The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences

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Partial sequences of the 16S ribosomal RNA genes of eleven autotrophic ammonia-oxidizing bacteria were determined by PCR amplification from small amounts of heat-lysed biomass followed by direct sequencing of PCR products. The sequences were aligned with those of representative Proteobacteria and phylogenetic trees inferred using both parsimony and distance matrix methods. This confirmed that the autotrophic ammonia-oxidizers comprise two major lines of descent within the Proteobacteria. Nitrosomonas spp., Nitrosococcus mobilis, and strains of Nitrosovibrio, Nitrosospira and Nitrosolobus were located in the beta-subdivision. The recovery of Nitrosococcus oceanus strains as a deep branch in the gamma-subdivision supported the RNA catalogue data which had indicated that the genus Nitrosococcus is polyphyletic. The autotrophic ammonia-oxidizing bacteria of the beta-Proteobacteria formed a coherent group which is interpreted as representing a single family. Within this clade, the genera Nitrosovibrio, Nitrosospira and Nitrosolobus exhibited very high levels of homology in their 16S ribosomal RNA gene sequences and can be accommodated within a single genus. Separation of these genera is currently based entirely on gross morphological differences and these can now be considered more appropriate for the identification of species within this group. It is therefore proposed that Nitrosolobus, Nitrosovibrio and Nitrosospira strains be reclassified in a single genus for which the name Nitrosospira has priority.

Introduction

Autotrophic ammonia-oxidizing bacteria are an ecologically important and physiologically specialized group. They are responsible for the oxidation of ammonia to nitrite, the reaction which drives the process of nitrification in a wide range of environments (Hall, 1986). The organisms are obligate chemoautotrophs and they all exhibit an essentially identical central metabolism. Carbon dioxide is fixed via the Calvin cycle, and ammonia is oxidized to nitrite to produce energy and reducing power. Their electron transport systems appear to be identical, as determined by difference spectroscopy (Giannakis et al., 1985; Koops et al., 1991).

Autotrophic ammonia-oxidizing bacteria are notoriously difficult to isolate in pure culture and, compared to chemoorganotrophs, grow to low cell densities and biomass yields in vitro. This, and their autotrophic physiology, means that they are largely intractable to traditional methods of phenotypic characterization (Koops & Möller, 1991). Consequently, ammonia-oxidizing bacteria are classified largely on the basis of morphological criteria such as cell shape and the arrangement of internal membranes (Watson et al., 1989; Bock et al., 1986). Chemosystematic studies of cellular lipids have provided little information of taxonomic utility, since all of the genera examined exhibit similar lipid profiles (Blumer et al., 1969).

Data from 16S rRNA catalogues (Woese et al., 1984b, 1985) first demonstrated that there are two phylogenetically distinct groups of autotrophic ammonia-oxidizing bacteria. One of these contained Nitrosococcus oceanus, and was within the gamma-subdivision of the Proteobacteria. The other contained Nitrosococcus mobilis and representatives of all of the other described genera of ammonia-oxidizers, and was located within the beta-subdivision of the Proteobacteria. The ammonia-oxidizing bacteria in the beta-subdivision formed three
taxonomic conclusions were further constrained by the fact that cataloguing methods only recover approximately 40% of the RNA sequence. However, represented by only a single strain and any deep branches; *Nitrosococcus mobilis*; *Nitrosomonas europaea*; and a third branch containing *Nitrosolobus, Nitrosovibrio* and *Nitrosospira* strains. Each genus was, however, represented by only a single strain and any taxonomic conclusions were further constrained by the fact that cataloguing methods only recover approximately 40% of the RNA sequence.

There have been a number of attempts at defining the taxonomic structure within the autotrophic ammonia oxidizers using DNA–DNA base pairing and mol% G+C determinations (Dodson *et al.*, 1983; Koops & Harms, 1985; Koops *et al.*, 1990, 1991). While groups of strains which could form the basis of species have been readily recognized using these approaches, they have proved of limited utility for the circumscription of genera. This is because the DNA pairing values between species are often very low and are close to the limits of resolution for DNA pairing methods (Huss *et al.*, 1983; Koops & Harms, 1985; Koops *et al.*, 1991).

The comparative analysis of complete or nearly complete 16S rRNA sequences provides greater resolution of higher level bacterial relationships than does DNA pairing or rRNA cataloguing (Wayne *et al.*, 1987; Stackebrandt, 1991). Furthermore, the development of the polymerase chain reaction (PCR; Saiki *et al.*, 1988) has made it possible to recover 16S rRNA gene sequences from uncultured and difficult to culture micro-organisms (Giovannoni *et al.*, 1990). The autotrophic ammonia-oxidizing bacteria are therefore excellent candidates for PCR-based phylogenetic analysis. In this paper, the higher level classification of ammonia-oxidizing bacteria was investigated by sequencing PCR-amplified 16S rRNA genes from representative strains of each of the currently recognized genera.

**Methods**

**Organisms and growth conditions.** The strains of ammonia-oxidizing bacteria used in this study are listed in Table 1. The organisms were grown autotrophically in the medium described by Watson (1971). Marine strains were grown in the same medium but containing 8.7 mg K2HPO4 l⁻¹ and artificial sea water (Tropic Marin, Dr Biener, GmbH Aquarientechnik) (8.3 g l⁻¹ *Nitrosococcus mobilis* Nc2; 30 g l⁻¹ *Nitrosococcus oceanus* C-27, *Nitrosococcus oceanus* C-107; 25 g l⁻¹ *Nitrosomonas marina* C-56). Erlemeyer flasks (500 ml) containing 200 ml of medium were inoculated with 200 μl of stationary phase culture and incubated in the dark at 30 °C until the medium acidified due to oxidation of ammonia to nitrite. This was indicated by a colour change to yellow of the phenol red incorporated in the medium. The culture was then neutralized with filter-sterilized Na2CO3 (5%, w/v) and incubated again until acidification had occurred. Cells were harvested for DNA extraction or further subcultured.

**DNA isolation, PCR amplification and sequencing of amplified 16S ribosomal RNA genes.** Cells from 200 ml of culture were harvested by centrifugation in a Sorval RC-5 centrifuge (10000 r.p.m., 20 min, GSA rotor). The cells were transferred to a microcentrifuge tube, washed three times with 1 ml of TE buffer (10 mm-Tris/HCl, 1 mm-EDTA, pH 8.0), pelleted and resuspended in 100 μl of lysis buffer (TE containing 1%, v/v, Tween 80). The cell suspension was placed in a boiling water bath for 1 min and then snap-frozen in dry ice. This procedure was repeated three times and the cell lysate was extracted with chloroform (200 μl). The aqueous layer, separated during centrifugation, was used as a source of DNA for PCR amplification. If amplification was unsuccessful, the DNA preparation was further

**Table 1. Strains of autotrophic ammonia-oxidizing bacteria used in this study**

All strains were provided by Professor S. W. Watson, Woods Hole Oceanographic Institute, USA, except C-31, C-71 and C-91, which were provided by Dr G. H. Hall of the Institute for Freshwater Ecology, Windermere, UK.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Strain designation*</th>
<th>Origin</th>
<th>GenBank accession no.</th>
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<tr>
<td><em>Nitrosomonas</em></td>
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<td><em>N. europaea</em></td>
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<td>Soil</td>
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<td>C-56 (Nm63)</td>
<td>Seawater</td>
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<td><em>Nitrosovibrio</em></td>
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<tr>
<td><em>N. mobilis</em></td>
<td>Nc2T</td>
<td>Seawater</td>
<td>M96403</td>
</tr>
<tr>
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<td>M96398</td>
</tr>
<tr>
<td><em>N. oceanus</em></td>
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<td>Seawater</td>
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* T. Type strain. Alternative strain designations of Koops & Harms (1985) are given in parentheses.
purified by centrifugal ultrafiltration using a Centricon-100 Microconcentrator (Amicon). A 2–4 μl volume of the retentate was generally sufficient for PCR amplification of almost full-length 16S rRNA genes using primers P4 and P2 (Edwards et al., 1989) and the PCR protocol of Embley (1991). The PCR products were sequenced using the linear PCR sequencing method of Embley (1991) and the sequencing primers described by Edwards et al. (1989).

Data analysis. The sequences from the ammonia-oxidizing bacteria were aligned by eye with published sequences from 41 strains chosen to represent the major proteobacterial subdivisions (Neefs et al., 1991; Olsen et al., 1991), and corrected for known secondary structure (Neefs et al., 1991). Subsets of this alignment were used in all subsequent analyses. Positions which could not be aligned unambiguously were omitted. The final alignments comprised 1014 bases (all subdivisions of Proteobacteria, gamma-subdivision Proteobacteria) and 1236 bases (beta-subdivision Proteobacteria). The alignment for the beta-subgroup comprised positions 97–834, 849–1023, 1032–1125 and 1140–1372, and that for the gamma-subdivision covered positions 99–182, 243–451, 486–818, 862–997, 1042–1125, 1140–1254 and 1302–1361 (numbered as in E. coli; Woese et al., 1983). Distance values were calculated using the Jukes and Cantor (1969) correction in the DNADIST program from PHYLIP 3.4 (J. Felsenstein, 1991, University of Washington). Phylogenetic trees were constructed using the ‘neighbor joining method’ (Saitou & Nei, 1987). In order to evaluate the robustness of the inferred trees, a bootstrap analysis consisting of 100 resamplings of the data was performed using SEQBOOT (PHYLIP 3.4) and a consensus tree was generated using neighbor joining and the program CONSENS (PHYLIP 3.4). Maximum parsimony analysis was carried out on the bootstrapped data using PAUP 3.0s (D. Swoford, 1990, Illinois Natural History Survey, Illinois).

Results and Discussion

The linear PCR method recovered almost full-length 16S rRNA gene sequences (1329–1448 nucleotides) for the eleven strains. The sequences have been deposited with GenBank and assigned the accession numbers given in Table 1. Preliminary analyses (data not shown) of the aligned sequences (1014 positions) from nine strains of ammonia-oxidizing bacteria, ten other Proteobacteria and Bacillus subtilis as an outgroup, confirmed the tree topology based on the 16S rRNA catalogue data of Woese et al. (1984a,b). Nitrososococcus oceanae C-27 and C-107 were recovered consistently as a deep branch within the gamma-Proteobacteria; all of the other strains, including Nitrososoccus mobilis NC2, formed a clade in the beta-subdivision of the Proteobacteria. Signature nucleotide analysis (Woese, 1987) of the sequences supported these groupings, although in the case of Nitrososococcus oceanus C-27 and C-107, 8 of the 58 signature positions examined differed from the consensus base for the gamma-Proteobacteria. This may be a reflection of the depth of the branch containing these strains. The Nitrososococcus oceanus strains contained the sequence AAACUCUAAGU, which has only been found in organisms from the gamma-Proteobacteria (Woese et al., 1985). The beta-subdivision diagnostic oligonucleotides AAAACCUCUACC and CYUUCACAU (Woese et al., 1984b) were present in all of the sequences from the beta-subdivision ammonia-oxidizers.

Gamma-subdivision ammonia-oxidizing bacteria

Only two Nitrososococcus oceanus strains were available for the present study. These organisms were found to be closely related to each other (96-4% identity over 1304 bp of unambiguously aligned sequence), thus confirming the results of DNA-DNA hybridization experiments (Koops & Harms, 1985; Koops et al., 1990). The strains share a common ancestry with the photosynthetic organisms Chromatium and Ectothiorhodospira (Fig. 1), as previously suggested by 16S rRNA catalogue data (Woese et al., 1985).

Beta-subdivision ammonia-oxidizing bacteria

In order to analyse in more detail the relationships between the ammonia-oxidizing genera recovered within the beta-Proteobacteria, longer stretches of 16S rRNA sequences from nine strains representing the genera Nitrosonomas, Nitrososococcus, Nitrosovibrio, Nitrosolobus and Nitrosospira, were examined. Alignment against eight other beta proteobacterial sequences and E. coli (gamma-subdivision) as an outgroup enabled 1236 sequence positions to be aligned unambiguously. The phylogenetic tree (Fig. 2) demonstrated that the autotrophic ammonia-oxidizing bacteria form a clade (100% support from bootstrap resampling) within the beta-subdivision. A single tree with identical topology was also recovered using parsimony analysis of the bootstrapped sequence data (not shown). No other beta-Proteobacteria were ever recovered within the radiation of nitriying organisms.

The depth of branching within the cluster of autotrophic ammonia-oxidizing bacteria is comparable to that within the family Neisseriaceae (Dewhirst et al., 1989), which is represented in this study (Fig. 2) by the genera Eikenella, Neisseria, Vitreoscilla and Chromobacterium. It could therefore be suggested that the beta-subdivision, autotrophic ammonia-oxidizers should form the basis of a single family. The family Nitrobacteraceae, which currently contains these taxa, is polyphyletic since it includes ammonia-oxidizing bacteria from both the gamma- and beta-subdivisions of the Proteobacteria, plus the nitrite-oxidizing organisms from the alpha-subdivision (Woese et al., 1984a). However, the published data are not exhaustive and we advocate the acquisition of more sequences from the gamma- and alpha-members of the family Nitrobiacteraeae before a formal taxonomic revision at the family level is undertaken.

The beta-subdivision ammonia-oxidizers could be
subdivided into two major lineages (Fig. 2). One lineage encompassed the strains of *Nitrosovibrio*, *Nitrosospira* and *Nitrosolobus* (100% support in bootstrap replicates), and the other contained the three *Nitrosomonas* species and *Nitrosococcus mobilis* (95% support). The taxonomy of the genus *Nitrosomonas* has recently been comprehensively overhauled and the two established species, *Nitrosomonas europaea* and *Nitrosomonas cryotolerans*, were joined by eight new species of *Nitrosomonas* on the basis of DNA–DNA pairing supported by a small number of phenotypic tests on type strains (Koops et al., 1991). Based on these criteria, *Nitrosomonas* strain C-56 was designated *Nitrosomonas marina* and strain C-91, *Nitrosomonas eutropha* (Koops et al., 1991). The separate taxonomic status of these two species was verified by our analyses (Fig. 2). Furthermore, on the basis of our data, *Nitrosococcus mobilis* Nc2 should also be transferred to the genus *Nitrosomonas*. The genus *Nitrosomonas* has recently been shown to contain both rods and true cocci (Koops et al., 1991). Our data confirm that these two different morphologies occur in closely related bacteria, and provide further evidence that simple morphological traits are not reliable indicators of higher relationships (van Niel, 1946; Stackebrandt & Woese, 1981).

The low level of sequence variation between the representatives of *Nitrosolobus*, *Nitrosospira* and *Nitrosovibrio* (96-1% identity between all five strains over 1335 bases of unambiguously aligned sequence) suggests that they are very closely related indeed. The DNA pairing data of Dodson et al. (1983) demonstrated that there was slightly higher DNA homology between the genera *Nitrosospira* and *Nitrosolobus* than there was between some species classified within the genus *Nitrosomonas*. Subsequent DNA pairing measurements, determined from renaturation kinetics (De Ley et al., 1970), gave mean hybridization values between representatives of these three genera of 33%, compared to average values within the genera of 33–42% (Koops & Harms, 1985). However, the renaturation method gives high background similarity values (typically 20 to 30%), because of the high baseline reassociation rate (Huss et al., 1983; Johnson, 1991). Values in this region should therefore be treated with caution and cannot be reliably used to resolve the generic status of *Nitrosovibrio*,

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**Fig. 1.** Phylogenetic tree of representatives of the Proteobacteria showing the position of *Nitrosococcus oceanus* within the gamma-subdivision. The tree was generated using the method of Saitou & Nei (1987). *Agrobacterium tumefaciens* (alpha) was used as an outgroup. The scale bar indicates 0.02 substitutions per nucleotide position.
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**Nitrosospira** and **Nitrosovibrio**. It is also well established that DNA hybridization values can be very low between organisms that otherwise share many phenotypic properties. Examples of this phenomenon can be found in *Streptomyces* (Mordarski et al., 1986), *Staphylococcus* (Freney et al., 1988) and *Legionella* (Fry et al., 1991). Additional data (to the rRNA sequences) that support a close relationship between *Nitrosovibrio*, *Nitrosospira* and *Nitrosolobus* include similar fatty acid and cytochrome compositions and the limited spread of mol\% G+C values (52.0 to 56.3\%)(Blumer et al., 1969; Giannakis et al., 1985; Watson et al., 1989).

The separation of *Nitrosovibrio*, *Nitrosospira* and *Nitrosolobus* into distinct genera is currently based solely on cell shape (Watson et al., 1989; Koops & Möller, 1991). We suggest that simple morphological differences are useful here for species classification and identification, but are insufficient to retain separate generic status for bacteria which on the weight of other phenotypic and genotypic evidence appear to be so closely related. Our assertion that cell shape alone is a poor criterion for the differentiation of autotrophic ammonia-oxidizing bacteria at the genus level, is supported by considering the taxonomy of Nitrosomonas, where cell shape varies between species. We therefore propose that *Nitrosovibrio*, *Nitrosospira* and *Nitrosolobus* be reclassified in a single genus for which the name *Nitrosospira* has nomenclatural priority. The genera *Nitrosospira*, *Nitrosovibrio* and *Nitrosolobus* each contain a single validly described nomenclature and/or type strains were examined in this study. Transfer of species from *Nitrosovibrio* and *Nitrosolobus* to the genus *Nitrosospira* results in the new combinations *Nitrosospira tenuis* and *Nitrosospira multiformis*. The species descriptions have been published previously (Watson et al., 1971, 1989; Harms et al., 1976).

**Emended description of the genus Nitrosospira**

*Nitrosospira* Winogradsky and Winogradsky 1933 emend. Cells are spiral, curved or lobate containing either no intracytoplasmic membranes or membranes which partially compartmentalize the cell. Spherical forms are present in early stationary phase cultures.
Gram-negative. Motile by flagella or non-motile. Aerobic. The major source of energy and reducing power is from the oxidation of ammonia to nitrite. Chemo-lithotrophs. May grow mixotrophically, but never heterotrophically. Carboxysomes and other inclusions are not observed. Occur in terrestrial and freshwater environments. Optimal growth occurs in mineral salts medium containing ammonium salts. Optimum temperature: 25–30 °C. Optimum pH 7.5. The mol% G + C of the DNA is 52.2 to 56.3 (Bd, Tm).

Type species: *Nitrosospira briensis*

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**References**


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