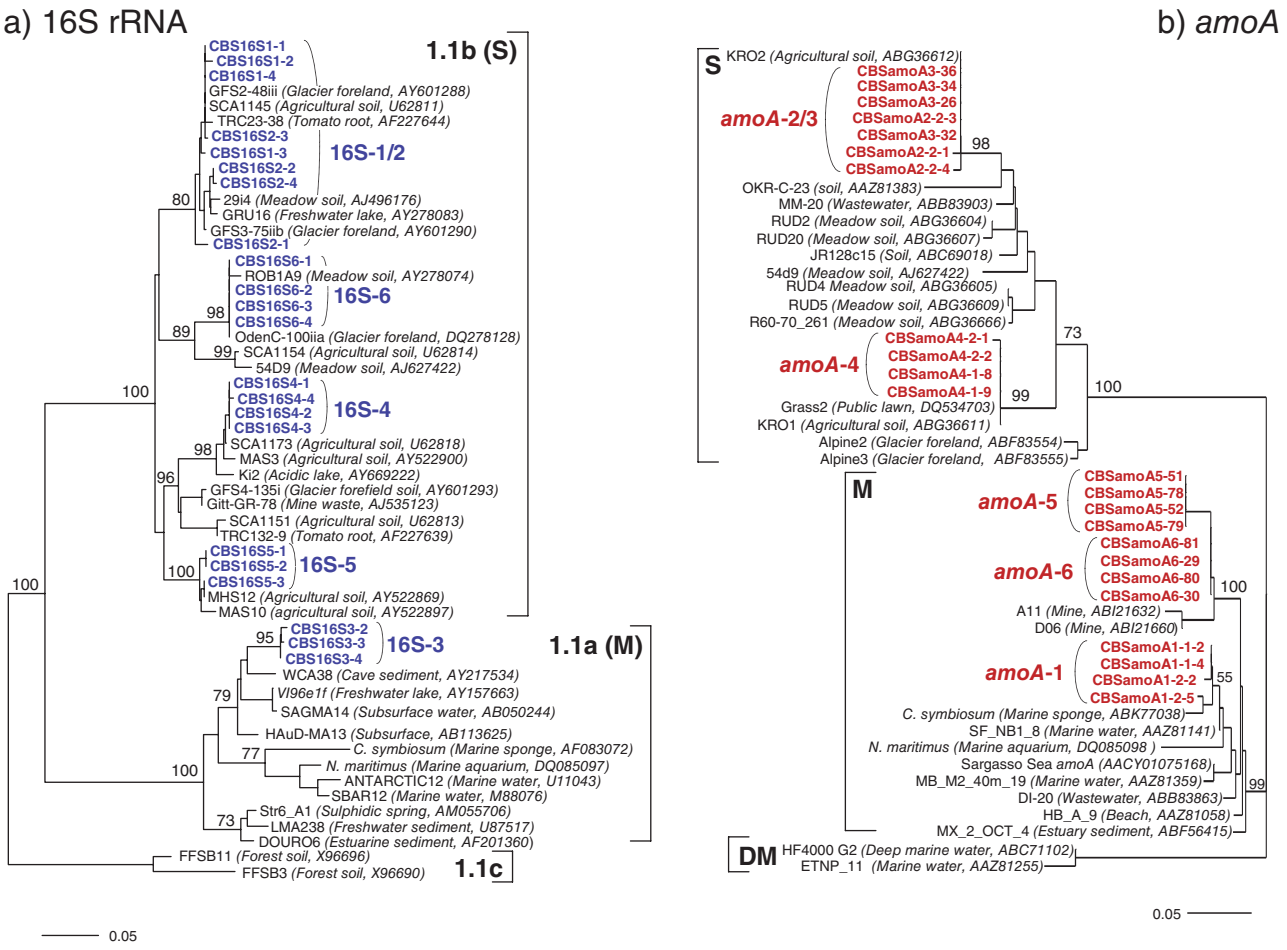

Fig. 4. Phylogenetic analysis of cloned crenarchaeal 16S rRNA and *amoA* gene PCR products.
A. 16S rRNA gene sequences representative of six DGGE band positions. LogDet/Paralinear pair-wise distances of unambiguously aligned positions were calculated using variable sites only (312 positions) estimated from a maximum-likelihood model. Bootstrap support was calculated using maximum-likelihood, distance and parsimony methods (1000 replicates respectively) with values at major nodes representing the most conservative value from all three methods (expressed as a percentage). Reference sequences are described as 'Name (environmental source, accession number)'. Clades labelled 1.1a (M) and 1.1b (S) are Group 1 16S rRNA-defined lineages dominated by sequences retrieved from marine and soil environments respectively. The tree was rooted with two sequences placed within the 1.1c lineage.
B. *amoA* genes representative of six DGGE band positions. Pair-wise distances of 159 aligned positions were calculated using the JTT substitution model with site variation (invariable sites and eight variable gamma rates). Bootstrap support was calculated using maximum-likelihood, distance and parsimony methods (1000 replicates) with values at major nodes representing the most conservative value from all three methods (expressed as a percentage). Clades labelled M and S are dominated by sequences retrieved from marine and soil environments respectively. The tree was rooted with two sequences belonging to a previously characterized deep marine water clade (DM).

community structure during nitrification. In contrast, there were significant changes in archaeal ammonia oxidizer community structure with incubation time which were accentuated at higher incubation temperatures. Intriguingly, most changes in community structure were associated with increases in relative abundance of sequences types associated with marine and subsurface archaea, rather than soil archaea. It is possible that, under conditions other than those used in the soil microcosms, bacterial ammonia oxidizers may be more active than archaeal ammonia oxidizers. Nevertheless, under the conditions used in this study (which are typical of soil microcosm nitrification studies) there were no signs of significant changes in bacterial ammonia oxidizer community structure during active nitrification, while there was significant evidence for changes in community structure of putative archaeal ammonia oxidizers. These results must be considered within the context of potential biases arising from molecular approaches. Nevertheless, they provide compelling evidence that non-thermophilic crenarchaea play a role in soil nitrification and suggest that further microcosm studies are required to determine the distinctive niches of bacterial and archaeal ammonia oxidizers.

Experimental procedures

Soil microcosms

Soil (pH 7.0) was collected from the upper 10 cm of an agricultural plot at the Scottish Agricultural College, Craibstone, Aberdeen, Scotland (Grid reference NJ872104) (see Kemp *et al*., 1992 for soil characteristics). Ten soil samples were pooled, sieved (3.35 mm mesh size) and stored at 4°C for 2 weeks before construction of microcosms. Microcosms consisted of 50 g of fresh soil in 250 ml sterile Erlenmeyer flasks. The soil had a water content of 37% (w/v) which was maintained throughout the experiment by weighing flasks at 1- to 2-day intervals and drop-wise addition of sterile distilled water to replace that lost through evaporation.

Soil sampling and analysis

Microcosms were sampled and soil samples (4 g) were stored at –80°C prior to chemical analysis and nucleic acid extraction. Combined nitrite and nitrate concentrations and ammonia concentration were determined colorimetrically by flow injection analysis (FIA star 5010 Analyser, Tecator, USA) (Allen, 1989). The initial ammonium concentration was 7 μg NH_4^+-N g^{-1} soil and total nitrogen and carbon contents were 0.4% (w/w) and 8.0% (w/w), respectively, determined using a Fisons NA-1500 NCS Analyser (Loughborough, UK). Total DNA and RNA were extracted from 0.5 g of soil samples as described previously (Griffiths *et al*., 2000) with some modifications (Nicol *et al*., 2005) and nucleic acid extracts were subdivided into two aliquots for the preparation of DNA or RNA templates.

(Reverse transcription) PCR amplification of 16S rRNA and amoA *genes and DGGE analysis*

To generate cDNA, 1 μg of total RNA was treated with RQ1 DNase (Promega, Southampton, UK) and cDNA was produced using Superscript II reverse transcriptase (Invitrogen, Paisley, UK). The reverse transcription (RT) reaction mixture included T4 gene 32 protein to a final concentration of 25 ng μl^{-1} to increase RT efficiency (Villalva *et al*., 2001). Random hexamer primers (Invitrogen) were used according to the manufacturer's instructions for all target genes except bacterial *amoA* genes, where a gene-specific primer (amoA5R, Table 1) was required for successful amplification. Two negative controls were performed with all reactions. The first control contained soil RNA template and all DNase/RT reagents except for the final addition of RT enzyme. This ensured that no residual DNA was present that could subsequently be amplified. A second control contained no template (water only) to ensure that all reagents were free of possible contaminants. In addition, after initial DNase treatment of all nucleic acid samples, 1 μl was added to a PCR to ensure that no DNA was present.

Polymerase chain reaction amplification was performed using reagents and concentrations described previously (Nicol *et al*., 2005) with sets of primers targeting 16S rRNA and *amoA* genes of either ammonia-oxidizing bacteria or ammonia-oxidizing crenarchaea from both DNA and cDNA templates (Table 1). A nested PCR strategy was used for

Table 1. Polymerase chain reaction (PCR) primer sets used in this study.

Target group	PCR primer set	Use	Gene position (rRNA or *amo*)	Reference or sequence (5′–3′) from this study
		16S rRNA genes of non-thermophilic crenarchaea		
Group 1.1b soil crenarchaea (and 1.1a)	G1.1b280f/957r	Cloning and nested PCR template	265–911[a]	Nicol *et al*. (2006)
Group 1 crenarchaea	771f/957r[c]	DGGE analysis	685–911[a]	Ochsenreiter *et al*. (2003)
		amoA genes of ammonia-oxidizing archaea		
Crenarchaeal *amoA*	CrenamoA23f/ CrenamoA616r	Cloning and DGGE[c]	7–631[a]	CrenamoA23f[d]: ATGGTCTGGCTWAGACG CrenamoA616r[d]: GCCATCCATCTGTATGTCCA
		16S rRNA genes of ammonia-oxidizing bacteria		
Betaproteobacterial ammonia oxidizers	CTOf/CTOr	Nested PCR template	189–654[b]	Kowalchuk *et al*. (1997)
Bacteria	P3/P2	DGGE analysis	341–534[b]	Muyzer *et al*. (1993)

a. Numbering according to soil crenarchaeal fosmid clone 54d9.
b. Numbering according to *E. coli*.
c. For DGGE, a GC clamp (Muyzer *et al*., 1993) was attached to 957r.
d. Crenarchaeal *amoA* primers were designed using the strategy reported elsewhere (e.g. Francis *et al*., 2005; Könneke *et al*., 2005; Treusch *et al*., 2005) by aligning sequences from soil fosmid 54d9 (Treusch *et al*., 2005) and the Sargasso Sea data set (Venter *et al*., 2004) and basing primers on conserved regions of sequence. CrenamoA23f is the same as that of Könneke *et al*. (2005). For all reactions, thermal cycling conditions were: 95°C for 5 min; followed by 10 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 25 cycles of 92°C for 30 s, 55°C for 30 s, 72°C for 1 min; followed by 72°C at 10 min. For all products ≥ 1 kb, the same conditions were used except an extension time of 2 min was used.

DGGE analysis of both bacterial ammonia oxidizer and crenarchaeal 16S rRNA gene sequences. For DGGE analysis of crenarchaeal *amoA* gene sequences, direct PCR products (620 bp in length) were resolved without the requirement of a GC clamp (Table 1).

Denaturing gradient gel electrophoresis of PCR products and excision and sequencing of DGGE bands (when required) was performed as described previously (Nicol *et al*., 2005). Relative (within lane) band intensities were quantified by densitometry analysis of normalized DGGE profiles using Phoretix one-dimensional gel analysis software (Phoretix International, Newcastle-Upon-Tyne, UK) as previously described (McCaig *et al*., 2001).

Cloning and sequence analysis of 16S rRNA and amoA *gene sequences*

First-round 16S rRNA and *amoA* gene PCR products were cloned into pGEM-T Easy vector (Promega, Southampton, UK) and screened by DGGE as described previously (Nicol *et al*., 2005). Selected clones were sequenced along both strands using M13f and M13r vector primers and assembled using Sequencher 4.1 (Gene Codes Corps, MI, USA). For 16S rRNA gene sequences, LogDet/Paralinear distances and tree construction were calculated in PAUP v4.01 (Swofford, 1998) as described previously (Nicol *et al*., 2005). Bootstrap support was calculated using PHYML (Guindon and Gascuel, 2003) and PHYLIP (Felsenstein, 2004). Distance analysis and bootstrap support of derived crenarchaeal *amoA* protein sequences were calculated using the Jones, Taylor and Thornton (JTT) substitution model with site variation (invariable sites and eight variable gamma rates) using PHYML (Guindon and Gascuel, 2003) and PHYLIP (Felsenstein, 2004). Sequences of chimeric origin were checked by analysing alignments using Ballerophon (Huber *et al*., 2004) and partial treeing analysis.

Statistical analysis

DGGE relative band intensities and nitrification process data from triplicate microcosms were analysed using a general linear model (GLM) of analysis of variance (ANOVA) using Minitab 14.

Accession numbers

All sequences have been deposited in the GenBank database with Accession No. EF450779 to EF450823.

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