Molecular analysis of enrichment cultures of marine ammonia oxidisers

Allison E. McCaig, T.M. Embley and J.I. Prosser

Department of Molecular and Cell Biology, University of Aberdeen, Marischal College, Aberdeen AB9 1AS, UK, and
Department of Zoology, Natural History Museum, Cromwell Road, London SW7 5BD, UK

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Abstract: Marine ammonia oxidising bacteria were enriched by incubation of sea water, amended with ammonium sulphate, and subsequent subculture in liquid inorganic medium. PCR primers were designed to be specific for rDNA sequences from ammonia oxidisers belonging to the β-sub-group of the proteobacteria. These primers were then used to amplify rRNA genes from ammonia oxidiser enrichment cultures containing heterotrophs. PCR products were recovered from all cultures in which complete ammonia oxidation occurred. Subsequent rDNA sequence analysis indicated the presence of three new lineages within the clade defined by sequences of cultured β-sub-group ammonia oxidisers. Two of the new lineages showed moderate similarity to sequences from pure cultures of ammonia oxidisers previously isolated from marine and brackish environments. The third lineage (AEM-3) was deep branching and occupied an intermediate position between clades defined by *Nitrosomonas* or *Nitrosospira*, which were isolated from soil or sewage. The phylogenetic analysis suggests that, in enrichment cultures, the primers are specific for members of the target group, the β-proteobacteria ammonia oxidisers. The results also indicate the presence of previously unknown ammonia oxidisers in marine samples. The approach enabled analysis of ammonia oxidiser enrichments at an early stage and without the requirement for isolation of pure cultures, significantly reducing the time required and facilitating quantitative assessment of relatedness of strains.

Key words: Nitrification; Marine ammonia oxidisers; 16S rRNA sequence analysis

Introduction

Nitrification in marine environments is carried out by autotrophic ammonia and nitrite oxidising bacteria and plays a central role in the marine nitrogen cycle. Analysis of the structure and diversity of communities of nitrifying bacteria in natural environments is, however, severely limited by two major technical problems. The first is difficulty in isolating pure cultures of nitrifying bacteria [1]. Ammonia is the sole energy source for ammonia oxidising bacteria, but the low energy yield on ammonia leads to low biomass yields and slow growth. Colony development on solid medium typically takes several months, colonies are small and difficult to transfer, and elimination of heterotrophic contaminants is difficult because of their higher growth rates. The second problem is identification and discrimination of strains following isolation. Identification is traditionally based on morphological characteristics, differentiating five genera of ammonia oxidisers.
However, recent molecular analysis [2] has demonstrated that morphology is a poor tool for defining genus-level relationships and *Nitrosovibrio* and *Nitrosolobus* have been reclassified as the genus *Nitrosospira*. Species identification is based on a limited number of physiological characteristics. Community analysis of ammonia oxidisers has therefore been hampered by both the time required and the limited amount of information which may be obtained using traditional techniques.

Recently 16S rDNA sequence analysis has been used to characterise microbial communities in marine environments [3]. This involves selective recovery and comparison of rRNA or rDNA sequences from nucleic acids extracted from the environment, either directly or following cell extraction. The recent publication of the rRNA sequences from culture collection strains of ammonia oxidisers [2] has provided the data for designing specific PCR primers which can be used to study marine ammonia oxidiser populations. Here we describe the use of β-proteobacterial ammonia oxidiser-specific PCR primers and combined use of rDNA sequence analysis to assess diversity within enrichment cultures of ammonia oxidisers obtained from a marine environment.

### Materials and Methods

#### Sample collection and enrichment for ammonia oxidisers

Samples of surface sea water were collected from the coast at either Balmedie or Aberdeen (map ref nos. OS 895 070 and OS 983 182 respectively). Within 1–2 h of collection, samples were prepared for enrichment of ammonia oxidisers by addition of ammonium sulphate (final ammonium concentration, 10 μg NH₄⁺-N ml⁻¹) to 100 ml sea water in a 250 ml Erlenmeyer flask. This process was repeated for several samples and for several sub-samples of sea water. All flasks were incubated in the dark at room temperature (15–23°C). Nitrification was assessed in samples of all cultures by spot tests for nitrite production (Griess Ilosvay's reagents I and II) or disappearance of ammonia (Nessler’s reagent). Subcultures were prepared by inoculation of 3 ml of a fully grown enrichment culture into 100 ml inorganic medium [4], containing 318 μg NH₄⁺-N ml⁻¹, in a 250 ml flask. Cultures were incubated in the dark at 25°C. The presence of heterotrophs in enrichment cultures was determined by streak plating onto nutrient agar and incubating for up to 3 weeks at 30°C. Enrichment cultures were also prepared by inoculating 100 ml inorganic medium [4] with 3 ml sea water and incubating as described above.

#### rRNA sequence analysis

Fully grown enrichment cultures, in which all ammonia had been converted to nitrite, were centrifuged (10000 × g, 15 min), pellets were resuspended in 100 μl of a 5% (w/v) aqueous suspension of Chelex® 100 (BioRad, Richmond, CA) [5] and cells were lysed by boiling for 5 min. After centrifugation, to precipitate debris and unlysed cells, the crude cell lysate was used directly for PCR. PCR amplifications were carried out using the method described by Embley [6]. Primers for ammonia oxidisers belonging to the β-sub-group of the proteobacteria were designed using published 16S rDNA sequence information [2] and unpublished sequences obtained from pure cultures (McCaig, unpublished data). The forward 16S rDNA ammonia oxidiser primer, βAMOₐ, bound at position 141–161 [7] of the *Escherichia coli* rDNA molecule and had the sequence 5'-TGGGGRATAACGCAYCGAAAG-3'. The reverse primer, βAMOᵣ, bound at position 1301–1320 and had the sequence 5'-AGACTCCGATCCGGACTACG-3'. These primers were tested for specificity on DNA extracted from pure cultures of ammonia oxidisers *Nitrosospira multiformis* (formerly *Nitrosolobus multiformis*) C71 NCIMB 11849, *Nitrosospira NpAV*, provided by Dr. E. Schmidt, University of Minnesota, *Nitrosomonas europaea* NCIMB 11850, *Nitrosomonas eutropha* and *Nitrosospira tenuis* (formerly *Nitrosovibrio tenuis*) 141 provided by Dr. I. Head, University of Liverpool, *Nitrosospira sp.*, provided by Dr. S. Burton, University of Aberdeen, and a representative selection of other bacteria. These were *Spirillum volutans*.
NCIMB 12341 (which is among the deepest branches in the tree for β-proteobacteria [8]), *Hafnia alvei* NCIMB 6578, *Listonella anguillarum* NCIMB 6, *E. coli* HB101 NCIMB 11668 (which have similar sequences to the ammonia oxidiser primer target sites) and *Pseudomonas fluorescens* NCIMB 10586. Eubacterial specific primers were used as positive controls [6]. The forward primer, BF, was 5'-TCAGAYGAACGCTGGCGG-3' and the reverse primer was 1541r, 5'-AAGGAGGTGATCCAGCC-3'. Sequencing of the PCR amplification product was carried out using the sequencing primer 16S537r and cycle sequencing as described by Embley [6].

Phylogenetic analysis was carried out by aligning by eye the partial 16S rRNA sequences from enrichment cultures with nine previously published sequences of ammonia oxidisers and reference β-proteobacteria contained in the Ribosomal RNA database project [2,8–10] and two sequences from pure cultures of ammonia oxidisers isolated by E. Schmidt and S. Burton. All data manipulations and phylogenetic analyses were achieved using the Genetic Data Environment software version 2.2 [8] operating on a SUN Workstation. The final alignments comprised 337 bases corresponding to *E. coli* positions 162 to 499 [7]. Phylogenetic trees were generated using the Olsen distance correction (G.J. Olsen, unpublished [8]) and a least-squares treeing method [11] or using the Jukes and Cantor correction [12] and neighbour-joining [13].

### Results and Discussion

Enrichment cultures from all sea water samples, obtained by direct amendment with ammonia, showed growth of ammonia oxidising bacteria within 4–6 weeks as judged by complete conversion of ammonia to nitrite. Those in which sea water was inoculated into defined inorganic medium did not show growth within 4 months. Heterotrophs were present in all cultures, although colony morphology indicated that each

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**Fig. 1.** Phylogenetic tree showing the position, within the β-proteobacteria, of partial sequences obtained from ammonia oxidiser enrichment cultures using primers designed to amplify sequences from the β-sub-group ammonia oxidising bacteria. Scale bar represents 2% estimated change. Accession numbers for the sequences from enrichment cultures are: AEM-1subm: U09542; AEm-2subm: U09543; AEM-3subm: U09544; AEM-4subm: U09545; AEM-5subm: U09546; AEM-6subm: U09547.
initial enrichment culture was dominated by a single heterotroph. No pure cultures of ammonia oxidisers were obtained within 18 months of establishment of enrichment cultures, demonstrating the problems associated with isolation of these organisms. A total of seven ammonia oxidiser enrichments were analysed using PCR primers designed to amplify 16S rRNA genes from β-proteobacteria ammonia oxidisers. Only one enrichment failed to give a PCR amplification product with these primers but it did give an amplification product using the control eubacterial primers.

The phylogenetic tree obtained is illustrated in Fig. 1. All sequences from enrichment cultures clustered with ammonia oxidisers from the β-proteobacteria but all were different from previously published ammonia oxidiser sequences. Phylogenetic analysis indicated three centres of variation, two of which fall near sequences from pure cultures of ammonia oxidisers isolated from marine (N. marina) or brackish (N. mobilis) environments. All other pure cultures were isolated from terrestrial environments, except N. eutropha which was isolated from sewage. The third new group, represented by a single sequence, AEM-3, was a deep branching organism and may represent a previously uncharacterised group. Its precise position relative to the two main clusters of ammonia oxidisers was not determined as it changed depending on the method of analysis (least squares or neighbour-joining). Sequences AEM-1, AEM-2, AEM-3 and AEM-4 were obtained from enrichments from Balmedie Beach and AEM-5 and AEM-6 were enriched from Aberdeen Beach. Two pairs gave identical sequences. AEM-1 and AEM-2 came from the same original sub-sample and AEM-5 and AEM-6 came from different sub-samples. Sequences of strains AEM-1 and AEM-2 and the group containing AEM-4, AEM-5 and AEM-6 showed 94% similarity to that of N. marina. Strain AEM-3 showed 90% similarity to the sequence from N. marina, 93% similarity to sequences from other marine enrichment sequences and 91% similarity with the terrestrial isolates N. tenuis and N. europaea.

This study represents the first analysis of enrichments of natural communities of ammonia oxidising bacteria using rDNA sequence analysis. The major advantage of this approach is the ability to identify and classify ammonia oxidisers without the requirement for isolation of pure cultures. PCR amplification, using primers specific to the β-sub-group ammonia oxidisers, enabled detection and characterisation of strains despite the presence of a substantial heterotrophic population. Subsequent identification and clustering were significantly easier than traditional methods. These require isolation of pure cultures which typically takes several months and which may not be possible for strains interacting closely with heterotrophs. Following isolation, characterisation is based on cell morphology and a limited number of physiological properties. These are time consuming and difficult to determine with confidence, and quantitative analysis of similarity between strains is not possible. Using the approach described above, some components of the community were identified within 2–3 weeks of establishment of initial or secondary enrichment cultures.

The use of enrichment cultures does not eliminate selection of strains suited to growth in laboratory media but avoids additional bias associated with removal of heterotrophs. Comprehensive community analysis, however, also requires analysis of DNA extracted directly from the environment and detection of cells in environmental samples using group and species specific probes. Further limitations are the restrictions imposed by the specificity of the primers used. Thus, for example, the single enrichment culture which did not produce a PCR product may have contained β-sub-group ammonia oxidisers whose sequences have reduced homology to the primers used, or strains belonging to other sub-groups of the proteobacteria. In particular, certain marine ammonia oxidiser strains are known to belong to the γ-sub-group of the proteobacteria [2], and these would not have been detected in this study. Nevertheless, the ability to amplify sequences based on ammonia oxidisers from the β-sub-group of the proteobacteria from all enrichment cultures indicates that they are present in marine environments. The study also demonstrates the advantages of rDNA analysis for analysis of bacterial communities, with particular relevance for organ-
isms, such as autotrophic ammonia oxidising bacteria, which are difficult to isolate.

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References


