Supporting Information

Tourna et al. 10.1073/pnas.1013488108

SI Materials and Methods

Soil enrichments. Five grams of grassland or garden soil (pH 8) were added as inoculum to 50 ml of medium for each of the 45 initial enrichment cultures. Variations included initial ammonium concentration (0.1, 0.2 and 0.5 mM), pH (6.4 and 7.5), incubation temperature (28 and 37°C) and supplements, i.e. autoclaved and filtered soil extract (10%), sand as a surface for cell attachment (10%), nitrite (0.1 mM) as a growth promoter, urea (0.5 mM) and streptomycin (50μg/ml). Cultures were monitored for ammonium consumption and subcultured with 20% inoculum. The presence of bacterial ammonia oxidizers was checked with PCR using specific AOB primers targeting the *amoA* gene (1). Contamination with fungi in some of the cultures was overcome by filtering through a 0.45 μm filter. Enrichments EN76 and EN123 stemmed from garden soil, incubated at pH 7.5 with 0.5 mM ammonium. For these enrichments an incubation temperature of 37°C was used, and the media contained streptomycin and 0.1 mM nitrite.

Cultivation of the pure culture. Fresh water medium (FWM) consisted of NaCl (1 g l^{-1}), $MgCl_2 \cdot 6H_2O$ (0.4 g l^{-1}), $CaCl_2 \cdot 2H_2O$ (0.1 g l^{-1}), KH_2PO_4 (0.2 g l^{-1}) and KCl (0.5 g l^{-1}). After autoclaving 1 ml non-chelated trace element mixture (2), 1 ml Fe-NaEDTA solution and 2 ml sodium bicarbonate (1M) were added as previously described (2). The pH of the medium was 7.5 with addition of 10 ml l^{-1} of HEPES buffer (1M HEPES, 0.6M NaOH). To determine the pH-optimum cultures were set up without HEPES and the pH was adjusted with 1M HCl or 1M KOH. Cultures were maintained routinely at 1 or 3 mM NH_4Cl either in 100 ml or in 20 ml medium and were incubated aerobically at 37°C without shaking. Medium for the pure culture was additionally supplemented with 0.5 mM pyruvate if not stated otherwise. Growth was assessed by measurements of ammonium consumption using a salicylic acid assay (3), measurements of nitrite production by using the Griess reagent system (Promega), microscopy and quantitative PCR. Purification trials included serial dilutions and passage of the cultures through different antibiotics (streptomycin, carbenicillin, ampicillin, kanamycin and tetracycline). For all experiments described cultures were grown in duplicate or triplicate in 100 or 20 ml medium.

Microscopy. Cell counting was done using phase contrast light microscopy (after concentrating the cells where necessary). For scanning electron microscopy cells were grown on poly-L-lysin coated glass slides inserted in the cultures during the exponential growth phase for 2 d. Cells were fixed for 1 h in 2.5% glutaraldehyde solution, washed (0.1M sodium cacodylate, pH 7.2, 2% sucrose) and post fixed with 1% OsO_4. Cells were dehydrated using an ethanol series (30%-100%) with a final dehydration step in acetone (100%), then critical point dried in liquid carbon dioxide. Slides were placed on conductive stubs and coated with gold (sputter coater, Agar), before analysis in a Philips XL30 scanning electron microscope (FEI) at 30keV. Images were processed with the Scandium Software (Olympus). For transmission electron microscopy cells were centrifuged and after fixation for 1h in 2.5% glutaraldehyde they were transferred on 200-mesh carbon/formvar-coated copper grids. Staining of cells was performed with 0.5% uranyl acetate for 2 min. A Philips EM 208 transmission electron microscope (FEI) operated at 70_keV and an attached megaview III camera (SIS) were used to record the micrographs.

Cloning/sequencing/phylogenetic analysis. PCR products of 16S rRNA and *amo*A and *amo*B genes were generated using the primer pairs A21F (4) and A1492R (5), CrenamoA-23F/CrenamoA-616R (6) and CrenAmo2.1F/CrenAmo2.1R (2), respectively. Cloning into pCR4-TOPO vector and sequencing with M13f and M13r vector primers was performed using standard procedures. A draft genome sequence of the enrichment EN76 was obtained by 454 pyrosequencing on a 454/FLX-Titanium sequencer (Roche) using 10 μg of input DNA which yielded 228,649 reads of an average length of 381 bp. Phylogenetic analysis of 16S rRNA gene sequences (1272 positions) and concatenated *amo*A and *amo*B protein sequences (365 positions) was performed using maximum-likelihood analysis calculated in PHYML (7) using the HKY85 with four-categories model of evolution and the LG model with eight categories, respectively. Bootstrap support was calculated 100 times for both analyses.

Quantitative PCR. Nucleic acid extraction from at least 1 ml culture was done as previously described (8). Quantification of archaeal *amo*A genes and bacterial 16S rRNA genes was performed in 20 μl volume containing 10 μl iQ™ SYBR Green Supermix (Bio-Rad), 1 μM of each primer and 0.2 mg ml^{-1} BSA. For archaeal standards a serial dilution of the linearized soil fosmid clone 54d9 was used and *amo*A gene copies were quantified using the primers 104F (5′-GCAGGAGACTACATMTTCTA-3′) and 616R (6) with an efficiency of 87%. For bacterial standards a serial dilution of the linearized plasmid (pCR4-TOPO, Invitrogen) containing the 16S rRNA gene of *Nitrosomonas europaea* was used and gene copies were quantified using the primers 338F and 518R (9) with an efficiency of 93%. Amplifications were performed in realplex (Mastercycler ep realplex, Eppendorf). Melting curve analysis was performed at the end of all qPCR runs to indicate amplification of specific products only, before confirmation by standard agarose gel electrophoresis. All qPCR data presented were from independent extractions from duplicate or triplicate cultures and duplicate independent PCR amplifications.

Isotope labeling studies. EN76 cultures were grown at 37 °C in 120 ml serum bottles in 50 ml medium containing 1 mM ammonium and 2 mM sodium bicarbonate, supplemented with either 0.5 mM fully labeled ^{13}C-pyruvate or 0.5 mM ^{12}C-pyruvate, respectively. Gas samples of the cultures at time point 0 h and 10 h were transferred to pre-evacuated glass vials and the stable carbon isotope ratios were analyzed in triplicate by continuous-flow isotope-ratio mass spectrometry on a Thermo Finnigan Delta V Advantage Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Finnigan Gas Bench.

For NanoSIMS analyses, cells from 4 ml of a stationary culture were harvested, washed several times in PBS, fixed with 3 % paraformaldehyde (in 1x PBS) for 1h at room temperature, transferred on boron-doped silicon wafer platelets (7 × 7 × 0.5 mm), rinsed with highly purified water, and stored in a vacuum drying cabinet at 46°C overnight. SIMS measurements from triplicate grown cultures were performed on a NanoSIMS 50L instrument from Cameca (France). See supplementary information for more details.

NanoSIMS analyses. Prior to image acquisition, the analysis areas were pre-sputtered by scanning an unfocused, high intensity Cs^+ primary ion beam (~150 pA beam current) over an area of

75 × 75 μm for 10 min. Pre-sputtering under these conditions leads to erosion of approx. half of the biomass and ensures that the subsequently acquired images originate from regions well within the cells and not from the cell surfaces. Secondary ion intensity distribution images were acquired by simultaneous detection of seven distinct negative ion species ($^{12}C^-$, $^{13}C^-$, $^{12}C^{14}N^-$, $^{16}O^-$, $^{19}F^-$, $^{31}P^-$, $^{32}S^-$) using a finely focused low intensity Cs^+ primary ion beam (~4 pA beam current), scanned over 40 × 40 μm with 512 × 512 pixels resolution and a dwell-time of 20 msec per pixel. The image data were evaluated using the WinImage software package provided by Cameca. Regions of interest (ROIs), referring to individual or agglomerated cells, were defined utilizing the CN^-, P^-, and S^- distribution images as indicators for biomass. The $^{13}C/^{12}C$ isotope-ratio of every ROI was then calculated by normalization of the total ion counts of $^{13}C^-$ to $^{12}C^-$ contained within the respective ROIs. Note that the relative isotopic composition ($c = {}^{13}C/({}^{13}C+{}^{12}C)$ c.f. Fig 4E) is related to the $^{13}C/^{12}C$ isotope-ratio R by $c = R/(1+R)$. Taking the median of the $^{13}C/^{12}C$ isotope-ratio of the cells from the control samples as a reference (R_r), the relative $^{13}C/^{12}C$ isotope-ratio deviation of each individual pixel (δ) was calculated from $\delta = (R_s-R_r)/R_r$, where R_s refers to the isotope ratio determined at each individual pixel. The results of these calculations were plotted as isotope ratio deviation maps, which provide a convenient overview on the homogeneity of the isotopic composition of the sampled area (c.f. Fig. 4 A and C). In the boxplot (Fig. 4E) the mean value is shown by a cross, the grey box marks the distance between Quartile 1 and Quartile 3 and the black lines represent the minimum and maximum values (in the case of ^{12}C-Control smaller than symbol size).

1. Rotthauwe JH, Witzel KP, Liesack W (1997) The ammonia monooxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl Environ Microbiol* 63:4704–4712.
2. Könneke M, et al. (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437:543–546.
3. Kandeler E, Gerber H (1988) Short-term assay of soil urease activity using colorimetric determination of ammonium. *Biology and Fertility of Soils* 6:68–72.
4. DeLong EF (1992) Archaea in coastal marine environments. *Proc Natl Acad Sci USA* 89: 5685–5689.
5. Lane DJ (1991) Nucleic acid techniques in bacterial systematics., eds Stackebrandt E, Goodfellow M (AcademicPress, Chichester, UK), pp 115–175.
6. Tourna M, Freitag TE, Nicol GW, Prosser JI (2008) Growth, activity and temperature responses of ammonia-oxidizing archaea and bacteria in soil microcosms. *Environ Microbiol* 10:1357–1364.
7. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *System Biol* 52:696–704.
8. Tourna M, Freitag TE, Prosser JI (2010) Stable isotope probing analysis of interactions between ammonia oxidizers. *Appl Environ Microbiol* 76:2468–2477.
9. Øvreås L, Torsvik V (1998) Microbial diversity and community structure in two different agricultural soil communities. *Microb Ecol* 36:303–315.

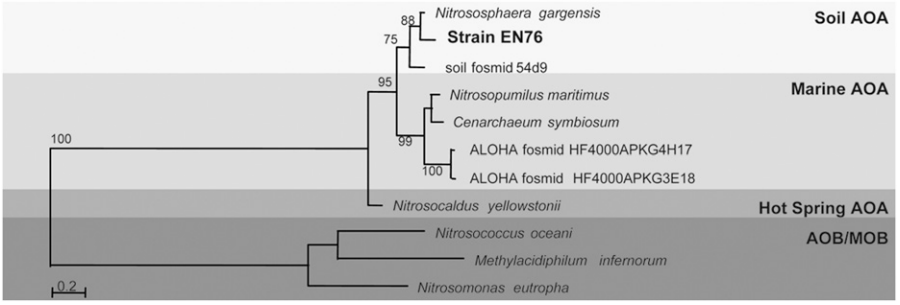

Fig. S1. Phylogenetic analysis of concatenated AmoA and AmoB protein sequences of strain EN76 and other AOA representatives including bacterial homologues of AMO and pMMO enzymes of ammonia and methane oxidizing bacteria respectively. The tree (365 aa positions) was constructed by using maximum-likelihood analysis with PhyML (LG eight categories). Bootstrap support was calculated 100 times. (Scale bar, 0.2 nucleotide changes per position.)

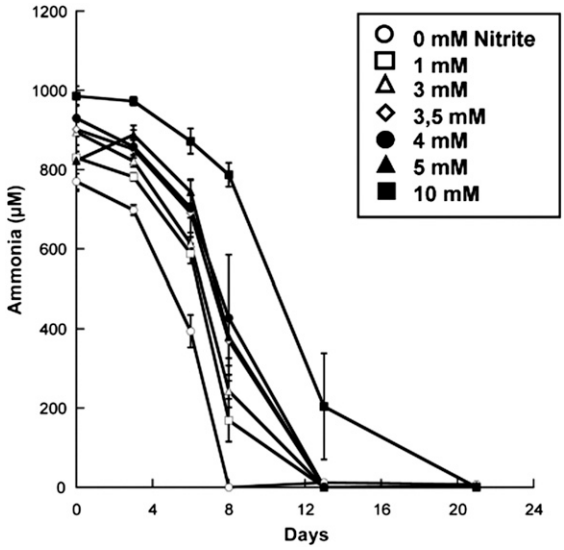

Fig. S2. Effect of nitrite on growth of strain EN76 as estimated by ammonia consumption measurements when grown in triplicate in inorganic medium containing 1 mM ammonia and 0.5 mM pyruvate and supplemented with different nitrite concentrations (0, 1, 3, 3.5, 4, 5, and 10 mM). Error bars represent standard errors.

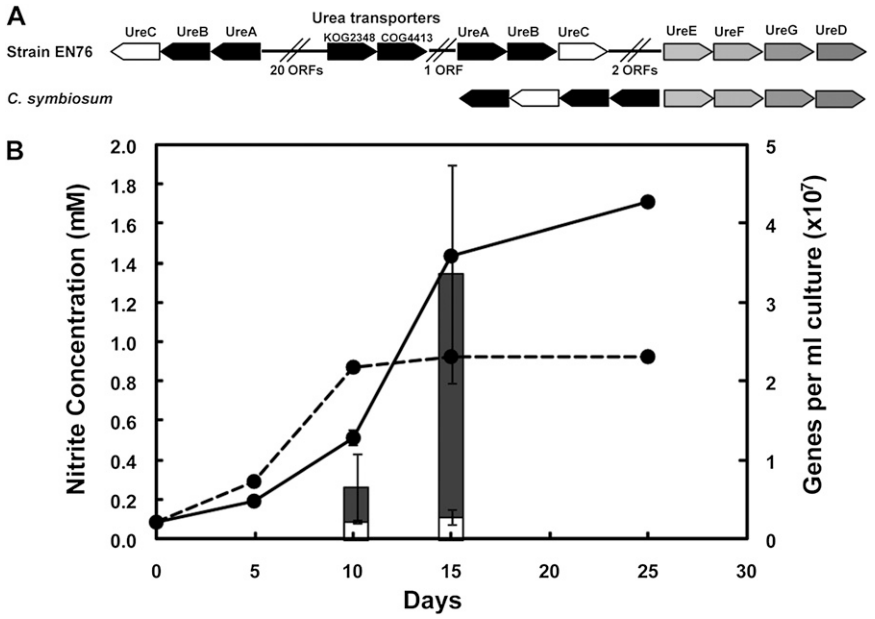

Fig. S3. (*A*) Schematic representation of potential urease gene cluster of strain EN76 assembled from the draft genome sequence and of *Cenarchaeum symbiosum*. (*B*) Growth of enrichment culture EN76 as estimated by nitrite production during growth on medium containing either 1mM NH_4^+ (dotted line) or 1mM urea (solid line) and correlation between nitrite production (solid line) and number of archaeal *amoA* genes (solid bars) and bacterial 16S rRNA genes (open bars) during growth on medium with urea. Plotted data represent means of duplicate incubations. Error bars represent standard errors.

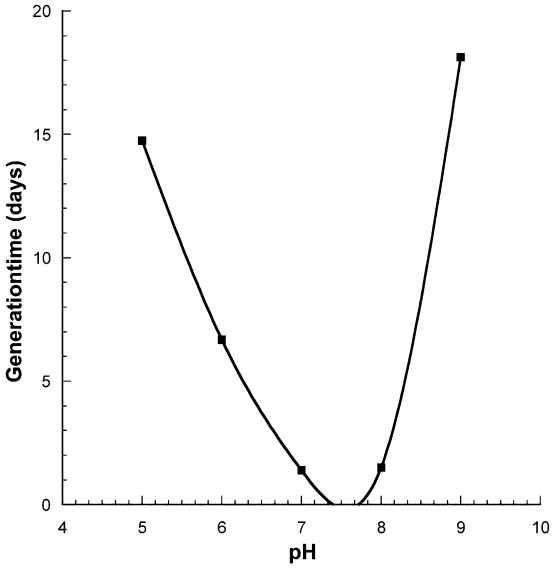

Fig. S4. Estimation of pH optimum of strain EN76. Triplicate cultures of strain EN 76 were grown in inorganic medium containing 1 mM ammonia and 0.5 mM pyruvate and under different pH (pH 5, 6, 7, 8, and 9) adjusted with either 1M HCl or 1M KOH and controlled and readjusted during growth, if necessary. Generation time was estimated as described in Fig. 1.

Table S1. Growth of strain EN76 with a range of substrates

Substrate	Growth score	Lag phase	Final cell density after 35 d
3 mM ammonia	+	Long (>25 d)	Low (1×10^7 cells/mL)
1 mM ammonia	+	Long (>25 d)	Very low (5×10^5 cells/mL)
1 mM ammonia + vitamins	–/+	—	—
3 mM ammonia + vitamins	+	Long (>25 d)	Very low
3 mM urea	–/+	—	—
1 mM urea	+	Long (>25 d)	Low
0.5 mM urea	+	Long (>25 d)	Low
1 mM ammonia + 5 mM bicarbonate	–/+	—	—
1 mM ammonia + 10 mM bicarbonate	–	—	—
1 mM ammonia + 0.05% peptone	–	—	—
0.05% peptone + vitamins	–	—	—
1 mM ammonia + 0.05% yeast extract	–	—	—
0.05% yeast extract + vitamins	–	—	—
1 mM ammonia + amino acid mix	–	—	—
1 mM amino acid mix + vitamins	–	—	—
1 mM ammonia + 1 mM glucose	+	Long (>25 d)	Very low
1 mM ammonia + 1 mM lactose	+	Long (>25 d)	Very low
1 mM ammonia + 1 mM arabinose	+	Long (>25 d)	Very low
1 mM ammonia + 1 mM malate	+	Long (>25 d)	Low
1 mM ammonia + 1 mM sucrose	+	Long (>25 d)	Very low
1 mM ammonia + 1 mM galactose	+	Long (>25 d)	Very low
1 mM ammonia + 1 mM acetate	+	Long (>25 d)	Very low
1 mM ammonia + 0.5 mM acetate	+	Long (>25 d)	Very low
1 mM ammonia + 0.25 mM acetate	+	Long (>25 d)	Very low
1 mM ammonia + 1 mM pyruvate	++	Short (5 d)	High (4.5×10^7 cells/mL)
1 mM ammonia + 0.5 mM pyruvate	++	Short (5 d)	High
1 mM ammonia + 0.25 mM pyruvate	++	Short (5 d)	High
0.5 mM urea + 0.5 mM pyruvate	++	Short (5 d)	High
1 mM ammonia + 0.5 mM pyruvate + 0.025% acetylene	–	—	—
1 mM pyruvate + vitamins	–/+	—	—
1 mM trimethylamine	–/+	—	—
1 mM ethanolamine	–/+	—	—
1 mM methanolamine	–	—	—

Growth of strain EN76 in inorganic medium containing 2 mM sodium bicarbonate (unless otherwise stated) and supplemented with a range of substrates. Evaluation of growth was estimated by regular ammonia consumption and nitrite production measurements as well as by cell counts after 35 days of incubation. Key to growth score: –, no growth; –/+, low nitrite formation (<250μM), single cells visible; +, increased nitrite formation (250-600μM), low cell density; ++, all ammonia converted to nitrite, high cell density. All incubations were done in duplicate.