

Nitrosospira spp. can produce nitrous oxide via a nitrifier denitrification pathway

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Summary

Nitrous oxide (N₂O) emission from soils is a major contributor to the atmospheric loading of this potent greenhouse gas. It is thought that autotrophic ammonia oxidizing bacteria (AOB) are a significant source of soil-derived N₂O and a denitrification pathway (i.e. reduction of NO₂⁻ to NO and N₂O), so-called nitrifier denitrification, has been demonstrated as a N₂O production mechanism in *Nitrosomonas europaea*. It is thought that *Nitrosospira* spp. are the dominant AOB in soil, but little information is available on their ability to produce N₂O or on the existence of a nitrifier denitrification pathway in this lineage. This study aims to characterize N₂O production and nitrifier denitrification in seven strains of AOB representative of clusters 0, 2 and 3 in the cultured *Nitrosospira* lineage. *Nitrosomonas europaea* ATCC 19718 and ATCC 25978 were analysed for comparison. The aerobically incubated test strains produced significant ($P < 0.001$) amounts of N₂O and total N₂O production rates ranged from 2.0 amol cell⁻¹ h⁻¹, in *Nitrosospira tenuis* strain NV12, to 58.0 amol cell⁻¹ h⁻¹, in *N. europaea* ATCC 19718. *Nitrosomonas europaea* ATCC 19718 was atypical in that it produced four times more N₂O than the next highest producing strain. All AOB tested were able to carry out nitrifier denitrification under aerobic conditions, as determined by production of ¹⁵N-N₂O from applied ¹⁵N-NO₂⁻. Up to 13.5% of the N₂O produced was derived from the exogenously applied ¹⁵N-NO₂⁻. The results suggest that nitrifier denitrification could be a universal trait in the betaproteobacterial AOB and its potential ecological significance is discussed.

Introduction

Nitrous oxide (N₂O) is a greenhouse gas with 296 times the global warming potential of carbon dioxide (Houghton *et al.*, 2001). Nitrous oxide also contributes to the destruction of the stratospheric ozone layer (Conrad, 1996). Atmospheric concentrations of N₂O have been increasing from pre-industrial values of 0.27 ppmv to current concentrations that approach 0.32 ppmv (Conrad, 1996). Both natural and agricultural soils are major sources of N₂O (Conrad, 1996; Houghton *et al.*, 2001) and have recently been estimated to contribute 57% to the total global N₂O budget (Mosier *et al.*, 1998; Kroeze *et al.*, 1999). It is thought that soil sources have contributed to the post-industrial revolution upturn in atmospheric N₂O loading through increased soil nitrogen availability as a result of nitrogen deposition and increased nitrogen fertilization and biological nitrogen fixation, through agricultural expansion and intensification (Nevison and Holland, 1997).

Microbial transformations of ammonium and nitrate are considered the main processes responsible for gaseous nitrogen emissions from soil. However, chemodenitrification may be important, particularly in acid soils (VanCleemput and Samater, 1996). The best characterized biotic pathway of N₂O production is heterotrophic denitrification in bacteria [reviewed in detail by Zumft (1997)], whereby, under conditions of low oxygen tension, nitrogen oxides serve in place of dioxygen as terminal electron acceptors in electron transport phosphorylation. Heterotrophs possessing the complete denitrification pathway produce N₂O as an intermediate in the stepwise dissimilatory reduction of nitrate to dinitrogen gas (NO₃⁻ → NO₂⁻ → NO → N₂O → N₂) with oxidation of an organic carbon source used as reducing power (Hochstein and Tomlinson, 1988). Thus denitrification completes the soil nitrogen cycle by returning dinitrogen gas to the atmosphere.

The ammonia oxidizing bacteria (AOB) also play a central role in the nitrogen cycle. They catalyse the first step in nitrification, the conversion of ammonia via hydroxylamine to nitrite. As chemolithoautotrophs, they use ammonia as a sole source of energy and reducing power and obtain carbon for biosynthesis by fixation of CO₂. Pure culture studies, mainly conducted with *Nitrosomonas europaea* strains (Ritchie and Nicholas, 1972; Hynes and Knowles, 1984; Poth and Focht, 1985; Anderson *et al.*, 1993; Kester *et al.*, 1997; Beaumont

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et al., 2002), but also with *Nitrosospira* spp. (Goreau *et al.*, 1980; Remde and Conrad, 1990; Jiang and Bakken, 1999a; Dundee and Hopkins, 2001; Wrage *et al.*, 2004a), have shown that AOB also produce N₂O. In fact, it has been estimated that ammonia oxidizers can contribute up to 80% to total soil N₂O emissions, depending on soil type, temperature and water content (Webster and Hopkins, 1996; Godde and Conrad, 1999).

The mechanism of N₂O production by nitrifiers is not completely characterized although traditionally two different routes have been proposed. The first relates to the activity of hydroxylamine oxidoreductase (HAO), which mediates the conversion of hydroxylamine to nitrite, the second step in ammonia oxidation. It has been shown, *in vitro*, that HAO can also catalyse the oxidation of hydroxylamine to N₂O (Hooper and Terry, 1979), possibly indirectly through the decomposition of unstable intermediates (Ritchie and Nicholas, 1972). The second N₂O-yielding route relates to the existence of a denitrification pathway, so-called nitrifier denitrification, where nitrite is reduced to nitric oxide and N₂O (Wrage *et al.*, 2001; Arp and Stein, 2003), as in the classical heterotrophic denitrification pathway. Recently, Schmidt and colleagues (2004) have identified denitrification as the major source of N₂O produced by *N. europaea* ATCC 19718, although mutants lacking the denitrification pathway released N₂O during oxidation of ammonia to nitrite (most likely as a result of auto-oxidation and chemodenitrification of hydroxylamine).

Several authors have considered the likely contribution of nitrifier denitrification to the total N₂O emission from soil and the factors that influence its relative importance, for example, local ammonium and oxygen concentrations

(Robertson and Tiedje, 1987; Webster and Hopkins, 1996; Wrage *et al.*, 2001, 2004b). However, the fundamental determinant of intrinsic nitrifier denitrification potential in soil will be the abundance of ammonia oxidizers with denitrifying ability (Wrage *et al.*, 2001). The nitrifier denitrification pathway has only been unequivocally demonstrated in *Nitrosomonas* spp. (Colliver and Stephenson, 2000), whereas there is mounting evidence that nitrosomonads are not representative of AOB groups found in soil (Arp and Stein, 2003). Specifically, analysis of clone libraries suggests that betaproteobacterial AOB in the *Nitrosospira* lineage may be most abundant (Stephen *et al.*, 1996; Kowalchuk and Stephen, 2001; Smith *et al.*, 2001). To our knowledge, it is not known whether nitrifier denitrification is universal among AOB or confined to the nitrosomonads. The aim of this study therefore was to determine N₂O production and nitrifier denitrification potential in AOB cultures more representative of phylotypes dominating soil AOB communities.

Results

Nine different cultures of AOB (Table 1) were incubated under ambient air concentrations of O₂ in medium containing ammonium and nitrite at initial concentrations of 3.5 mM and 1 mM, respectively, and the production of N₂O and nitrite was quantified during incubation for 8 h. Total (¹⁴⁺¹⁵N) N₂O production was quantified and the ability of the AOB strains to produce ¹⁵N-N₂O by reduction of exogenously applied ¹⁵N-NO₂⁻ during nitrifier denitrification was determined. Results are exemplified with assay data obtained with *Nitrosospira* sp. strain En13, because this strain has not previously been the subject of physio-

Table 1. Details and 16S-rRNA gene-based phylogenetic affiliation of AOB strains used in this study.

| AOB strain | NCBI accession number for 16S rRNA gene sequence; isolation details (if known) and available literature references ^a | 16S-rRNA gene – based phylogenetic cluster/lineage ^b |
|--|--|--|
| <i>Nitrosomonas europaea</i> ATCC 19718 | BX321856; Isolated from soil; Source: H. J. E. Beaumont, Vrije University, Amsterdam, the Netherlands (Beaumont <i>et al.</i> , 2002, 2004a,b; Chain <i>et al.</i> , 2003) | <i>Nitrosomonas europaea</i> / <i>Nitrosococcus mobilis</i> ' lineage |
| <i>Nitrosomonas europaea</i> ATCC 25978 | NB070982; Isolated by soil enrichment; (Lewis and Pramer, 1958). | <i>Nitrosomonas europaea</i> / ' <i>Nitrosococcus mobilis</i> ' lineage |
| <i>Nitrosospira briensis</i> strain 128 | L35505; (Teske <i>et al.</i> , 1994) | <i>Nitrosospira</i> lineage, cluster 3 |
| <i>Nitrosospira multififormis</i> ATCC 25196 | L35509; Isolated from soil, Surinam, South America; (Watson <i>et al.</i> , 1989). | <i>Nitrosospira</i> lineage, cluster 3 |
| <i>Nitrosospira tenuis</i> strain NV12 | M96405 | <i>Nitrosospira</i> lineage, cluster 3 |
| <i>Nitrosospira</i> sp. strain 40KI | X84656; Isolated from cultivated clay loam soil (pH 6.5), Norway; (Jiang and Bakken, 1999a,b). | <i>Nitrosospira</i> lineage, cluster 0 |
| <i>Nitrosospira</i> sp. strain B6 | X84657; Isolated from pebbles (pH 6.3), nitrifying reactor, sewage treatment plant, Norway (Jiang and Bakken, 1999a,b). | <i>Nitrosospira</i> lineage, cluster 2 |
| <i>Nitrosospira</i> sp. strain En13 | Isolated from Craibstone soil (pH 4.5), Aberdeen, UK; This study. | <i>Nitrosospira</i> lineage, cluster 3 |
| <i>Nitrosospira</i> sp. strain NpAV | Y10127; Isolated from soil, Apple Valley, Minnesota; (McCarty <i>et al.</i> , 1991) | <i>Nitrosospira</i> lineage, cluster 3 |

a. Unless otherwise stated, the immediate source of the strains was the AOB culture collection at the University of Aberdeen, UK.

b. According to the classification proposed by Purkhold and colleagues (2003). Not all 16S rRNA gene sequences of the strains used in the present study were included in the analysis of Purkhold and colleagues (2003). Accordingly, the 16S-rRNA gene-based phylogenetic tree of Purkhold and colleagues (2003) was reconstructed to include sequences for those strains not previously analysed. The resulting tree is available as *Supplementary material* (Fig. S1).

logical characterization. The results of a typical assay are shown in Fig. 1. Over the time course, significant ($P < 0.001$) N_2O (Fig. 1A) and nitrite (Fig. 1B) were produced in comparison to the non-inoculated control. All other strains tested also produced concentrations of N_2O significantly above ($P < 0.05$) control levels. *Nitrosospira* sp. B6 was found to be contaminated with nitrite oxidizers, as determined by detection of nitrate in the growth media and successful polymerase chain reaction (PCR)-amplification of 16S rRNA gene fragments using nitrite oxidizer-specific primers (T. Freitag and J.I. Prosser, unpublished). Therefore, this strain was not used for quantitative analysis of N_2O and ^{15}N - N_2O production rates, but the ability of this co-culture to produce ^{15}N - N_2O from ^{15}N - NO_2^- was assessed. For the remaining eight strains, the kinetics of both cumulative N_2O and nitrite production could be estimated using a linear function (see Fig. 1A and B for strain

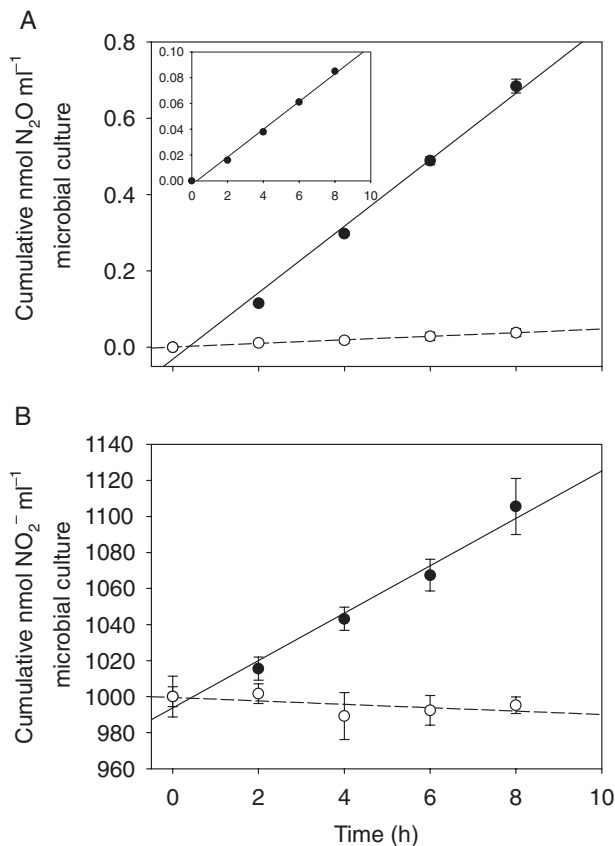


Fig. 1. Typical kinetics of (A) $^{14+15}N$ - N_2O and (B) nitrite production by *Nitrosospira* sp. En13 cell suspensions (●) in comparison to non-inoculated controls (○). The initial cell concentration of *Nitrosospira* sp. En13 in the assay was $1.63 \pm 0.09 \times 10^7$ cells ml^{-1} . Data points are mean \pm SE ($n = 4$). Solid lines represent linear regression function ($P < 0.001$, $R^2 \geq 0.991$) $Y = \beta_1 X + \beta_0$; where Y = cumulative concentration of N_2O or NO_2^- produced ($nmol\ ml^{-1}$), X = time, β_1 = linear rate ($nmol\ ml^{-1}\ h^{-1}$) and β_0 = intercept ($nmol$). Inset in A shows production of ^{15}N - N_2O in medium containing 1 mM ^{15}N - NO_2^- at 25 atom% excess ^{15}N .

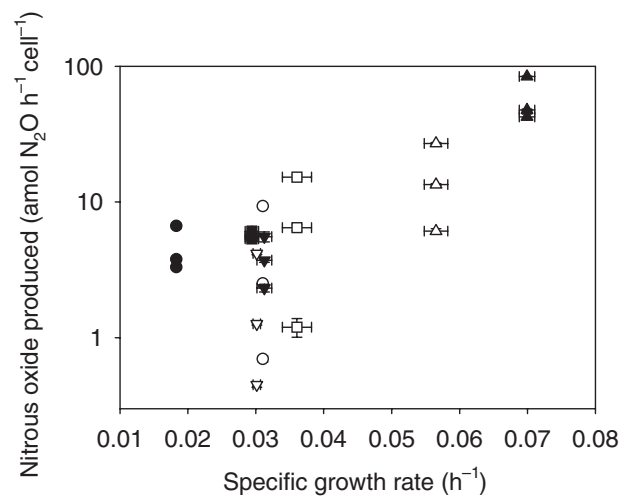


Fig. 2. Production of N_2O by ammonia oxidizing bacterial strains: *Nitrosospira* sp. 40KI (●), *Nitrosospira briensis* 128 (○), *Nitrosospira* sp. En13 (■), *Nitrosospira multiformis* ATCC 25196 (□), *Nitrosomonas europaea* ATCC 19718 (▲), *Nitrosomonas europaea* ATCC 25978 (△), *Nitrosospira* sp. NpAV (▼) and *Nitrosospira tenuis* NV12 (▽). The N_2O production rate is expressed as a function of the maximum specific growth rate for each strain. The estimated linear rate of N_2O produced during the 8-h assay was corrected for the initial number of cells in each assay. Each data point represents the mean ($n = 4$) N_2O production rate for cells harvested from an independent flask. Bars represent ± 1 SEM.

En13; $R^2 \geq 0.92$, $P < 0.01$ in all cases), the exception being nitrite production by *Nitrosospira* sp. 40KI, for which the rate parameter was not significantly different from zero.

For each strain the N_2O production assay illustrated in Fig. 1A was repeated three times with different batches of cells and data were used to estimate the linear rate of N_2O production, correcting to take account of the initial cell number in each repetition. These data are presented in Fig. 2 as a function of maximum specific growth rate, which ranged from $0.018\ h^{-1}$ (*Nitrosospira* sp. 40KI) to $0.070\ h^{-1}$ (*N. europaea* ATCC 19718). The rate of N_2O production ranged from $0.45 \pm 0.02\ amol\ cell^{-1}\ h^{-1}$ in *Nitrosospira briensis* strain 128– $85 \pm 0.5\ amol\ cell^{-1}\ h^{-1}$ in *N. europaea* ATCC 19718: a 186-fold difference. Analysis of variance using all data (specific N_2O production rate for eight AOB strains, repeated three times with four within-repetition replicates, i.e. 95 d.f.) revealed that both AOB strain ($P < 0.001$) and repetition ($P < 0.001$) had a highly significant effect on the specific N_2O production rate. One-way ANOVA using the repetition means (23 d.f.) also supported the highly significant effect ($P < 0.001$) of AOB strain on N_2O production rate, but pairwise comparison of repetition means (Table 2) revealed that this was due to the significantly ($P < 0.05$) elevated N_2O production rate of *N. europaea* ATCC 19718; there was no difference in N_2O produced between the remaining strains. When expressed as a percentage of the nitrite produced during

Table 2. Ammonia oxidizer $^{14+15}\text{N-N}_2\text{O}$ and $^{15}\text{N-N}_2\text{O}$ production rates and molar yields expressed as a percentage of the NO_2^- and $^{14+15}\text{N-N}_2\text{O}$ production rate respectively.[†]

| AOB strain | Total N_2O production rate (amol $^{14+15}\text{N-N}_2\text{O h}^{-1} \text{ cell}^{-1}$) [‡] | Yield of $^{14+15}\text{N-N}_2\text{O}$ on a nitrite basis [§] (%) | $^{15}\text{N-N}_2\text{O}$ production rate (amol $^{15}\text{N-N}_2\text{O h}^{-1} \text{ cell}^{-1}$) [§] | Yield of $^{15}\text{N}_2\text{O}$ on a total ($^{14+15}\text{N-}$) N_2O basis [§] (%) |
|-------------------------------------|---|---|---|---|
| <i>N. europaea</i> ATCC 19718 | 58.0 ^{a*} | 0.45 ^a | 3.29 ^a | 7.8 ^a |
| <i>N. europaea</i> ATCC 25978 | 15.5 ^b | 0.19 ^b | 0.34 ^b | 5.6 ^b |
| <i>N. briensis</i> strain 128 | 4.2 ^b | 0.03 ^c | 0.02 ^c | 3.5 ^c |
| <i>N. multiformis</i> ATCC 25196 | 7.6 ^b | 0.08 ^d | 0.20 ^d | 3.1 ^c |
| <i>N. tenuis</i> strain NV12 | 2.0 ^b | 0.06 ^d | 0.06 ^e | 4.5 ^d |
| <i>Nitrosospira</i> sp. strain 40KI | 4.6 ^b | — ^{**} | 0.88 ^f | 13.5 ^e |
| <i>Nitrosospira</i> sp. strain En13 | 5.7 ^b | 0.70 ^e | 0.66 ^g | 12.4 ^f |
| <i>Nitrosospira</i> sp. strain NpAV | 3.9 ^b | 0.29 ^f | 0.33 ^b | 8.9 ^g |

*Mean values in columns superscripted by different letters are significantly different ($P > 0.05$).

**Rate of nitrite production by *Nitrosospira* sp. 40KI was not significantly different from 0 ($P > 0.05$); therefore, percentage yield of $^{14+15}\text{N-N}_2\text{O}$ on a nitrite basis was not calculated.

[†]Calculations are based on N_2O concentration measured in the headspace; no correction is made for the concentration of N_2O dissolved in the medium.

[‡]Values are the means of three independent experiments.

[§]Values are the means ($n = 4$) for cells harvested from a single independent flask.

each assay, N_2O production ranged from $0.03 \pm 0.002\%$ (*N. briensis*) to $0.70 \pm 0.08\%$ (*Nitrosospira* sp. En13).

All of the strains tested produced $^{15}\text{N-N}_2\text{O}$ at concentrations significantly ($P < 0.05$) greater than the non-inoculated control. As in the case of total N_2O production, kinetics of $^{15}\text{N-N}_2\text{O}$ production could be fitted to a linear function, an example of which is shown in Fig. 1A (inset). Estimated specific rates of $^{15}\text{N-N}_2\text{O}$ production are presented in Table 2. *Nitrosospira* sp. B6 also produced significant amounts of $^{15}\text{N-N}_2\text{O}$, but this culture was contaminated with nitrite oxidizers. Freitag and colleagues (1987) have shown that *Nitrobacter* strains can grow by dissimilatory nitrate reduction with production of N_2O . Thus the role of the contaminating nitrite oxidizers in $^{15}\text{N-N}_2\text{O}$ production in incubations with *Nitrosospira* sp. B6 can not be ruled out and quantitative data are not presented. With regard to total ($^{14+15}\text{N}$) N_2O production, *N. europaea* ATCC 19718 had the highest specific $^{15}\text{N-N}_2\text{O}$ production rate, which was more than double that of the next highest producing strain (*Nitrosospira* sp. 40KI). When the amount of $^{15}\text{N-N}_2\text{O}$ was expressed as a percentage of the $^{14+15}\text{N-N}_2\text{O}$ produced (Table 2), mean values for the AOB strains varied fourfold from $3.1 \pm 0.04\%$ for *Nitrosospira multiformis* to $13.5 \pm 0.3\%$ for *Nitrosospira* sp. 40KI.

Discussion

Most previous attempts to characterize the physiology of N_2O production by AOB have concentrated on *N. europaea* strains (Ritchie and Nicholas, 1972; Hynes and Knowles, 1984; Poth and Focht, 1985; Anderson *et al.*, 1993; Kester *et al.*, 1997; Beaumont *et al.*, 2002). Here, we quantified N_2O production in aerobic incubations by six AOBs representing different phylogenetic clusters in the *Nitrosospira* lineage [clusters 0, 2 and 3 in the classifica-

tion according to Purkhold and colleagues (2003) based on cultured strains, Table 1] in addition to two different strains of *N. europaea*. All eight AOB strains tested produced N_2O at detectable levels in the experimental system employed. Results show that, in addition to nitrosomonads, nitrosospiras also produce N_2O , in agreement with observations reported by Goreau and colleagues (1980), Remde and Conrad (1990), Jiang and Bakken (1999a), Dundee and Hopkins (2001) and Wrage and colleagues (2004a).

Nitrous oxide production by our strains was not always comparable to production reported elsewhere in the literature. Published data on a marine *Nitrosomonas* sp., studied by Goreau and colleagues (1980), lead to a N_2O production rate of $46 \text{ amol h}^{-1} \text{ cell}^{-1}$, a value similar to those reported here. However, data obtained by Remde and Conrad (1990) for *N. europaea* (strain 28) suggest a production rate of $229 \text{ amol N}_2\text{O h}^{-1} \text{ cell}^{-1}$, i.e. fourfold and 15-fold higher than rates reported here for *N. europaea* strains ATCC 19718 and ATCC 25978 respectively. Similarly, data in Hynes and Knowles (1984) suggest a value for a *N. europaea* strain of $833 \text{ amol N}_2\text{O cell}^{-1} \text{ h}^{-1}$. The discrepancies in the rate of N_2O production between studies probably reflect differences in incubation conditions used because the amount of N_2O formation depends on ammonium concentration (Hynes and Knowles, 1984), dissolved oxygen concentration (Goreau *et al.*, 1980) and cell density (Remde and Conrad, 1990). Low oxygen conditions are known to induce clear increases in N_2O production rates by *Nitrosomonas* sp. (Goreau *et al.*, 1980; Kester *et al.*, 1997; Dundee and Hopkins, 2001). The discussed data sets were all obtained using aerobic incubations. However, *N. europaea* (strain 28) was incubated in medium containing 10 mM ammonium (compared with 3.5 mM in this study), and Hynes and Knowles (1984)

conducted assays at cell densities of 1.2×10^9 ml⁻¹ (compared with $\sim 1 \times 10^7$ in this study). In contrast to N₂O production rate, the values for molar yield of N₂O expressed as a percentage of moles of NO₂⁻ produced are of the same order of magnitude (0.03–0.7%) as those reported by Remde and Conrad (1990) for *N. europaea* strain 28 (0.05–1.95%) and by Jiang and Bakken (1999a) for *Nitrosomonas* ATCC 25978 (0.2%) and *Nitrospira* sp. strains (~0.05–1%).

One characteristic of the N₂O assays employed in this study was the significant variation in the rate of N₂O production between different repeats of the assay for some of the AOB strains. Variation between assay repeats (and for within-repeat replicates) has been recorded previously during experiments to quantify consumption of NO by *N. europaea* ATCC 19718 (Beaumont *et al.*, 2004a) and the effect of inhibitors on N₂O production by *N. europaea* ATCC 19718 and *N. briensis* strains (Wrage *et al.*, 2004a). It is known that N₂O production depends on cell density (Remde and Conrad, 1990) and differing initial cell concentrations in assays has been suggested as a reason for variable N₂O production (Wrage *et al.*, 2004a). In our assays the initial cell concentration was between 0.63 and 2.6×10^7 ml⁻¹, but did not consistently influence the N₂O production rate (data not shown). An alternative explanation is variation in the activity of different batches of cells, although care was taken to ensure that cells used were in a comparable physiological state, by using those harvested in the same growth phase (75–90% of the available ammonium used, as judged by nitrite analysis). However, in spite of the variation between cell batches, *N. europaea* ATCC 19718 consistently produced significantly ($P < 0.001$) greater amounts of N₂O (four times more than the next highest producing strain). The genome of *N. europaea* ATCC 19718 has recently been sequenced (Chain *et al.*, 2003) and this strain is becoming a model for reverse genetics approaches to study the role of denitrification-like genes in inorganic nitrogen metabolism (Beaumont *et al.*, 2002, 2004a; Schmidt *et al.*, 2004). The atypical behaviour of *N. europaea* ATCC 19718 in terms of N₂O production should be borne in mind if extrapolating findings gained with this strain to other soil AOB.

Most strikingly, all of the strains tested, both nitrosomonads and nitrospiras, were able to reduce nitrite to N₂O, i.e. to carry out nitrifier denitrification. Application of ¹⁵N-NO₂⁻ (25 atom percentage excess ¹⁵N) enabled distinction of N₂O produced by nitrite reduction from that potentially produced by other mechanisms, such as the formation of N₂O by abiotic reactions involving intermediates of ammonia oxidation. The ability of *N. europaea* to mediate nitrifier denitrification has been demonstrated previously, either through use of ¹⁵N analysis (Poth and Focht, 1985; Remde and Conrad, 1990) or artificial electron donors (Remde and Conrad, 1990;

Anderson *et al.*, 1993). Genes putatively involved in nitrifier denitrification [*nirK* (*aniA*) and *norB*, encoding nitrite reductase and nitric oxide reductase, respectively] have been annotated on the *N. europaea* ATCC 19718 genome (Chain *et al.*, 2003) and detected by PCR amplification in pure cultures of marine *Nitrosomonas* and *Nitrosococcus* spp. (Casciotti and Ward, 2001, 2005). It should be noted that Arp and Stein (2003) suggest, based on evidence gained using a *nirK*⁻ deficient *N. europaea* ATCC 19718 mutant, that the *nirK* gene product is not wholly responsible for nitrite reduction. However, recent experiments show that *N. europaea* ATCC 19718 *nirK*⁻ and *norB*⁻ mutants are incapable of ¹⁵N-gas production from ¹⁵N-NO₂⁻, indicating a key role for NirK and NorB in nitrifier denitrification (Schmidt *et al.*, 2004). Interestingly, despite the efforts of Casciotti and Ward (2001, 2005), *nirK* and *norB* homologues have yet to be identified in *Nitrospira* spp. by PCR amplification. By contrast, genomic DNA from *Nitrospira* sp. NpAV and *Nitrospira* (formerly *Nitrosolobus*) sp. 24-C has been shown to hybridize at low stringency to probes specific for *nirK* in *Pseudomonas* sp. G-179 (Bruns *et al.*, 1998). Regardless of the molecular basis of nitrifier denitrification in *Nitrosomonas* and *Nitrospira* spp., this is the first demonstration of the ability of nitrospiras to undertake this function.

Up to 13.5% (*Nitrospira* sp. strain 40KI, Table 2) of the N₂O produced was enriched in ¹⁵N derived from exogenously applied ¹⁵N-NO₂⁻. If enzymes responsible for nitrite reduction reside in the periplasmic space, as seems most likely from knowledge of other denitrification systems (Zumft, 1997), the exogenously -applied ¹⁵N-NO₂⁻ must have been able to cross the outer membrane for the first step in the denitrification pathway to occur. The pK_a of nitrous acid (HNO₂) is 3.3; therefore, at the pH of medium used in this study (7.5), less than 0.01% of exogenous nitrite would have been present in the protonated form, enabling free passage through the outer membrane (Moir and Wood, 2001). It is more likely that the majority of the nitrite entered the periplasmic space via outer membrane porins (Nikaido, 2003). A gene putatively encoding general diffusion outer membrane porins has been identified on the *N. europaea* ATCC 19718 genome (<http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=30248031>). Given the fact that ¹⁵N-NO₂⁻ was applied at only 25 atom%, the actual percentage of total (¹⁴+¹⁵N) N₂O produced through denitrification of total exogenous (¹⁴+¹⁵N) NO₂⁻ may have been much higher (i.e. four times that of the values obtained on a ¹⁵N-N₂O basis, assuming negligible isotopic discrimination against ¹⁵N). Thus, up to 54% of the N₂O produced may have been derived from denitrification of exogenous nitrite. Whether the remaining N₂O was produced by reduction of nitrite produced endogenously (by ammonia oxidation) or through other sources

(e.g. abiotic reaction of intermediates of ammonia oxidation) is not known.

Detection of nitrifier denitrification activity in six nitrosospiras (possibly a seventh, including strain B6), which are representatives of three major phylogenetic clusters (Purkhold *et al.*, 2003) in the cultured *Nitrosospira* lineage, in addition to the previously known activity of nitrosomonads, suggests that the ability to denitrify is a widespread, if not ubiquitous, trait in ammonia oxidizers, at least in those phylogenetic clusters with cultured representatives. The widespread nature of the denitrification trait prompts conjecture regarding the ecological significance of nitrifier denitrification. The benefits to ammonia oxidizers are beginning to be understood. One suggestion is that reduction of nitrite may be a strategy to reduce competition for oxygen from nitrite oxidizers by removing their substrate (Poth and Focht, 1985). Another suggestion is that nitrite is reduced to conserve oxygen and produce energy in low oxygen environments (Poth and Focht, 1985; Schmidt and Bock, 1997) as in classical heterotrophic denitrification. If the role of denitrification in ammonia oxidizers is the same as in heterotrophs, the pathway should be regulated in the same way, i.e. by concentrations of available oxygen and nitrite (Zumft, 1997). However, NorB in *N. europaea* ATCC 19718 is expressed under aerobic conditions (Beaumont *et al.*, 2004a) and NirK is expressed aerobically and in response to increasing concentrations of nitrite (Beaumont *et al.*, 2004b). The latter finding suggests that a major role for the NirK enzyme is to help to protect *N. europaea* cells from toxic nitrite produced during nitrification (Beaumont *et al.*, 2002, 2004b; Arp and Stein, 2003).

Several studies have reported oxygen sensitivity of N₂O production in ammonia oxidizers (Goreau *et al.*, 1980; Hynes and Knowles, 1984; Poth and Focht, 1985; Remde and Conrad, 1990; Kester *et al.*, 1997; Dundee and Hopkins, 2001). For example, N₂O production was shown to be greatly stimulated under anaerobic conditions (approximately 700 times greater than in aerobic conditions) in both *N. europaea* strain 28 and *Nitrosospira* (formerly *Nitrosovibrio*) strain K71 (Remde and Conrad, 1990). Thus, the potential of the *N. europaea* and *Nitrosospira* spp. strains tested here for N₂O production may also be significantly greater at oxygen tensions lower than those studied. Increased N₂O production by ammonia oxidizers at low oxygen tensions has previously been attributed to increased rates of nitrifier denitrification (Dundee and Hopkins, 2001); presumably even if oxygen concentration does not significantly repress the expression of denitrifying enzymes in AOBs (Beaumont *et al.*, 2004b), denitrification activity could be greater under low O₂ due to reduced competition with nitrite from O₂ as a preferred electron acceptor. However, studies have not specifically quantified the contribution of nitrifier denitrification above

other possible processes that could be occurring. Here, the application of ¹⁵N-NO₂⁻ as a tracer, has demonstrated that significant nitrite reduction occurs in well-aerated cultures (in one experiment, the dissolved oxygen content was estimated as 5.6 ± 0.1 mg l⁻¹); a finding in agreement with the studies with *N. europaea* (Beaumont *et al.*, 2004a,b) which demonstrate the aerobic expression of denitrification pathway enzymes. Thus, soil-based studies which attempt to identify the contribution of various biological pathways to total N₂O production from soils through use of oxygen suppression of denitrification (Webster and Hopkins, 1996; Wrage *et al.*, 2004b) should consider the possibility that this approach may not completely suppress the nitrifier denitrification pathway.

Experimental procedures

Ammonia oxidizer bacteria (AOB) strains and maintenance

Nine betaproteobacterial AOB strains were used in this study. Details of their source and affiliation with ammonia oxidizer 16S rRNA gene-based phylogenetic clusters proposed by Purkhold and colleagues (2003) are given in Table 1. All strains were maintained as static batch liquid cultures at 28°C in modified Skinner and Walker (1961) (S and W) medium containing (per litre): (NH₄)₂SO₄ (0.235 g); KH₂PO₄ (0.200 g); CaCl₂·2H₂O (0.040 g); MgSO₄·7H₂O (0.04 g); FeSO₄ (0.5 mg); Na₂EDTA (0.5 mg) and phenol red (0.5 mg) as a pH indicator. After autoclaving, a sterile solution of Na₂CO₃ (5% w/v) was added dropwise to the medium until the colour changed from yellow to pink, corresponding to a pH of 7.5–8. Growth of AOB cultures results in acidification of the medium (through production of nitrous acid) and a change in colour of the indicator from pink to yellow. Cultures were checked weekly for pH indicator colour change and, if required, the pH was adjusted by addition of Na₂CO₃. Ammonia oxidizing bacteria cultures were subcultured into fresh S and W medium after the pH had been adjusted three times. Cultures were maintained in at least triplicate and, at each subculture, were screened for heterotroph contamination by drop plating on nutrient agar. Nutrient agar plates were incubated at 28°C for 28 days. Cultures showing contamination were discarded.

Determination of AOB specific growth rates

Stationary phase cultures, maintained in S and W medium, were used to inoculate (1%, v/v) quadruplicate 250 ml conical flasks containing 150 ml SWH medium, consisting of S and W medium with added HEPES (free acid, Sigma-Aldrich, Dorset, UK) buffer (20 mM, pH 7.5) and without phenol red. After autoclaving, the medium was amended with 1.78 ml l⁻¹ of 5% (w/v) Na₂CO₃ solution. Flasks were incubated at 28°C in the dark on an orbital shaker (120 r.p.m.). At regular intervals, aliquots (500 µl) were removed for quantification of nitrite production (see below) as a surrogate measure of growth.

Ln nitrite concentration was plotted against time and the specific growth rate (μ) estimated over the linear range by regression analysis.

Colorimetric quantification of nitrite

Nitrite concentrations in culture supernatant samples were determined using a miniaturized version of the assay of Keeney and Nelson (1982). Briefly, 20 μ l diazotizing reagent (50 mg sulfanilamide ml^{-1} in 2.4 M HCl) was added to 40 μ l sample diluted in 920 μ l d.d. H_2O . After vortexing and incubation for 5 min, 20 μ l of coupling reagent [30 mg N-(1-naphthyl)-ethylenediamine HCl ml^{-1} in 0.12 M HCl] was added and the solution vortexed and incubated for a further 10 min prior to determination of absorbance at 540 nm. A standard curve was constructed using NaNO_2 in the range 0–5 $\mu\text{g NO}_2\text{-N ml}^{-1}$.

Assay of $^{14+15}\text{N-N}_2\text{O}$ and $^{15}\text{N-N}_2\text{O}$ production by AOB strains

Ammonia oxidizing bacteria cultures for N_2O assays were grown up in 250 ml SWH medium in 1-l conical flasks at 28°C and shaken at 120 r.p.m. (Innova 4330 refrigerated incubator shaker, New Brunswick Scientific). Cells were harvested by filtration (0.2 μm cellulose acetate filter) using a vacuum manifold when 75–90% of the available ammonium had been utilized (as judged by analysis of nitrite and, for strain B6, nitrate and nitrite). Cells were washed off the filter and resuspended in an equal volume of fresh SWH medium. Aliquots (50 ml) of the resulting cell suspensions were decanted to quadruplicate 125 ml narrow-necked bottles. A solution of $\text{Na}^{15}\text{NO}_2$ (200 μ l, 250 mM, 25 atom percentage ^{15}N) was pipetted into each bottle, resulting in a final NO_2^- concentration of 1 mM. $\text{Na}^{15}\text{NO}_2$ was purchased at 98–99 atom percentage ^{15}N (Sercon, Cheshire, UK) and mixed with NaNO_2 to give the desired ^{15}N enrichment. The bottles were sealed with gas-tight silicone rubber septa and placed on a rotary shaker (120 r.p.m., 28°C). The headspace contained ambient air concentrations of O_2 . After incubation for 2, 4, 6 and 8 h, headspace samples (12 ml) were removed using a gas-tight syringe and stored in 12 ml evacuated gas-vials. From the 12 ml of sample, 1 ml was taken for total ($^{14+15}\text{N}$) N_2O determination by gas chromatography (GC) and the remainder was used for $^{15}\text{N-N}_2\text{O}$ analysis by isotope ratio mass spectrometry (IRMS). After gas sampling, the bottles were opened, a subsample (300 μ l) of the culture removed for nitrite analysis and the headspace allowed to exchange with the atmosphere before resealing. The initial number of cells used in each assay was determined by direct counts using epifluorescence microscopy and staining with 4',6-diamidino-2-phenylindole (DAPI) (Hobbie *et al.*, 1977).

Gas subsamples were analysed for total ($^{14+15}\text{N}$) N_2O using an Agilent 6890 GC equipped with a ^{63}Ni electron capture detector and a Haysep Q 60–80 mesh packed 8 ft (2.44 m) column. The carrier gas was N_2 and the isothermal column and detector temperatures were 40°C and 250°C respectively. The GC was calibrated using laboratory air, 1.1 and 5.2 ppmv standards.

Isotope ratio mass spectrometry analysis was conducted by cryofocusing using an ANCA TGII trace gas preparation module connected to a 20–20 isotope ratio mass spectrometer (PDZ/Europa). Headspace sample in the 12-ml vial was transferred to pre-evacuated and He-flushed gas-tight 125-ml glass bottles and cut with laboratory air prior to analysis. $^{15}\text{N-N}_2\text{O}$ atom percentage values, volume of headspace and sample analysed and total concentration of N_2O in the culture headspace, as determined by GC analysis, were used to calculate the μmoles of $^{15}\text{N-N}_2\text{O}$ produced in the culture headspace. The concentration of $^{15}\text{N-N}_2\text{O}$ was then converted to a per cell basis using the DAPI count data.

Statistical treatment of data

Analysis of Variance (General Linear Model) and regression analysis were performed using Minitab v. 13.1.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Phylogenetic trees showing the placement of nine strains (in bold) used in this study within the *Nitrosospira* (A) and *Nitrosomonas* (B) lineages of the *Betaproteobacteria* subgroup ammonia oxidizing bacteria.