Selective inhibition of ammonia oxidising archaea by simvastatin stimulates growth of ammonia oxidising bacteria

Jun Zhao 1, Marcus O. Bello 2, Yiyu Meng, James I. Prosser, Cecile Gubry-Rangin *

School of Biological Sciences, University of Aberdeen, Cruickshank Building, St. Machar Drive, Aberdeen, AB24 3UU, UK

ARTICLE INFO

Keywords:
Activity
Nitrification Inhibitor
Ammonia
Thaumarchaeota
Stable isotope probing

ABSTRACT

The desire to understand and distinguish the relative growth and activity of ammonia oxidising archaea (AOA) and ammonia oxidising bacteria (AOB) in soil nitrification has increased the search for selective inhibitors of these two groups. This study aimed to investigate the potency and specificity of simvastatin as a specific AOA inhibitor in pure cultures and in soil and to determine the effect of AOA inhibition on both ammonia oxidation activity and growth of AOB, under the hypothesis that AOB growth is higher when competition for NH₄⁺ from AOA is removed. Simvastatin selectively inhibited pure cultures of all tested AOA at concentrations of 8–100 μM. In soil microcosms incubated for 21 days with low and high NH₄⁺ concentrations, AOA but not AOB were selectively inhibited by simvastatin in both acidic (pH 4.5) and near-neutral (pH 6.5) soils. Additionally, growth of AOB significantly increased at both NH₄⁺ concentrations following inhibition of AOA by simvastatin, suggesting that competition for substrate between AOA and AOB is a key factor restraining AOB growth in NH₄⁺ limited soils. Simvastatin can therefore be used as a selective AOA inhibitor to investigate kinetic characteristics of AOB in soils and to study competition between AOA and AOB in complex environments.

1. Introduction

Ammonia oxidising archaea (AOA) and bacteria (AOB) and more recently discovered complete ammonia oxidisers (comammox) perform the initial step of nitrification (oxidation of ammonia to nitrite). There is evidence that AOA and AOB have different preferences for ammonia source and concentration, with AOA being favoured when NH₄⁺ is generated by mineralisation of organic N, while AOB prefer supply of high concentrations of inorganic NH₄⁺ (Di et al., 2009; Hink et al., 2018; Hofferle et al., 2010; Leviciuk-Hofferle et al., 2012; Stopnisek et al., 2010; Verhamme et al., 2011). This was previously explained by higher ammonia affinity and sensitivity of AOA compared to AOB (Prosser and Nicol, 2012), but recent studies have challenged this theory following the isolation of several Ca. Nitrososococcus strains of AOA (Jung et al., 2016; Lehtovirta-Morley et al., 2016a; Sauder et al., 2017) that can grow at high ammonium concentrations, similar to those supporting growth of typical soil AOB. Recent studies have also failed to find major differences between AOA and AOB affinities for NH₃ (Hink et al., 2017a; Kits et al., 2017). In addition, stimulation of AOA growth has recently been demonstrated at high soil ammonium concentration following specific inhibition of AOB (Hink et al., 2018). These findings suggest that both AOA and AOB are able to grow at both low and high NH₄⁺ concentrations, while competition for ammonia may be the major factor differentiating growth of AOA and AOB in soil.

Soil pH is another key factor controlling niche specialisation of both AOA and AOB (Aigle et al., 2019; Gubry-Rangin et al., 2011, 2015, 2018; Nicol et al., 2008). While AOA can dominate ammonia oxidation in both acidic and neutral soils (Gubry-Rangin et al., 2010; Zhang et al., 2010, 2012), information on AOB growth and activity in acidic soils is limited. However, the potential activity of AOB at low pH is indicated by the presence of AOB phenotypes in acidic soils and laboratory cultivation and isolation of AOB from such soils (Aigle et al., 2019; Allison and Prosser, 1991; Carnol et al., 2002; De Boer et al., 1995; Jiang and Bakken, 1999; Long et al., 2012; Nicol et al., 2008; Petersen et al., 2012; Wertz et al., 2012), including recent isolation of an acid-tolerant AOB strain (Hayatsu et al., 2017). Accumulating evidence for physiological adaptation of AOB to low pH and potentially similar substrate affinities of soil AOA and AOB provide the basis for hypotheses that i) AOB growth

* Corresponding author.
E-mail address: c.rangin@abdn.ac.uk (C. Gubry-Rangin).
1 Present address: Institute for Food and Agricultural Sciences (IFAS), Department of Microbiology & Cell Science, University of Florida, 3205 College Avenue, Fort Lauderdale (Davie), FL33314, USA.
2 Present address: Department of Microbiology, Faculty of Science, Adekunle Ajasin University Akungba Akoko, Nigeria.

https://doi.org/10.1016/j.soilbio.2019.107673
Received 23 August 2019; Received in revised form 15 November 2019; Accepted 16 November 2019
Available online 18 November 2019
0038-0717/© 2020 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (http://creativecommons.org/licenses/by/4.0/).
occurs in soils of low pH and/or low NH₄ concentration and ii) alleviation of AOA competition for ammonia will stimulate AOB growth in soil.

Testing these hypotheses requires an inhibitor of AOA that does not inhibit AOB. Several nitrification inhibitors have been tested for selective inhibition of AOA or AOB and there is strong evidence that the aliphatic alkane 1-octene is an efficient inhibitor of AOB, which does not reduce AOA activity and growth in both pure culture and soil (Hink et al., 2017b, 2018; Taylor et al., 2013). Several selective inhibitors of AOA have also been tested, but the majority of these compounds are unsuitable due to either weak inhibitory effect or high toxicity to AOB (Shen et al., 2013). PTIO (2-phenyl-4,4,5,5-tetramethylimidazolidine-1-oxyl 3-oxide) is currently the most commonly used inhibitor for archaeal ammonia oxidation in pure culture (Kozlowski et al., 2016; Martens-Habbena et al., 2015; Shen et al., 2013) and environmental samples (Duan et al., 2018; Meinhardt et al., 2018; Sauder et al., 2017). This inhibitor scavenges NO, an important intermediate of archaeal ammonia oxidation (Walker et al., 2010; Yan et al., 2012). However, PTIO showed no or incomplete inhibition of AOA nitrification in several environmental samples, including soil (Fu et al., 2018; Sauder et al., 2016), and might affect the activity and growth of AOB, following evidence that NO may also be an obligate intermediate for ammonia oxidation by AOB (Caranto and Lancaster, 2017). Therefore, an alternate AOA inhibitor with distinct mechanism of action is needed to replace PTIO.

Statins are a class of molecules that interfere with archaebal cell membrane biosynthesis by inhibiting 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, a crucial enzyme in the mevalonate pathway (Lam and Doolittle, 1992; Miller and Wolin, 2001). Statins do not inhibit growth of bacteria, which use an alternative pathway for cell membrane biosynthesis (Jain et al., 2014). Statins have been demonstrated as effective inhibitors of several archaea, including methanogens from animal and human intestines (Gottlieb et al., 2016), but have not been tested on AOA in culture or in soil. The initial aim of this study was therefore to assess the effect of a commercially available statin derivative drug, simvastatin, on the specific growth rate of pure cultures of several AOA and AOB and on soil communities. The second aim was to test the hypotheses stated above, that AOB growth is stimulated in the absence of AOA growth, regardless of ammonium concentration and soil pH. This was investigated by analysis of nitrification rate and changes in growth and activity of AOA and AOB in microcosms containing soils of different pH and in the presence and absence of urea and simvastatin.

2. Materials and methods

2.1. Inhibition of ammonia oxidisers in laboratory culture

The effect of simvastatin was assessed on growth of four AOA strains, Candidatus Nitrosotalea devanaterra (Lehtovirta-Morley et al., 2011), Candidatus Nitrosotalea sinensis (Lehtovirta-Morley et al., 2014), Nitrososphaera viennensis (Tourna et al., 2011) and Candidatus Nitroscocmics franklandii (Lehtovirta-Morley et al., 2016a), and three AOB strains, Nitrosomonas europaea (ATCC, 19718), Nitrosospira multiformis (ATCC 25196) and Nitrosomonas eutropha. Ca. N. devanaterra and Ca. N. sinensis were cultivated in freshwater medium (FWM) at pH 5.0 as described by Lehtovirta-Morley (2011), modified by adding 2.5 mM final concentration 2-(N-morpholino) ethanesulfonic acid buffer (MES buffer) (pH 5.35) and 4 mM sodium bicarbonate. Ca. N. franklandius and N. viennensis grew in FWM at pH 7.5 as described by Lehtovirta-Morley (2016a) and Tourna et al. (2011), respectively. N. europaea, N. multiformis and N. eutropha were cultivated in Skinner and Walker medium at pH 7.9 (Skinner and Walker, 1961). Cultures were incubated for 4–17 days in 100-ml Duran bottles containing 10 ml of liquid medium. Autoclaved growth media were supplemented with HPLC-grade simvastatin (Sigma-Aldrich, UK), dissolved in dimethyl sulfoxide (DMSO), to reach final simvastatin concentrations of 0, 8, 16, 32 or 100 μM. The amount of DMSO was adjusted to give a final concentration of 0.0098% (for simvastatin concentrations of 8, 16 and 32 μM) or 0.17% (v/v) (for a simvastatin concentration of 100 μM). Potential toxicity of DMSO was assessed in cultures supplemented with DMSO at 0.0098% or 0.17%. All cultures were incubated in triplicate in the dark without shaking and 100 μl of culture was sampled daily for assessment of growth through changes in nitrite concentration.

Ammonia oxidiser maximum specific growth rate was calculated as the slope of semi-logarithmic plots of temporal increases in nitrite concentration. Effects of a range of simvastatin concentrations (8–100 μM) were quantified as the proportional decreases in specific growth rate in comparison to the control (culture with same DMSO concentration).

2.2. Inhibition in soil microcosms

Microcosms consisted of 250-ml serum bottles containing agricultural soil at pH 4.5 or 6.5 collected from field plots at SRUC, Craibstone, Scotland (grid reference NJ872104). Details of the sampling sites and soil characteristics were described by Kemp et al. (1992). Triplicate microcosms were constructed for each soil with the following combined treatments: i) with or without urea amendment, ii) with or without addition of simvastatin and iii) incubation under 5% (v/v) 13C-2 CO2 or isotopically labelled 15C-CO2 in the headspace gas, resulting in a total of 8 treatments. The urea in 13C-CO2 amended microcosms was 99 atom % 13C-labelled (Sigma-Aldrich). Soil microcosms were established in sterile 250-ml serum bottles containing 23 g equivalent dry soil and sterile distilled water to achieve an initial moisture content of 30% (w/w, with respect to soil dry weight). For the urea treatment, water was replaced by urea solution to provide an initial concentration of 200 μg urea-N g⁻¹ dry weight soil. Urea was rapidly converted to ammonium and ammonium-N concentration was monitored weekly in 1-g soil samples and microcosms were further supplemented with urea at the beginning of the third week to restore a concentration of 200 μg N g⁻¹ soil. Simvastatin was added by thoroughly mixing solid Acros Organics™ simvastatin (Thermo Fisher Scientific, USA) with soil at a mass ratio of 12.5 mg g⁻¹ dry weight soil before construction of the microcosms. This proportion is based on a preliminary experiment performed on the pH 4.5 soil (see supplementary results and Fig. S1). All bottles were sealed with a butyl rubber stopper and aluminium cap during incubation in the dark at 28 °C for 21 days. Bottles were aerated by removing caps at 3–4-day intervals to ensure adequate O2 supply for nitrification and were re-supplied with 13C-CO2 to avoid dilution by 12C-CO2 gas originating from soil respiration. All microcosms were destructively sampled after incubation for 21 days and collected soils were frozen at ~80 °C before use.

2.3. Chemical analysis

Nitrite concentration in samples of growth media was measured colorimetrically as described by Lehtovirta-Morley et al. (2011) and the increases in NO2 concentration (μM) over time during exponential growth were used to calculate the maximum specific growth rate of AOA and AOB. Soil NH4 and NO3 (NO2 plus NO3) concentrations were determined by colorimetric analysis as previously described using 1 g of soil (Hink et al., 2018).

2.4. DNA extraction and quantification of amoA genes

DNA extraction was performed on 0.5 g soil as described by Griffiths et al. (2000). The quantity and quality of DNA extracts were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The abundance of archaeal and bacterial amoA genes was estimated by quantitative PCR (qPCR) of the DNA extracts (diluted to 2–10 ng μl⁻¹) using the primer pairs amoA23f/amoA616r (Tourna et al., 2008) and amoA1F/amoA2R (Rothsauwe et al., 1997), respectively. qPCR
conditions and standards were as described in Hink et al. (2017b). To test whether comammox also contributed to nitrification and were stimulated when AOA growth is inhibited, the abundance of comammox amoA gene was estimated in 20-μl reaction mixes consisting of 10 μl IQ® SYBR® Green Supermix, 0.4 μg BSA, and 0.5 μM each of the primers Ntsp-amoA 16F2/359R (Fowler et al., 2018). qPCR conditions were optimised as: 95°C for 5 min, 40 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min followed by measurement of fluorescence. The standards for comammox qPCR contained an equal-molar mix of 23 out of 40 sequenced clones of PCR amplicons by Ntsp-amoA 16F2/359R, to cover the degeneracy of the primers as much as possible, and a dilution series containing 10^1–10^8 genes per assay was used for quantification of all genes. The efficiencies of the AOA, AOB and comammox amoA qPCR assays were 0.89–1, 0.82–0.91 and 0.93–1.02, respectively, with R^2 values >0.99. Melting curve analysis and standard agarose gel electrophoresis were used to assess amplification specificity. Growth of ammonia oxidisers was estimated as the difference between initial and final gene abundances for each sample. Relative contributions of growing AOA and AOB to nitrification were then estimated using reported maximum specific cell activities of AOA (0.57 fmol NH₃ cell⁻¹ h⁻¹) and AOB (23 fmol NH₄⁺ cell⁻¹ h⁻¹) (Prosser and Nicol, 2012).

2.5. Stable isotope probing

Isopycnic density gradient centrifugation was performed on the extracted DNA from microcosm samples after incubation for 21 days as previously described (Zhang et al., 2010). Briefly, 1 μg of DNA was mixed with CsCl solution (dissolved in TE buffer, 1.710 g ml⁻¹ density) in an 8-ml quick-seal polyallomer tube (Beckman Coulter, USA) before centrifugation in a MLN-80 rotor (Beckman Coulter) at 100,000 g (45,000 rpm) for 60 h at 20°C. DNA in each tube was then separated into 15 fractions (500 μl each). DNA in each fraction was precipitated by incubating at 4°C overnight with 1 ml polyethylene glycol 6000 and 10 μg glycogen (Thermo Fisher Scientific) followed by centrifugation at 16,000 g for 45 min. The precipitated DNA was washed by 1 ml 70% ethanol and re-suspended in 30 μl sterile water. The archaean, bacterial and comammox amoA genes in each DNA fraction (fractions 2–14) were then quantified by qPCR. Autotrophic growth of ammonia oxidisers was determined by comparing 12C-CO₂ and 13C-CO₂ incorporation profiles, i.e. when the relative gene abundance was higher in 13C-CO₂- than 12C-CO₂-amended treatments in the “heavy fractions” (with buoyant density of 1.721–1.739 g ml⁻¹ for AOA, 1.733–1.755 g ml⁻¹ for AOB and 1.730–1.750 for comammox). The abundances of growing AOA, AOB and comammox were estimated as the proportions of cells incorporating 13C-CO₂ multiplied by the total corresponding amoA gene abundance, estimated by qPCR in each sample. The relative contributions of autotrophic AOA and AOB to nitrification were estimated using the reported maximum specific cell activity of ammonia oxidisers described above, but the absence of cultivated terrestrial comammox prevented their inclusion.

2.6. Statistical analysis

All statistical analyses were performed on Statistics 23 (SPSS, Chicago, IL, USA). For pure cultures, one-way ANOVA was performed on the proportional decrease in maximum specific growth rates with simvastatin concentration as a fixed factor. For soil microcosms, two-way ANOVA was performed to assess the effect of NH₄⁺ concentration and inhibitor on soil nitrification rate (measured as NO₂⁻ production after incubation for 21 days) and growing AOA, AOB and comammox abundances (calculated as the difference between initial and final gene abundance). Tukey HSD multiple post-hoc tests were used to assess the significance of the differences among the means.

The relative proportion and abundance of 13C-labelled archaean, bacterial and comammox amoA genes in heavy fractions were analysed by independent Student’s t-tests between corresponding microcosms in the absence and presence of simvastatin.

3. Results

3.1. Effect of simvastatin on ammonia oxidiser growth in liquid culture

Inhibition of ammonia oxidation activity of four AOA and three AOB isolates by simvastatin was determined by calculating the proportional decrease in maximum specific growth rate in medium supplemented with a series of simvastatin concentrations. Ammonia oxidation activity of Ca. N. devanaterra and Ca. N. sinensis was completely inhibited by simvastatin at a concentration of 8 μM (Fig. 1), with no detectable increases in nitrite concentration after incubation of simvastatin-amended cultures for 17 and 11 days, respectively. Ca. N. franklandus and N. viennensis growth were not inhibited at 8–32 μM simvastatin but were inhibited at the higher concentration of 100 μM after incubation for 10 and 7 days, respectively (Fig. 1). In contrast, growth of the three AOB strains was not inhibited at any simvastatin concentration tested in this study (8–100 μM) after incubation for 4 days (Fig. 1). Growth of all AOA and AOB isolates tested was not inhibited by 0.0098 or 0.17% (v/v) DMSO (Fig. S2).

3.2. Effect of simvastatin on nitrification and ammonia oxidiser growth in soil

Ammonium concentration remained low (<10 μg N g⁻¹ dry soil) during incubation of microcosms containing non-amended agricultural soil with pH 4.5 or 6.5 (Fig. S3) and nitrification (measured as NO₂⁻ production) occurred at rates of <55 and <30 μg N g⁻¹ dry soil, respectively (Fig. 2a and b). Nitrification rate was not significantly inhibited by addition of simvastatin (Fig. 2a and b). In urea-fertilised soils, NH₄⁺ concentration increased rapidly after urea supplementation, due to the rapid urea hydrolysis and release of ammonium, and CO₂-assimilation (Fig. S3) and nitrification rate were greater than in the unfertilised soils (Fig. 2a and b). Simvastatin supplementation significantly increased and decreased NO₂⁻ production in urea-amended pH 4.5 and pH 6.5 soils, respectively (Fig. 2a and b).

Growth of AOA occurred in both soils in the absence of simvastatin and was not affected by urea amendment (Fig. 2c and d). Growth of AOA was not detectable in soil microcosms after addition of simvastatin, except in urea-amended pH 4.5 soil, where growth was significantly reduced (Table 1 and Fig. 2c and d). AOB growth was not detected in either unfertilised soil, in the presence or absence of simvastatin. However, AOB growth was significant in urea-supplemented soils and, importantly, was significantly greater in microcosms amended with simvastatin (Fig. 2e and f). Comammox growth was not detected under any conditions in either soil (Table 1 and Fig. S4).

DNA-SIP confirmed selective inhibition of AOA growth. Autotrophic AOA growth was detected in all soils in the absence of simvastatin (Fig. 3), but was not detected in microcosms amended with simvastatin, with the exception of urea-amended pH 4.5 soil, in which simvastatin supplementation significantly reduced the relative abundance of 13C-labelled AOA from 10.2% to 1.5% (Fig. 3). Integration of these percentages with AOA total abundance indicated that some autotrophic growth occurred in the absence of simvastatin in both soils (Table 1). In both soils, autotrophic growth of AOB was not decreased or inhibited by simvastatin addition. DNA-SIP provided evidence for AOA growth in both unamended and urea-amended pH 4.5 soil, and growth of AOB in unamended simvastatin-treated soil (Fig. 3 and Table 1). DNA-SIP also indicated potential autotrophic growth of comammox in urea-fertilised pH 4.5 soil, following inhibition of AOA growth by simvastatin (Fig. 3 and Table 1).

The estimation of relative contributions of AOA and AOB, either by the autotrophic ammonia oxidiser abundance (13C-labelled genes in heavy fractions) or by the total increase in ammonia oxidiser abundance after incubation for 21 days, indicated unaccounted sources of
nitrification in low NH$_4$ soils after inhibition of AOA growth (Fig. S5), suggesting growth of other ammonia oxidisers.

4. Discussion

This study is the first to investigate the inhibitory effect of simvastatin on ammonia oxidisers and provided evidence for selective inhibition of AOA growth in all pure cultures tested at varying concentrations that do not affect AOB growth (Fig. 1). This is consistent with previous inhibition by statins of many non-AO archaeal isolates (Cabrera et al., 1986; Gottlieb et al., 2016; Lam and Doolittle, 1992; Matsumi et al., 2007; Wendoloski et al., 2001; Zheng et al., 2012). The minimum inhibitory concentration (MIC) of simvastatin for the two acidophilic Thaumarchaeota (8 μM) was similar to that for the Euryarchaeota.

Fig. 1. The proportional changes in maximum specific growth rate of ammonia oxidiser cultures without inhibitor (C) or in the presence of simvastatin at concentrations of 8, 16, 32 and 100 μM. Activity of Ca. N. devanaterra and Ca. N. sinensis was not detectable at 8 and 16 μM of simvastatin and higher concentrations were not investigated. Error bars represent standard errors of means from triplicate cultures and the different letters in the graphs indicate significant differences ($p < 0.05$) within each plot.

Fig. 2. Increases in nitrite plus nitrate (NO$_x$) concentration (a–b) and archaeal (c–d) and bacterial (e–f) amoA gene abundances after incubation of microcosms containing soils of pH 4.5 and 6.5 for 21 days. “no SVS” and “+ SVS” refer to incubation of soil microcosms in the absence and presence of simvastatin, respectively, while “no urea” and “+ urea” refer to soils without and with urea fertilisation, respectively. Error bars represent standard errors of means from triplicate microcosms, with different letters above bars indicating a significant difference ($p < 0.05$) within each plot. “NS” refers to no significant increase after microcosm incubation.

J. Zhao et al.
Table 1

Estimated abundance of growing AOA, AOB and comammox (amoA genes g⁻¹ dry soil). The abundance was either calculated as the increased abundance of total AOA, AOB or comammox amoA genes after incubation (qPCR) or estimated as the abundance of ¹⁵N-labelled amoA genes in heavy fraction (SIP). *no SVS* and *- SVS* refer to incubation of soil microcosms in the absence and presence of.sinvastatin, respectively, while *no urea* and *- urea* refer to soils without and with urea fertilisation, respectively. *NS* indicates absence of significant increase. Symbol *†* indicates significant difference between *no SVS* and *- SVS* treatments based on t-test statistics.

|                | AOA qPCR | AOB qPCR | Comammox qPCR |
|----------------|----------|----------|---------------|
| pI 4.5 (no urea) |          |          |               |
| no             | 2.5      | *3.9*    | NS            |
| SVS            | 10²      | 10⁶      | NS            |
| SVS            | NS       | NS       | 1.7           |
| pI 4.5 (urea)  |          |          |               |
| no             | *3.4*    | *6.2*    | 1.4           |
| SVS            | 10²      | 10⁶      | 1.2 10⁷       |
| SVS            | *8.8*    | 10⁶      | 1.6           |
| pI 6.5 (no urea)|         |          |               |
| no             | *9.9*    | 8.6      | NS            |
| SVS            | 10⁶      | 10⁶      | *2.4*         |
| pI 6.5 (urea)  |          |          |               |
| no             | *9.6*    | *2.3*    | *1.3*         |
| SVS            | 10²      | 10⁶      | *1.2* 10⁷     |

Thermococcus kodakarenensis (5 µM) (Matsumi et al., 2007), while the MIC observed for the two neutrophilic thaumarchaeotal strains tested (100 µM) was much higher than the highest reported archaeal inhibitory concentration for a wild-type archaeal strain (16 µM) for the crenarchaeotal Sulfolobus islandicus, Zheng et al., 2012). The reasons for different sensitivities to sinvastatin in AOA strains are not known, but may be due to differences in membrane composition, as sinvastatin inhibits the rate-limiting enzyme of the pathways producing the main component (isoprenoid) of the archaeal lipid layer (Delmotte and Delmotte-Plaquee, 1953; Siriton, 2014). All AOA membranes studied contain a characteristic glycerol dialkyl glycerol tetraether lipid, crenarchaeol, that is unique to Thaumarchaeota (Elling et al., 2017; Schouten et al., 2013), but the proportion of crenarchaeol is much higher in all tested neutrophilic AOA (including *N. viennensis*) than the acidiphilic *Ca. Nitrosotalea* devanaterra strains (Lehtovirta-Morley et al., 2016b). This suggests that the majority of thaumarcheotal genera (except *Ca. Nitrosotalea*) would be inhibited at similarly high concentrations (>100 µM), considering the phylogenetic divergence of the *Ca. Nitrosotalea* genus (Gubry-Rangin et al., 2015; Macqueen and Gubry-Rangin, 2016). However, additional screening of other AOA is required to generalise this finding. The mechanism of action of sinvastatin on AOA is different from that of previous inhibitors of ammonia oxidation in AOA and AOB, inhibiting membrane synthesis rather than using a competitive substrate (CO, methane and n-alkynes (McCarty, 1999; Ruser and Schulz, 2015; Taylor et al., 2013)) to inhibit oxidation of ammonia to hydroxylamine, deactivating the AMO enzyme (DDC, DMPP and ATU (McCarty, 1999; Ruser and Schulz, 2015)), or preventing the conversion of hydroxylamine to nitrite by inhibiting the hydroxylamine dehydrogenase or acting as a nitric oxide scavenger (PTIO, caffeic acid and methylene blue hydrate (Sauder et al., 2016)).

Autotrophic growth of AOA was detected in all soil microcosms in the absence of sinvastatin irrespective of NH₄ concentration (Fig. 3), demonstrating that growth of AOA in soil is not inhibited by high ammonium concentration (>100 µg N g⁻¹) and supporting previous physiological and ecological studies on high NH₄ tolerance of AOA (Hink et al., 2017b, 2018; Jung et al., 2016; Lehtovirta-Morley et al., 2016a; Sauder et al., 2017). Urea amendment increased growth of both AOA and AOB (Fig. 3). AOB dominated autotrophic ammonia oxidation in soils with high NH₄ concentrations, while AOA appeared to be the more active ammonia oxidisers in soils with low NH₄ concentrations (Fig. 3). These results collectively support previous findings (Hink et al., 2018), suggesting that niche differentiation is not due to inability of AOA or AOB to grow under high and low concentrations, respectively, but rather to differences in the ability of AOA and AOB to compete for NH₄ within the soil.

Further support for this proposal comes from microcosms in which ammonia was supplied through mineralisation of native soil organic nitrogen. In the absence of sinvastatin, this favoured growth of AOA, which is consistent with previous studies (Ji et al., 2010; Hink et al., 2018; Levincik-Hoferle et al., 2012; Stopnisek et al., 2010; Verhamme et al., 2011). This has been explained by resultant lower ammonium concentration and higher ammonia substrate affinity of AOA, as indicated by early studies of cultivated isolates (Prosser and Nicol, 2012), but more recent studies indicate that cultivated representatives of soil AOA and AOB have similar KM values (Hink et al., 2017a, 2018; Kits et al., 2017) and direct evidence for niche specialisation through differences in high NH₄ affinity is therefore lacking. However, this study suggests that preferential growth of AOA could be due to higher NH₃ affinity, but estimation of ammonia affinity on a larger range of AOA strains is still required, in particular for the strains thriving in acidic soils such as those affiliated to the Nitrosotalea lineage. Although there was no detectable increase in AOB abundance in these unamended soils, some autotrophic AOB growth was demonstrated by DNA-SIP in pH 4.5 but not pH 6.5 soil, demonstrating that DNA-SIP provided greater sensitivity than changes in amoA abundance when determining AO growth. Interestingly, DNA-SIP demonstrated that growth of AOB was higher in each unamended soil when AOA were inhibited by sinvastatin. Thus, when relieved of competition from AOA, AOB but not comammox growth was significantly stimulated and nitrification rate was unaltered at low ammonium concentration, indicating that low substrate affinity was a potential limiting factor for AOB but not comammox growth. Inhibition of AOA also stimulated AOB growth in urea-amended soils where NH₄ concentration was high (Fig. 2e and f), while AOA activity in urea-amended soils in the absence of sinvastatin (Fig. 3) provided further evidence for the activity of, and competition between AOA and AOB at both high and low ammonium concentration.

Surprisingly, DNA-SIP also provided evidence for AOB growth in unfertilised pH 4.5 soil even without inhibition of AOA (Fig. 3 and Table 1), which was significantly stimulated following a urea fertilisation, indicating physiological adaptation of AOB to grow at low pH. Analysis of the betaproteobacterial *Nitrosospira* amoA genes present in this soil showed that a single phylogenetic cluster was highly abundant (Aigle et al., 2019) and strains affiliated to this phylogenetic cluster have been isolated from acidic soils, including *Nitrosospira* sp. II17, O13, O4 and AH1B1 (Aakra et al., 1999; De Boer and Laanbroek, 1989; Jiang and Bakken, 1999). In addition, a survey of 48 UK soils over a range of pH (3.5–9.0) showed that AOB phenotypes within this cluster were frequently detected in low pH soils (Aigle et al., 2019; Laverman et al., 2001; Nurgolo et al., 2007). Therefore, this study provides evidence for AOB-mediated ammonia oxidation in acidic terrestrial ecosystems, possibly through pH-independent urea hydrolysis (Burton and Prosser, 2001).

Comammox were detected in both soils but did not increase in abundance during incubation at either high or low NH₄ concentration, and the inhibition of AOA stimulated primarily autotrophic growth of AOB, suggesting that competition for NH₄ is not the most critical factor in differentiating comammox from other ammonia oxidisers in soils. Comammox amoA was 43-fold more abundant in pH 6.5 than in pH 4.5 soil and their abundance was lower than that of AOA and either similar
Fig. 3. Distribution of the relative abundance of archaeal, bacterial and comammox amoA genes in CsCl gradients of DNA for 13C-CO2- and 13C-CO2-treated microcosms. “no SVS” and “SVS” refer to incubation of soil microcosms in the absence and presence of simvastatin, respectively, while “no urea” and “urea” refer to soils without and with urea fertilisation, respectively. The plotted values are the relative abundances of archaeal, bacterial or comammox amoA genes in each fraction as a proportion of total abundance across the gradient. Vertical error bars represent standard errors of the relative abundances from triplicate microcosms. Horizontal error bars represent standard errors of buoyant density of the same order fraction from triplicate microcosms. The shaded region represents the ‘heavy DNA’ CsCl fractions, enriching 13C-enriched ammonia oxidiser DNA, at buoyant densities in the ranges of 1.721–1.739, 1.733–1.755 and 1.730–1.750 g ml−1 for AOA, AOB and comammox, respectively. The percentage within each grey bar indicates the relative abundance of 13C-labelled amoA genes for each incubated soil.

Declaration of competing interest

None.

Acknowledgements

The authors would like to thank Dr Robin Walker for access to the experimental plots at the SRUC, Craibstone Estate, Aberdeen. JZ was funded by a Natural Environment Research Council grant (NE/K016342/1), MOB by a University of Aberdeen Elphinstone Scholarship and by TETFund through Adekunle Ajasin University Akungba (AAUA) Nigeria, YM by a NERC grant (NE/R001529/1) and CGR by a Royal Society University Research Fellowship (URF150571).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.107673.

References

Aakra, A., Utaker, J.B., Nes, I.F., Bakken, L.R., 1999. An evaluated improvement of the extinction dilution method for isolation of ammonia-oxidizing bacteria. Journal of Microbiological Methods 30, 25–31.
Aigle, A., Prosser, J.J., Gubry-Rangin, C., 2019. The application of high-throughput sequencing technology to analysis of amoA phylogeny and environmental niche specialization of terrestrial bacterial ammonia-oxidizers. Environmental Microbiology 14.

Allison, S.M., Prosser, J.J., 1991. Urease activity in neutrophilic autotrophic ammonia-oxidizing bacteria isolated from acid soils. Soil Biology and Biochemistry 23, 45–51. Burton, S.A.Q., Prosser, J.J., 2001. Autotrophic ammonia oxidisers in low pH through urea hydrolysis. Applied and Environmental Microbiology 67, 2952–2957.

Cabrera, J.A., Bolda, J., Shields, P.E., Havel, C.M., Watson, J.A., 1986. Isoperoxidase synthesis in Haloarcula halobium. Modulation of 3-hydroxy-3-methylglutaryl coenzyme A concentration in response to mevalonate availability. Journal of Biological Chemistry 261, 3578–3583.

Caranton, J.D., Lancaster, K.M., 2017. Nitric oxide is an obligate bacterial nitrification intermediate produced by Rhodococcus hydroxymolybdate reductase. Proceedings of the National Academy of Sciences 114, 8217–8222.

Carlucci, M., Kowalchuk, G.A., De Boer, W., 2002. Nitrosomonas europaea -like bacteria detected as the dominant β-subclass Proteobacteria ammonia oxidizers in reference and limed acid forest soils. Soil Biology and Biochemistry 34, 1047–1050.

Daims, H., Lebedeva, E.V., Pjevac, P., Han, P., Herbold, C., Albertsen, M., Jehmlich, N., Palatinzky, M., Viehjell, I., Bulaev, A., Kirkegaard, R.H., von Bergen, M., Rattei, T., Bendering, B., Nielsen, P.H., Wagner, M., 2015. Complete nitrification by Nitroposti bacteria. Nature 528, 504–509.

De Boer, W., Gunniewicz, P.A.K., Lanbroek, H.J., 1995. Ammonium-oxidation at low pH by a chemolithotrophic bacterium belonging to the genus Nitrospira. Soil Biology and Biochemistry 27, 127–132.

De Boer, W., Lanbroek, H.J., 1989. Ureolytic activity at low pH by Nitrospira species. Archives of Microbiology 152, 178–181.

Delmonte, P., Delmonte-Plaquer, J., 1953. A new antifungal substance of fungal origin. Nature 171, 544.

Di J., H.J., Kowalchuk, C.C., Shen, J.P., Winefield, C.S., O’Callaghan, M., Bowatte, S., He, J.Z., 2010. Ammonia-oxidizing bacteria and archaea grow under contrasting soil nitrogen conditions. FEMS Microbiology Ecology 72, 386–394.

Di J., H.J., Kowalchuk, C.C., Shen, J.P., Winefield, C.S., O’Callaghan, M., Bowatte, S., He, J.Z., 2010. Nitrification dynamics and not archaea in nitrogen-rich grassland soils. Nature Geoscience 2, 621–624.

Duan, P., Wu, Z., Zhang, Q., Fan, C., Xiong, Z., 2018. Thermodynamic responses of ammonia-oxidizing archaea and bacteria explain N2O production from greenhouse gas emissions. Biogeochemistry and Biological Soil Degradation 120, 37–47.

Ebling, F.J., Konke, M., Nicol, G.W., Stieglmeier, M., Bayer, B., Sippel, E., de la Torre, J.R., Becker, K.W., Thomm, M., Prosser, J.J., Hernd, G.J., Schleper, C., Hinrichs, K-U., 2017. Chemotaxonomic characterisation of the thauamarchaeal lineage. The EMBO Journal 36, 35–45.

Lehtovirta-Morley, L.E., Ge, C., Ross, J., Yao, H., Nicol, G.W., Prosser, J.J., 2014. Characterisation of terrestrial acidophilic archaeal ammonia oxidisers and their inhibition and stimulation by organic compounds. FEMS Microbiology Ecology 89, 80–92.

Lehtovirta-Morley, L.E., Ross, J., Hink, I., Weber, E.B., Gubry-Rangin, C., Thion, C., Prosser, J.J., Nicol, G.W., 2016a. Isolation of ‘Candidatus Nitrosococcus franklandii’, a novel ureolytic soil archaeal ammonia oxidiser with tolerance to high ammonia concentration. FEMS Microbiology Ecology 92, 1–10.

Lehtovirta-Morley, L.E., Sayavedra-Soto, L.A., Gallois, N., Schouten, S., Steen, L.Y., Prosser, J.J., Nicol, G.W., 2016b. Identifying potential mechanisms enabling acidophily in the ammonia-oxidising archaeon ‘Candidatus Nitrososphaera stenoxerotherma’. Applied and Environmental Microbiology 82, 2608–2619.

Lehtovirta-Morley, L.E., Stoecker, K., Vilcinskas, A., Prosser, J.J., Nicol, G.W., 2011. Cultivation of an obligate acidophilic ammonia oxidizer from a nitrifying acid soil. Proceedings of the National Academy of Sciences 108, 15892–15897.

Levkin-Hoferle, S., Nicol, G.W., Ause, L., Mandic-Mulec, I., Prosser, J.J., 2012. Stimulation of thauamarchaeal ammonia oxidation by ammonia derived from organic nitrogen but not added inorganic nitrogen. FEMS Microbiology Ecology 80, 114–123.

Long, X., Chen, C., Xu, Z., Oren, R., He, J., 2012. Abundance and community structure of ammonia-oxidizing bacteria and archaea in a temperate forest ecosystem under ten years elevated CO2. Soil Biology and Biochemistry 46, 163–171.

McCleery, D.J., Gubry-Rangin, C., 2000. Rapid molecular adaptation of ammonia monoxygenase during independent pH specialization in Thauamarchaeota. Molecular Microbiology 26, 1986–1999.

Martens-Habbena, W., Qin, W., Horak, E.A.R., Urakawa, H., Schauer, A.J., Moffett, J.W., Arnosti, C.E., Ingalls, J.A., Stahl, D.A., 2016. Oxidation of nitric oxide by marine ammonia-oxidising archaea and inhibition of ammonia oxidation by a nitric oxide scavenger. Environmental Microbiology 17, 2261–2274.

Matsumi, R., Manabe, K., Fukui, T., Atomi, H., Imanaka, T., 2007. Disruption of a sugar transporter gene cluster in a heterotrophic archaeon using a host-marker system based on antibiotic resistance. Journal of Bacteriology 189, 2683–2691.

McGarty, W.G., 1999. Modes of action of nitrification inhibitors. Biology and Fertility of Soils 29, 1–9.

Meinhardt, R.A., Kostnop, I., Nunn, M.W., Strand, S.E., Fransen, S.C., Casciotti, K.L., Stahl, D.A., 2018. Ammonia-oxidizing bacteria are the primary N2O producers in an ammonia-oxidising archaea dominated alkaline agricultural soil. Environmental Microbiology 20, 2195–2206.

Miller, T.R., Wollin, M.J., 2001. Inhibition of growth of methane-producing bacteria of the ruminant rumenostach from hydroxymethylglyutaroyl–SCoA reductase inhibitors. Journal of Dairy Science 84, 1445–1448.

Nicol, G.W., Leininger, S., Schleper, C., Prosser, J.J., 2008. The influence of soil pH on the diversity, abundance and transcriptional activity of ammonia oxidizing archaea and bacteria. Environmental Microbiology 10, 2966–2978.

Nugroho, R.A., Roling, W.F.M., Laverman, A.M., Verhoef, H.A., 2007. Low nitrification rates in acid Scots pine forest soils are due to pH-related factors. Microbial Ecology 53, 89–97.

Offre, P., Prosser, J.J., Nicol, G.W., 2009. Growth of ammonia-oxidising archaea in soil microcosms is inhibited by acetate. FEMS Microbiology Ecology 70, 99–108.

Peterman, D.G., Blazewicz, S.J., Firestone, M., Herman, D.J., Turetsky, M., Waldrop, M., 2012. Abundance of microbial taxa associated with nitrogen cycling as indices of biochemical process rates across a vegetation gradient in Alaska. Environmental Microbiology 14, 993–1008.

Pichl, M., Schachberger, C., Poghosyan, L., Herbold, C.W., van Kessel, M.A.H.J., Daebeler, A., Steinberger, M., Jetten, M.S.M., Lückner, S., Wagner, M., Daims, H., 2017. AmoA-targeted polymerase chain reaction primers for the specific detection
and quantification of commamon Nitrospira in the environment. Frontiers in Microbiology 8, 1–11.
Prosser, J.I., Nicol, G.W., 2012. Archaeal and bacterial ammonia-oxidisers in soil: the quest for niche specialisation and differentiation. Trends in Microbiology 20, 523–531.
Rotthauwe, J., Witzel, K., Liesack, W., 1997. The ammonia monoxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. 63, 4704–4712.
Ruser, R., Schulz, R., 2015. The effect of nitrification inhibitors on the nitrous oxide (N2O) release from agricultural soils—a review. Journal of Plant Nutrition and Soil Science 178, 171–188.
Saunder, L.A., Albertsen, M., Engel, K., Schwarz, J., Nielsen, P.H., Wagner, M., Neufeld, J. D., 2017. Cultivation and characterization of Candidatus Nitrosocomicus exaquare, an ammonia-oxidizing archaeon from a municipal wastewater treatment system. The ISME Journal 11, 1142–1157.
Saunder, L.A., Ross, A.A., Neufeld, J.D., 2016. Nitric oxide scavengers differentially inhibit ammonia oxidation in ammonia-oxidizing archaea and bacteria. FEMS Microbiology Letters 363, 1–8.
Schouten, S., Hopmans, E.C., Damste, J.S.S., 2013. The organic geochemistry of glycerol dialkyl glycerol tetraether lipids: a review. Organic Geochemistry 54, 19–61.
Shen, T., Stieglmeier, M., Dai, J., Urih, T., Schleper, C., 2013. Responses of the terrestrial ammonia-oxidizing archaean Ca. Nitrosoarchaea viennensis and the ammonia-oxidizing bacterium Nitrospira multiformis to nitrification inhibitors. FEMS Microbiology Letters 344, 121–129.
Sirtori, C.R., 2014. The pharmacology of statins. Pharmaceutical Research 88, 3–11.
Skinner, F., Walker, N., 1961. Growth of Nitrirammonas europaea in batch and continuous culture. Archiv für Mikrobiologie 38, 339–349.
Stopniek, N., Gabry-Raygin, C., Hofferle, S., Nicol, G.W., Mandic-Mulec, I., Prosser, J.I., 2010. Thaumarchaeal ammonia oxidation in an acidic forest peat soil is not influenced by ammonium amendment. Applied and Environmental Microbiology 76, 7626–7634.
Taylor, A.E., Vajrala, N., Giguer, A.T., Gitelman, A.I., Arp, D.J., Myrlood, D.D., Sayavedra-Soto, L., Bottomley, P.J., 2013. Use of aliphatic n-alkynes to discriminate soil nitrification activities of ammonia-oxidizing thaumarchaea and bacteria. Applied and Environmental Microbiology 79, 6544–6551.
Tourna, M., Freitag, T.E., Nicol, G.W., Prosser, J.I., 2008. Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. Environmental Microbiology 10, 1157–1164.
Tourna, M., Stieglmeier, M., Spang, A., Konneke, M., Schleper, C., Engels, M., Schlote, M., Wagner, M., Richter, A., Schleper, C., 2011. Nitrospumare viennensis, an ammonia oxidising archaeon from soil. Proceedings of the National Academy of Sciences 108, 8420–8425.
van Kessel, M.A.H.J., Speth, D., Albertsen, M., Nielsen, P.H., Op den Camp, H.J.M., Kartal, R., Jetten, M.S.M., Lücker, S., 2015. Complete nitrification by a single microorganism. Nature 528, 555–559.
Verhamme, B.T., Prosser, J.I., Nicol, G.W., 2011. Ammonia concentration determines differential growth of ammonia-oxidising archaea and bacteria in soil microcosms. The ISME Journal 5, 1067–1071.
Walker, C.B., Torre, J.R., De, Klitz, M.G., Urakawa, H., Pinel, N., Arp, D.J., Brochier-armanet, C., Chain, P.S.G., Chan, P.P., Gollabgir, A., Hemm, J., Hügler, M., Karr, E.A., Konneke, M., Shin, M., Lawton, T.J., Lowe, T., Martens-Habbena, W., Sayavedra-Soto, L.A., Lang, D., Sievert, S.M., Rosenzweig, A.C., Manning, G., A, S.D., 2010. Nitrospumare maritimus genome reveals unique mechanisms for nitrification and autotrophy in globally distributed marine crenarchaea. Proceedings of the National Academy of Sciences 107, 8818–8823.
Wendeloski, D., Ferrer, C., Dyal-Smith, M.I., 2001. A new simvastatin (mevinolin) resistance marker from Haloarcula hispanica and a new Haloflexx volcanii strain cured of plasmid pHV2. Microbiology 147, 959–964.
Wertz, S., Leigh, A.K.K., Grayston, S.J., 2012. Effects of long-term fertilization of forest soils on potential nitrification and on the abundance and community structure of ammonia oxidizers and nitrite oxidizers. FEMS Microbiology Ecology 79, 142–154.
Yan, J., Haisier, S.C.M., Den Camp, H.J.M.O., Van Niftrik, L., Stahl, D.A., Konneke, M., Rush, D., Damste, J.S.S., Hu, Y.Y., Jetten, M.S.M., 2012. Mimicking the oxygen minimum zone: stimulating interaction of aerobic archaeal and anaerobic bacterial ammonia oxidizers in a laboratory-scale model system. Environmental Microbiology 14, 3146–3158.
Zhang, L.-M., Offre, P.R., He, J.-Z., Verhamme, D.T., Nicol, G.W., Prosser, J.I., 2010. Autotrophic ammonia oxidation by soil thaumarchaea. Proceedings of the National Academy of Sciences 107, 17240–17245.
Zhang, L.M., Hu, H.W., Shen, J.P., He, J.Z., 2012. Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. The ISME Journal 6, 1032–1045.
Zheng, T., Huang, Q., Zhang, C., Ni, J., She, Q., Shen, Y., 2012. Development of a simvastatin selection marker for a hyperthermophilic acidophile, Sulfolobus islandicus. Applied and Environmental Microbiology 78, 568–574.