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Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North Pacific Subtropical Gyre

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Summary

The recent isolation of the ammonia-oxidizing crenarchaeon *Nitrosopumilus maritimus* has expanded the known phylogenetic distribution of nitrifying phenotypes beyond the domain *Bacteria*. To further characterize nitrification in the marine environment and explore the potential crenarchaeal contribution to this process, we quantified putative nitrifying genes and phylotypes in picoplankton genomic libraries and environmental DNA samples from coastal and open ocean habitats. Betaproteobacteria ammonia monooxygenase subunit A (*amoA*) gene copy numbers were low or undetectable, in stark contrast to crenarchaeal *amoA*-like genes that were broadly distributed and reached up to 6×10^4 copies ml^{-1}. Unexpectedly, in the North Pacific Subtropical Gyre, a deeply branching crenarchaeal group related to a hot spring clade (pSL12) was at times abundant below the euphotic zone. Quantitative data suggested that the pSL12 relatives also contain archaeal *amoA*-like genes. In both coastal and open ocean habitats, close relatives of known nitrite-oxidizing *Nitrospina* species were well represented in genomic DNA libraries and quantitative PCR profiles. Planktonic *Nitrospina* depth distributions correlated with those of *Crenarchaea*. Overall, the data suggest that *amoA*-containing *Crenarchaea* are more phylogenetically diverse than previously reported. Additionally, distributional patterns of planktonic *Crenarchaea* and *Nitrospina* species suggest potential metabolic interactions between these groups in the ocean's water column.

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Introduction

Microbial activities drive biogeochemical dynamics in the world's oceans (Karl, 2002). Key elements of the nitrogen cycle such as ammonia oxidation, nitrite oxidation, nitrogen fixation and denitrification are uniquely microbial. While the general biogeochemistry and thermodynamics of nitrogen cycling are well understood, there have been a number of recent unanticipated discoveries regarding the distribution of specific processes and the types of microbial species involved. For example, the anaerobic oxidation of ammonium (anammox) has only recently been recognized as a prevalent process in marine oxygen minimum zones (Dalsgaard *et al*., 2003; Kuypers *et al*., 2003; Jetten *et al*., 2005), with members of the bacterial order *Planctomycetales* mediating this process.

Aerobic nitrification of ammonia to nitrate is one of the more important bioelemental transformations in the sea. It is believed to be a two-step process performed by two distinct groups of chemolithoautotrophic microbes (Kowalchuk and Stephen, 2001; Arp and Stein, 2003). One step oxidizes ammonium to nitrite (beta- or gammaproteobacterial groups typically), and another oxidizes nitrite to nitrate (typically alpha-, gamma-, deltaproteobacteria, or *Nitrospira* species). Until recently, only members of the domain *Bacteria* were thought to be capable of autotrophic ammonia oxidation. Several new lines of evidence, however, have suggested a role for non-thermophilic crenarchaea in ammonia oxidation. Isotopic tracer studies coupled with lipid biomarker or microautoradiographic analyses have indicated that planktonic crenarchaea use bicarbonate as a major carbon source (Pearson *et al*., 2001; Wuchter *et al*., 2003; Herndl *et al*., 2005; Ingalls *et al*., 2006).

Metagenomic surveys of soil or marine samples provided the first genetic evidence of putative crenarchaeal ammonia oxidation (Venter *et al*., 2004; Treusch *et al*., 2005). PCR-based gene cloning surveys of ammonia monooxygenase subunit A (*amoA*) genes indicated their

Table 1. Quantitative macroarray probing data.

Site	Sample depth (m)	Library	Crenarchaeal *amoA*	Group I SSU	Bacterial *amoA*	*Nitrospina* SSU
Hawaii, Station ALOHA	10	HF10	ND	ND	ND	ND
Hawaii, Station ALOHA	70	HF70	ND	ND	ND	ND
Hawaii, Station ALOHA	130	HF130	2	ND	ND	ND
Hawaii, Station ALOHA	200	HF200	3	2	ND	4
Hawaii, Station ALOHA	500	HF500	11	11	ND	3
Hawaii, Station ALOHA	770	HF770	15.75	15	ND	4
Hawaii, Station ALOHA	4000	HF4000	6.5	4	ND	2
Monterey Bay, Station M1	0	EB000	ND	ND	ND	ND
Monterey Bay, Station M1	80	EB080	ND	ND	ND	1
Monterey Bay, Station M1	100	EF100	ND	ND	ND	1
Monterey Bay, Station M1	500	EF500	4	1	ND	ND

Quantitative analyses of specific clone categories. For the fosmid library, the data are normalized to the total amount of cloned DNA that was screened, ~332 Mbp for each assay (9216 clones). HF and EF indicate fosmid libraries, and EB indicates BAC. Approximately 668 and 682 Mbp DNA were screened for EB000 and EB080 libraries respectively. ND, not detected. All positives were confirmed by sequencing of target genes amplified from library clone by PCR (see *Experimental procedures*).

high allelic diversity, the clustering of specific ecotypes, and presence of this gene in marine sediments, estuaries, terrestrial soil samples (Francis *et al*., 2005) and wastewater treatment reactors (Park *et al*., 2006). Metabolic pathway reconstruction in the crenarchaeon *Cenarchaeum symbiosum* (Preston *et al*., 1996) identified more genes potentially involved in ammonia oxidation, including *amoA*, *B* and *C*, as well as genes encoding urease, urea uptake, ammonia transport, nitric acid reductase, and nitrite reductase (Hallam *et al*., 2006a,b). In addition, genes encoding a presumptive modified 3-hydroxypropionate autotrophic carbon assimilation pathway were identified, suggesting a potential pathway for non-thermophilic crenarchaeal autotrophy (Hallam *et al*., 2006a,b).

The recent cultivation of the autotrophic, ammonia-oxidizing *Nitrosopumilus maritimus* provided definitive proof that some marine planktonic crenarchaea are capable of the first step of nitrification (Könneke *et al*., 2005). With this discovery, a North Sea surface water enrichment study suggested that naturally occurring *Crenarchaea* were capable of ammonia oxidation, evolving nitrite as the end-product (Wuchter *et al*., 2006). The same study also showed that archaeal-like *amoA* gene numbers were at least an order of magnitude higher than bacterial *amoA*-like genes in coastal North Sea and North Atlantic waters (Wuchter *et al*., 2006). Similar results were reported in soil ecosystems, where crenarchaeal putative *amoA*-like gene counts were found to range from about equal, to as much as three orders of magnitude higher than detectable bacterial *amoA* depending upon soil type measured (Leininger *et al*., 2006). In total, these studies have identified some specific lineages within the *Crenarchaea* that appear to function, at least in part, as autotrophic ammonia oxidizers.

The distribution and abundance of nitrite-oxidizing bacteria in the ocean is less well known (Zehr and Ward, 2002). Early studies employed immunofluorescent cell counting methods in attempts to enumerate these groups in marine plankton (Ward and Carlucci, 1985). More recently, small subunit (SSU) rRNA gene surveys of marine picoplankton DNA libraries suggested a significant number of rRNA containing clones closely related to *Nitrospina* (Suzuki *et al*., 2004; DeLong *et al*., 2006), a genus whose cultivated members so far all appear to be obligate nitrite oxidizers (Watson and Waterbury, 1971). Among the known nitrite oxidizers, genomic and ribosomal RNA surveys have detected *Nitrospina*, but generally not other nitrite-oxidizing bacterial groups in the coastal and open ocean habitats investigated.

In this study, we report quantitative spatial and temporal profiles of planktonic crenarchaeal SSU rRNA and *amoA*-like genes in Monterey Bay and North Pacific Subtropical Gyre (NPSG) habitats. These surveys revealed the unanticipated abundance of a deeply branching crenarchaeal clade in the open ocean. Archaeal *amoA*-like and SSU rRNA gene distributions were compared with those of betaproteobacterial *amoA* genes in the same samples. In parallel, we report a full sequence of a 64 kb BAC clone, and the DNA insert termini sequences of fosmid and BAC clones, from *Nitrospina*-like *Bacteria.* Additionally, we quantified the distribution and abundance of the *Nitrospina* group by Q-PCR profiling in the water column.

Results

Archaeal SSU rRNA and amoA*-like genes in environmental DNA libraries*

Environmental genomic libraries were screened by hybridization for genes similar to bacterial and crenarchaeal *amoA*, and *Nitrospina*-related SSU rRNA (Table 1). Station ALOHA fosmid libraries had been pre-

viously screened for archaeal SSU rRNA genes (DeLong *et al*., 2006), and those results are included here for comparison (Table 1). All hybridization-positive clones were confirmed by PCR amplification and sequencing of specific genes of interest. Fosmid and BAC libraries (Monterey, EF, EB; Station ALOHA, HF notation) screened for crenarchaeal *amoA*-like and SSU rRNA genes displayed a range of 1 to > 15 positive clones per 332 Mbp DNA screened, corresponding to a range of about 0.6–9.5% genome equivalents, assuming a 2 Mbp average genome size (Table 1). In the case of EB000, 668 Mbp of DNA were screened due to a larger average clone insert size of 80 kb, and 8352 clones total. The EB080 library consisted of 9216 clones of 74 kb average insert size resulting in 682 Mbp total screened. Crenarchaeal *amoA*-like and SSU rRNA genes displayed a one-to-one gene stoichiometry in the different environmental DNA libraries, suggesting that the *amoA*-like gene is present in one copy per genome. [All available evidence indicates that planktonic *Crenarchaea* contain only one rRNA operon per genome (Béjà *et al*., 2002; DeLong *et al*., 2006; Hallam *et al*., 2006a).] No positive hybridization signals were obtained even at low hybridization stringencies for bacterial *amoA* genes, using probes targeting *Nitrosococcus*, *Nitrosomonas* and *Nitrosospira* groups.

A number of BAC and fosmid clones were identified to be close relatives of *Nitrospina* species, by macroarray hybridization and subsequent rRNA sequence analysis (Table 1, see below). Evidence from other experiments (see below) suggested that the rRNA operon in *Nitrospina* is present at two copies per genome.

Distribution of archaeal cells, amoA*-like and rRNA genes in Monterey Bay*

In general, planktonic crenarchaeal cells became detectable (10 000–30 000 cells ml^{-1}, Fig. 1A) along the trace of the nitracline, roughly coinciding where nitrate is 2 μM (Fig. 1D). The crenarchaeal cells reached a maximum of about 2×10^5 cells ml^{-1}.

Conversely, planktonic euryarchaeal cell numbers typically reached their maxima in surface waters, where nitrite levels were relatively high, and appeared to form an intense bloom in late April, 1998 (about 6×10^5 cells ml^{-1}) (Fig. 1B and C). This single bloom event coincides with a relatively low abundance of crenarchaeal cells.

Analysis of the same water column samples indicated that the crenarchaeal *amoA*-like and SSU rRNA gene copy numbers were within one standard deviation of replicate error to one another, supporting a one-to-one gene stoichiometry (Fig. 2A–D). The concentrations of crenarchaeal *amoA*-like or rRNA genes measured in seawater were lower (~25–60%) (at times significantly) than the absolute crenarchaeal cell counts. The correspondence of crenarchaeal *amoA* and rRNA genes when compared with the cell count data suggested that these lower values in the Q-PCR assays were probably due to overall losses in DNA extraction and purification. An alternate explanation for the above discrepancy could be that the cell counts overestimated the abundances. Even though the primer combinations that we employed were developed to detect as many different subclades of the Group I *Crenarchaea* as possible, it could be likely that some were not detected by our Q-PCR method.

The SSU rRNA gene copy number of *Nitrospina*-like *Bacteria* were most abundant at depths of 100–200 m in Monterey Bay where counts ranged from unity to one-fifth that of crenarchaeal *amoA* and SSU rRNA gene copies. *Nitrospina* depth profiles showed roughly similar trends, increasing with depth as did the crenarchaeal distributions, with specific exceptions at 200 m depths in the June and July 1998 samples (Fig. 2B and C).

Primers targeting the *Nitrosomonas*- and *Nitrosospira*-related *amoA* genes yielded Q-PCR values that ranged from about 50 to 1000 genes ml^{-1} seawater, to below our detection limit, with at times high deviation from the mean (Table S1). Bacterial *amoA* gene copy numbers were low in comparison with those observed for archaeal *amoA*-like genes, that were at times two orders of magnitude higher. These data are consistent with the low abundance of these groups in environmental fosmid and BAC libraries, where one *Nitrosomonas*-related clone was detected at 80 m depth from an SSU rRNA survey of the Monterey BAC library EB080 (Suzuki *et al*., 2004).

Diversity and stoichiometry of crenarchaeal rRNA and amoA*-like genes at station ALOHA*

Q-PCR profiles of crenarchaeal *amoA*-like and SSU rRNA genes revealed discrepancies in gene stoichiometries at particular depths. Specifically, crenarchaeal *amoA* gene copy numbers were several orders of magnitude higher than crenarchaeal SSU rRNA genes, particularly at 200 m (Fig. 3A). To investigate whether groups not targeted by our crenarchaeal rRNA gene primers might account for this disparity, archaeal SSU rRNA genes were amplified using universal primers, cloned and sequenced from the 200 m DNA sample. Of the archaeal rRNA gene clones, 72% were crenarchaeal, with 32 clones affiliated with marine Group I *Crenarchaea* while surprisingly another 17 clustered with the pSL12-related clade (Fig. 4). While only qualitative inferences can be drawn from library data, they are consistent with the relative proportions of the pSL12 in the subsequent Q-PCR (see below). The remainder of the clones were derived from planktonic Group II *Euryarchaea* (8), Group III *Euryarchaea* (10) and Group IV *Euryarchaea* (1) (data not shown). The deeply branching pSL12 clade, originally discovered in a hot spring in Yel-

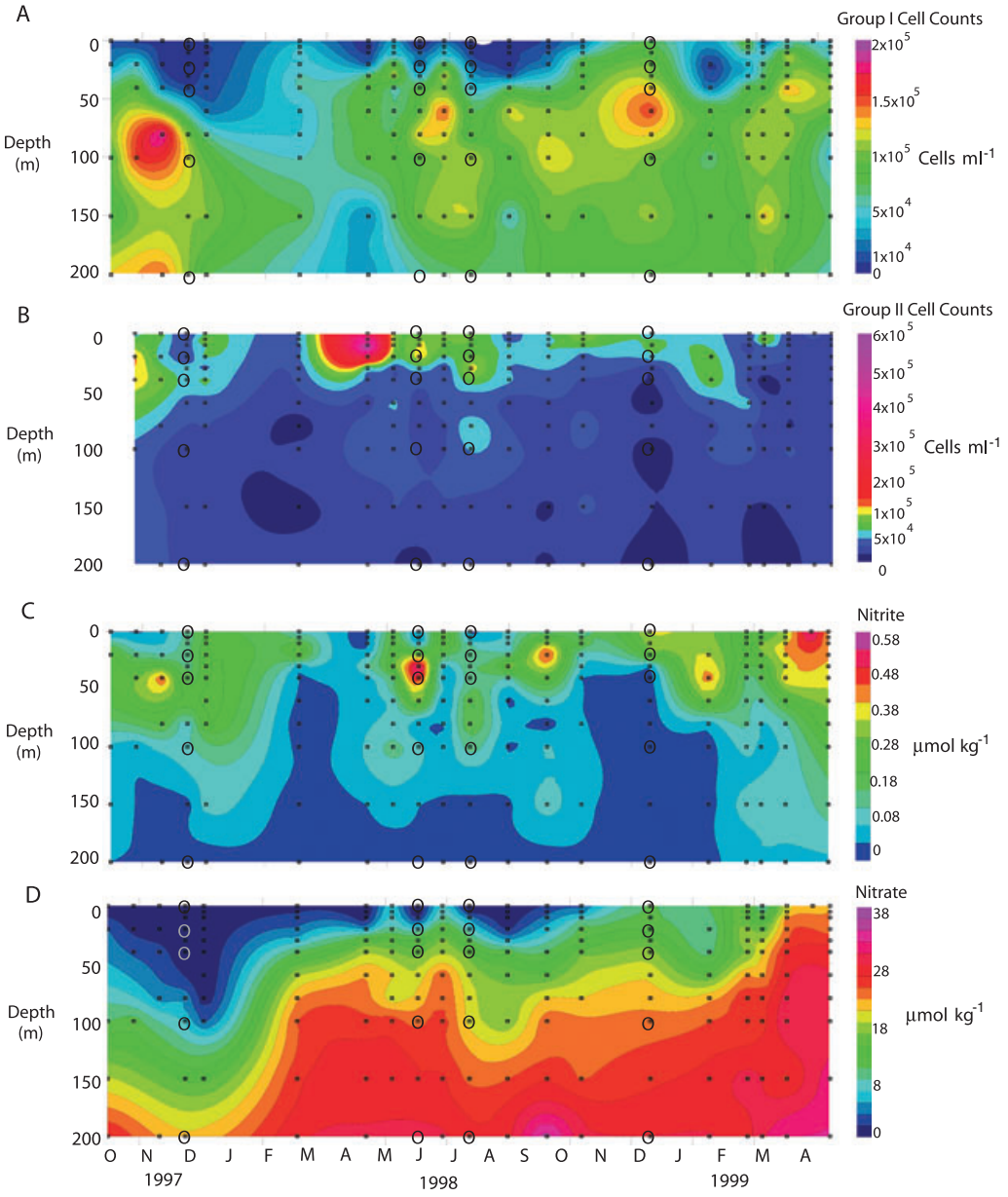

Fig. 1. Time-series data from Monterey Bay, California of archaeal cell counts and environmental data collected from October, 1997 to April, 1999.
A. Marine Group I crenarchaeal cell counts performed using polyFISH method.
B. Marine Group II euryarchaeal cell counts performed using polyFISH method.
C. Nitrite in µmol kg^{-1}.
D. Nitrate in µmol kg^{-1}.
Small black squares indicate sample data points. Circled data points indicate samples characterized further using Q-PCR.

lowstone National Park (Barns *et al*., 1996), currently has no cultivated representatives. The high abundance of these organisms was unanticipated, because members of the pSL12 clade have only been rarely encountered in the marine environments such as deep ocean sediments (Vetriani *et al*., 1999), deep-sea brine salterns (van der Wielen *et al*., 2005), and a mesopelagic Mediterranean Sea sample (Zaballos *et al*., 2006).

We designed and deployed a pSL12-targeted Q-PCR primer set to better quantify this crenarchaeal clade in the water column. The sum of the rRNA gene copy numbers derived independently from the crenarchaeal Group I and pSL12-like clades, showed good agreement with the total crenarchaeal *amoA*-like gene copy number (Fig. 3B). These data suggested that the pSL12-like *Crenarchaea* may account for the original discrepancy between

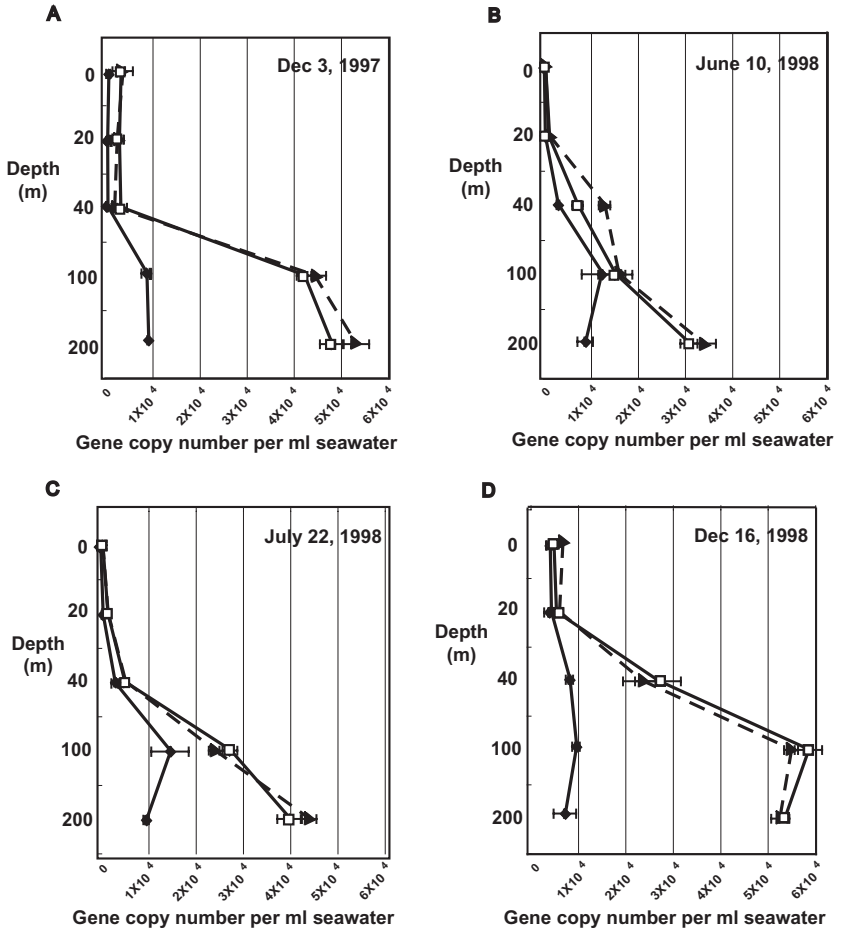

Fig. 2. Crenarchaeal ammonia monooxygenase subunit A (*amoA*), SSU rRNA and *Nitrospina* SSU rRNA gene Q-PCR profiles of samples indicated in Fig. 1.
A. 3 December, 1997.
B. 10 June, 1998.
C. 22 July, 1998.
D. 16 December, 1998.
Open squares joined by a solid line indicate crenarchaeal *amoA*-like gene copy numbers per ml, black triangles joined by a dashed line indicate Group I crenarchaeal SSU rRNA gene copy number per ml, and diamonds joined by a solid line indicate *Nitrospina* SSU rRNA gene copy number per ml seawater. Error bars represent one standard error from a mean of three replicate reactions.

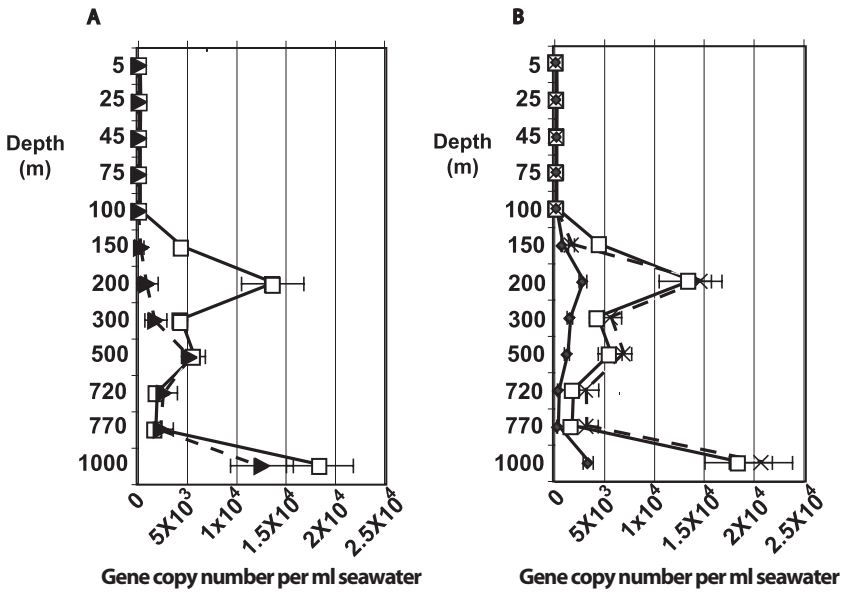

Fig. 3. Q-PCR of environmental DNA samples from Station ALOHA, collected November, 2005. All error bars indicate one standard error from a mean of three replicate reactions.
A. Comparison illustrating gene count discrepancies between the crenarchaeal *amoA* gene copy number per ml (open square joined by a solid line), and marine Group I crenarchaeal SSU rRNA gene copy number per ml (black triangles joined by a dashed line).
B. Sum of Group I and pSL12-like SSU rRNA gene copy numbers per ml (black X joined by a dashed line), compared with crenarchaeal *amoA* (open squares) and *Nitrospina* SSU rRNA (black diamonds joined by a solid black line) gene copy number per ml.

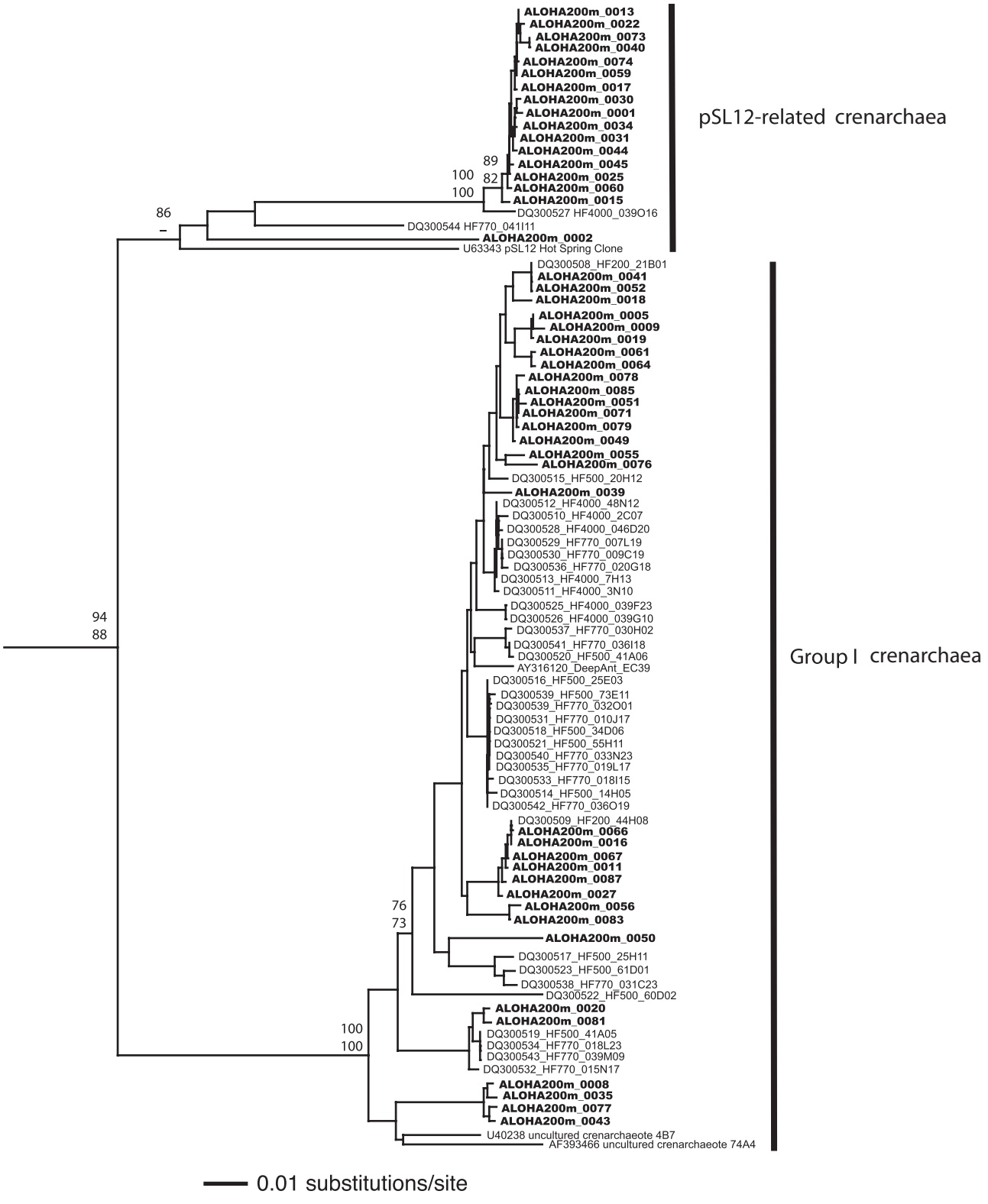

Fig. 4. Phylogenetic tree based on 950 bp of unambiguous SSU rRNA gene sequence illustrating marine Group I and pSL12-related archaeal SSU rRNA gene clone diversity. Clone library was constructed using the 200 m environmental DNA from Station ALOHA and 'universal' archaeal primers Ar20F, Ar1390R. Distance (top) and parsimony (bottom) bootstrap values are indicated at nodes.

crenarchaeal *amoA*-like and Group I SSU rRNA gene counts, and further suggest that these atypical planktonic *Crenarchaea* also contain *amoA*-like genes.

In an attempt to identify *amoA*-like gene variants that might be associated with pSL12 *Crenarchaea*, we cloned archaeal *amoA* PCR amplicons from the same 200 m sample. The presumed crenarchaeal *amoA* clone sequences (40 total) displayed a mol G + C content ranging from 44% to 48%. Phylogenetic analyses of the *amoA* amino acid sequences provided weak support for a specific clade clustering with *C. symbiosum amoA*, having a slightly elevated G + C content (47–48% mol G + C, data not shown). The SSU rRNA gene sequences of the pSL12-like clones from the 200 m sample ranged from 54% to 55% mol G + C, compared with an average mol G + C for the marine Group I *Crenarchaea* of 51–53% over the same 1100 bp region. The phylogenetic grouping together with the higher percentage mol G + C provide some evidence that these alleles of *amoA* may belong to the pSL12-related cluster of *Archaea*, but no obvious candidates were revealed.

Phylogenetic analyses of crenarchaeal amoA, B *and* C *subunits from library clones*

Figure S2A and B show significant phylogenetic partitioning of crenarchaeal *amoA* and *B* respectively. These data suggest a physical linkage of *amoAB* to the *c*. 35–40 kb genomic DNA fragment that they reside. PCR amplification of the *amoC* gene was only successful for members of the shallow clade (Fig. S2C). Amplification of the more numerous deep clade clones (500–4000 m) was attempted; however, no product was detected. A crenarchaeal *amoC* gene sequence obtained from an HF4000 library fosmid was analysed to assure proper PCR primer sequence design. Again, none of the fosmids from 500, 770 or 4000 m containing *amoA* yielded an *amoC* gene amplification product, suggesting that the *C* subunit is not linked in the deeper subpopulation of Group I *Crenarchaea*.

Nitrospina*-related fosmid and BAC clones*

Hybridization to macroarrays using a *Nitrospina* SSU rRNA-specific probe revealed 19 positive genomic clones in Monterey Bay and Station ALOHA fosmid and BAC libraries. The positive clones were confirmed by sequencing the SSU rRNA gene of each large insert clone. The SSU rRNA genes from *Nitrospina*-like clones were about 93–95% similar to *Nitrospina gracilis* cultivars (Fig. 5).

To characterize the *Nitrospina*-like bacterioplankton further, one *Nitrospina*-related BAC clone (EB080L20_F04) was fully sequenced and annotated (Table S2). Of the 88 protein-encoding open reading frames (ORFs) identified, approximately 22% shared no significant homology to current database (GenBank) entries. Most other ORFs were only distantly related to other previously sequenced genes and genomes, with identities ranging from 27% to 65% at the amino acid sequence identity. The full NADH biosynthetic operon, a terminal substrate for reverse electron transport in nitrite oxidizers, shared highest similarity with homologues from the deltaproteobacteria (*Anaeromyxobacter dehalogens*, and *Geobacter* spp.) and the planctomycete Candidatus *Kuenenia stuttgartiensis*. Comparison of the ORFs found in Table S2 to the environmental whole genome shotgun database env_nt using TBLASTX displayed a majority of hits to the following: Sargasso Sea data set (61% of all hits), farm surface soil (14% of all hits), and whale fall microbial mat/bone collection of data sets (7% of all hits). About 8% of all ORFs displayed no significant similarity to any env_nt entries at the time of analysis, 18 July, 2006.

Only about half (11 of 19) of the *Nitrospina*-like SSU rRNA containing fosmid clones shared at least one terminus with 60% or greater nucleotide identity to the fully sequenced BAC clone above (Fig. S1). In addition, the putative syntenic versus non-syntenic clones were evenly distributed in each different sample depth, with similar stoichiometry (Fig. 5). These data suggest the presence of two rRNA operons per genome in these planktonic *Nitrospina*. Fosmid termini did not reveal any obvious gene context relevant to the nitrite-oxidizing phenotype (Table S3).

One single *Nitrospina*-like SSU rRNA gene sequence was found in the Sargasso Sea whole genome shotgun sequence data set (Fig. 5). Its origin, sample 3 of the Sargasso data set, collected in the winter during a period of deep water mixing (Venter *et al*., 2004), also contained the highest representation of planktonic Group I *Crenarchaea* metabolic and SSU rRNA genes (Hallam *et al*., 2006b).

Discussion

Nitrification is a key process in the marine environment that in part sustains the large standing stock of ~25–40 μM nitrate in the ocean's subeuphotic zone worldwide (Dore and Karl, 1996; Zehr and Ward, 2002). Axenic cultures of more than 25 species of bacterial ammonia oxidizers and eight species of nitrite-oxidizing bacteria (Koops and Pommerening-Roser, 2001) have provided much physiological insight into this microbially mediated process. Culture-based and field observations indicating marine-nitrifying microorganisms may be susceptible to photoinhibition, for example, could partially explain their distribution below the euphotic zone (Horrigan *et al*., 1981; Olson, 1981; Ward *et al*., 1982; Guerrero and Jones, 1996). Complicating this picture slightly, nitrifying

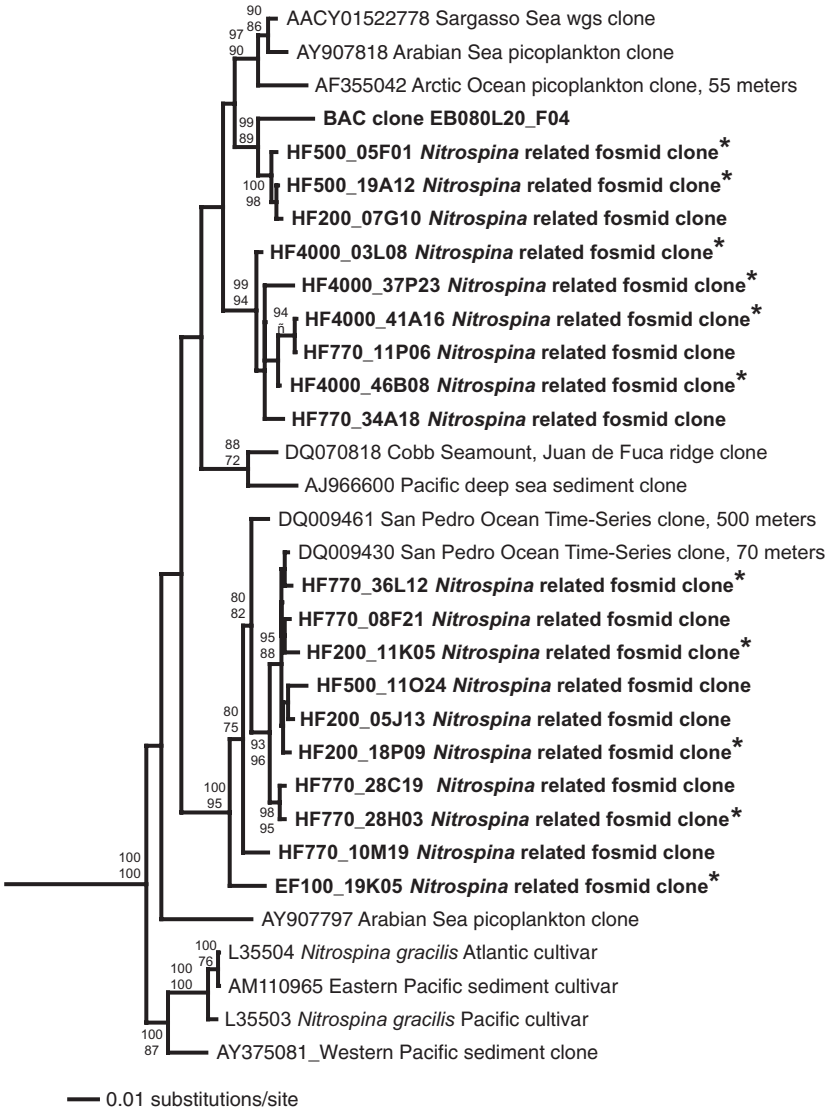

Fig. 5. Phylogenetic tree based on 800 bp of unambiguous SSU rRNA gene sequence displaying all *Nitrospina*-related fosmid and BAC clones from libraries in Table 1 (bold font). Asterisks (*) indicate fosmid clones possessing termini sequences with ≥ 60% nucleotide similarity to the fully sequenced BAC clone EB080L20_F04. Altogether, 11 of 19 fosmid clones mapped to the fully sequenced BAC (Fig. S1), suggesting that these *Nitrospina* clade members harbour two rRNA operons per genome. Distance (top) and parsimony (bottom) bootstrap values are indicated at nodes.

bacteria have also been observed to tolerate low light levels when they are cycled with dark incubation periods (Guerrero and Jones, 1996). This tolerance is somewhat consistent with observed depth distributions of planktonic *Crenarchaea* (Massana *et al*., 1997; Murray *et al*., 1998; Karner *et al*., 2001), and surface waters of Antarctica in winter (Murray *et al*., 1998; Church *et al*., 2003).

Our data show distributions of *Crenarchaea* and *Nitrospina* predominantly below the euphotic zone. Only during times of deep mixing such as in winter do these planktonic microorganisms appear to reach appreciable numbers near the surface (Massana *et al*., 1997; Murray *et al*., 1998; Hallam *et al*., 2006a). In contrast, planktonic euryarchaeal distributions in Monterey Bay tended to be inversely distributed relative to planktonic *Crenarchaea*. The high euryarchaeal abundance in the surface waters coincided with a nitrite maximum, perhaps indicating the potential utilization of this nutrient by planktonic *Euryarchaea*. While the specific physiological properties of planktonic *Euryarchaea* are not well known, their distribution in the open ocean euphotic zone coincides with the presence of proteorhodopsin in their genomes (Frigaard *et al*., 2006). Both light- and surface-derived nutrients therefore appear critical in the ecophysiology of these marine *Euryarchaea*.

Previous non-quantitative cloning studies based upon the functional gene *amoA* have shown that *Betaproteobacteria* ammonia oxidizers could be detected in coastal marine sediments (Nold *et al*., 2000), and the Monterey Bay water column in particular (Nold *et al*., 2000; O'Mullan and Ward, 2005). While betaproteobacterial *amoA* genes were detectable in our study, they were significantly lower than crenarchaeal *amoA*-like genes. Consistent with this, using *amoA* probes derived from the obligate ammonia

oxidizers *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus*, yielded negative results in the picoplankton BAC and fosmid DNA libraries screened. These results corroborate recent data reported by Wuchter and coworkers, who found that betaproteobacterial *amoA* signal was at least 10-fold below the crenarchaeal *amoA* gene counts in the North Atlantic and North Sea (Wuchter *et al*., 2006).

The significant phylogenetic partitioning of putative *amoA* and *B* crenarchaeal genes (Figure S2A and B) could be due to photoinhibition-resistance adaptations. As the *amo* genes are known to be membrane spanning, this enzymatic complex could experience significant exposure to light and particular residue substitutions could compensate for this. This phylogenetic separation might also be due to hydrostatic pressure adaptation, although this is unlikely because measurable piezophilic adaptations generally occur at depths greater than 500–1000 m (Yayanos, 1995).

A one-to-one relationship was observed between crenarchaeal SSU rRNA and their *amoA*-like genes by macroarray hybridization. The libraries from Station ALOHA show this most clearly at depths 500 and 770 m, due to the high representation of planktonic *Crenarchaea*. The same was true for our Q-PCR analyses when the entire crenarchaeal SSU rRNA assemblage was accounted for.

The unanticipated abundance of the pSL12-related *Crenarchaea* open ocean samples suggests these organisms may have significant environmental influence. The high representation of pSL12-related members in some NPSG Station ALOHA environmental DNA samples (about 10^4 per ml seawater) contrasts with the negligible signal obtained in Monterey Bay. As the pSL12-related rRNA signal accounts for the crenarchaeal *amoA* gene copy number these organisms likely also contain an *amoA*-like gene, and may be involved in oceanic ammonia oxidation. Genomic reconstruction of the pSL12-related clones from fosmid libraries could provide more insight into their metabolic potential and natural history.

The presence and distribution of *Nitrospina*-related clones in cultivation-independent picoplankton surveys (Brown *et al*., 2005; Fuchs *et al*., 2005) suggest a subeuphotic zone distribution similar to that of planktonic *Crenarchaea*. Previous attempts to quantify *Nitrospina* using immunofluorescence approaches were reportedly difficult, due to the pleomorphic nature of this group (Ward and Carlucci, 1985). The data reported here represent some of the first quantitative information on *Nitrospina* distributions in the water column, and suggest these bacteria are abundant in both coastal and open ocean habitats. The BAC clone sequences we report here also provide a first glimpse into the genomes of naturally occurring relatives of nitrite-oxidizing *Nitrospina* isolates.

In Monterey Bay *Nitrospina*-like SSU rRNA gene copy number correlated well with planktonic crenarchaeal abundance. In the open ocean, the *Nitrospina* profiles showed the same relationship, at depths where high nitrification rates have been observed (Dore and Karl, 1996). The correspondence between planktonic *Crenarchaea* and *Nitrospina* distributions indicates a possible metabolic coupling of ammonia and nitrite oxidation between these two groups.

Interestingly, there are several other phylogenetic groups with distributions similar to those of planktonic *Crenarchaea* and *Nitrospina* that possibly contribute to nitrification processes in the water column, including ammonia oxidation, nitrite oxidation, or both. Potential groups that might possibly be involved include SAR202, an abundant environmental clade related to *Chloroflexi*, that accounts for reportedly 10% of bacterioplankton between 500 and 4000 m depth (Morris *et al*., 2004). Other *Bacteria* with similar distributions include a subclade of the deltaproteobacteria environmental cluster SAR324 (Wright *et al*., 1997), novel deltaproteobacteria observed in fosmid libraries (Moreira *et al*., 2006), and the Agg47 gammaproteobacterial cluster (DeLong *et al*., 1993; 2006).

Marine *Crenarchaea* may prove to be important contributors to the first step in oceanic nitrification (transformation of NH_3 to NO_2^-), and so may have significant impact on marine biogeochemical cycles. They may exert other influences beyond ammonia oxidation as well. For example, under anoxic conditions, ammonia-oxidizing bacteria exhibit a 30-fold increase in the production of N_2O, a potent greenhouse gas (Goreau *et al*., 1980; Codispoti *et al*., 2001). As planktonic *Crenarchaea* encode genes required for N_2O production (Hallam *et al*., 2006a,b), they may also be involved in both nitrogen removal and greenhouse gas production in the marine water column.

The observations we report here add to a picture of nitrification in the marine environment, where members of the *Crenarchaea*, including planktonic marine Group I and the pSL12-related clade may be responsible for significant amounts of ammonia oxidation. *Nitrospina* species, and potentially other groups, likely consume the archaeal-produced nitrite thereby replenishing the nitrate pool. Further investigation of the dynamics and activities of these microbial groups, and their relationship and potential metabolic interactions with anammox bacteria in oxygen-limited environments, should enhance our understanding of the microbial drivers of these critical transformations in the marine nitrogen cycle.

Experimental procedures

Seawater collection, library preparation and environmental DNA purification

Seawater samples for fosmid libraries were collected at 22°45′-N, 158°00′-W, Station ALOHA, *c.* 100 km north of

Oahu, Hawaii October, 2002 (HF10–HF500 libraries) and December 2003 (HF770 and HF4000 libraries) as described (DeLong *et al*., 2006). Coastal California fosmid and BAC libraries were collected at 36°45′-N, 122°01′-W, station M1, Monterey Bay, CA, on the following dates: EB000, 17 March, 1999; EB080, 23 July, 1999; EF100, 21 February, 2002; EF500, 11 April, 2000. All fosmid and BAC libraries were constructed as described (Béjà *et al*., 2000; Suzuki *et al*., 2004; DeLong *et al*., 2006).

Monterey Bay seawater samples for environmental DNA used in Q-PCR were collected as singletons in large volumes (1–9 l) from CTD casts at station M1 from depths of 0, 20, 40, 100 and 200 m from 1 October, 1997 to 11 April, 1999. Samples were harvested using 0.2 μm sterivex filters (Millipore, Billerica, MA) following a GFA prefiltration (Whatman, Florham Park, NJ). Picoplankton samples for environmental DNA preparation from Hawaii, Station ALOHA were collected in the same manner. Crude nucleic acid extracts were prepared as previously described (Suzuki *et al*., 2000). Subsequent clean-up of crude nucleic acid preparations was performed using a modification of the DNA extraction protocol supplied with the 96 well DNeasy columns from Qiagen (Chatsworth, CA) as follows: crude nucleic acids (~5–10 μg) brought up to 200 μl volume in water were added to 20 μl Buffer AW1 and 500 μl Buffer AW2, mixed completely and loaded onto the DNeasy columns. The 96 well columns were spun in a swinging bucket rotor at 6000 *g* for 10 min, put in new collection tubes, washed with another 600 μl Buffer AW2 and spun again at 6000 *g* for 10 min. The washed columns were dried at 70°C for 15 min, put in new collection tubes and nucleic acids eluted three times with 200 μl aliquots of Buffer AE, spinning after each addition of buffer for 2 min at 6000 *g*. DNA eluates were concentrated using a 96 well size-exclusion-based ExcelaPure 96 well UF plate vacuum filtration system from EdgeBioSystems (Gaithersburg, MD). DNA was filtered to dryness, resuspended in 200 μl dilute TE buffer (1 mM Tris pH 8, 0.1 mM EDTA), quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and stored at −20°C.

Gene probing and analysis of macroarrayed clone libraries

Large-insert fosmid or BAC clones were spotted in duplicate onto Hybond-N+ nylon filters (Amersham Biosciences, Piscataway, NJ) using a Genetix Q-Pix2 arrayer (Genetix Limited, Hampshire, UK), cells were lysed and nucleic acids were irreversibly bound to membranes following standard protocols supplied from the manufacturer. Hybridization and washes were carried out at 55°C (relatively low-stringency conditions) with PCR-generated DNA probes using the AlkPhos Direct Labeling and ECF Chemifluorescent Detection kits (Amersham Biosciences, Piscataway, NJ) all according to the manufacturer's instructions.

PCR-generated fragments for probes were fractionated by gel electrophoresis and purified using a QIAquick Spin Kit (Qiagen, Chatsworth, CA). Crenarchaeal *amoA* gene probes were generated using primer pair CrenAmoAModF (5′-TGGCTAAGACGMTGTA) CrenAmoAModR (5′-AAGC GGCCATCCATCTGTA), fosmid templates HF130_12J19 and HF500_19K23 were prepared in separate reactions and combined as an equimolar cocktail. Bacterial *amoA* probes were generated from genomic DNA isolated from pure cultures of *Nitrosomonas europaea* ATCC 19718, and *Nitrosospira multiformis* ATCC 25196 using primers amoA-1F and amoA-2R (Rotthauwe *et al*., 1997). The *amoA* probe derived from *Nitrosococcus oceani* ATCC 19707 genomic DNA was amplified using the primers NocAmoA_F (5′-CCGGAGG AAGCTGCTAAA) and NocAmoA_R (5′-AGAACCAGCGG CCAACAA). The bacterial *amoA* probe fragments were labelled and used in an equimolar cocktail. Positive control DNA samples were spotted and dried onto nylon membrane strips and included in macroarray hybridization experiments and purified and labelled as detailed above.

The probe targeting *Nitrospina*-related SSU rRNA genes was generated using NitSSU130F (5′-GGGTGAGT AACACGTGAATAA) and universal bacterial primer 530R (5′-GTGCCAGCMGCCGCGG) and the BAC EB080L20_F04 as template using routine PCR amplification conditions.

Clones positive for the crenarchaeal *amoA* gene by hybridization were amplified using the PCR with the following primer pairs: CrenAmoAModF and CrenAmoAModR; CrenAmoBF (5′-ATGATTCAAACTGCTGAAGGA) and CrenAmoBR (5′-CCAAGGTCGTGTTGCRAA); CrenAmoCF (5′-TGGCAC AGATGCCCGCTTTAA) and CrenAmoCR (5′-GGTATCCGA TCTGCTGTACAA). PCR products were purified using the ExcelaPure 96 well UF plate vacuum filtration system from EdgeBioSystems (Gaithersburg, MD) and sequenced directly using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK).

Clones displaying a positive hybridization with the *Nitrospina* SSU rRNA gene probe were amplified using the standard eubacterial primers F27 (AGAGTTTGATCMTGG CTCAG) and R1492 (TACGGYTACCTTGTTACGACTT), and sequenced directly. To prevent trace amounts of *E. coli* host DNA present in fosmid DNA preparations from co-amplifying, samples were pretreated with Plasmid Safe nuclease using the manufacturer's protocol (Epicentre Technologies, Madison, WI).

Environmental PCR amplicon clone library construction and analysis

To survey archaeal SSU rRNA diversity, environmental clone libraries were constructed. Five nanograms of purified environmental DNA from Station ALOHA at 200 m depth was amplified with the PCR using primers Ar20F (5′-TTCCGGTTGATCCYGCCRG) and 1390R (5′-GACGGGC GGTGTGTRC) and the following parameters: 5 min initial denaturation; 25 cycles of, 94°C for 30 s, 60°C for 30 s, 72°C for 1 min; 7 min final extension at 72°C using the EasyA high-fidelity thermostable polymerase (Stratagene, LaJolla, CA). Amplification products were cloned using the Topo TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Colony PCR of clones was performed using M13F and M13R primers and purified amplicons were sequenced directly using primers Ar20, 958R and 1390R as previously described (DeLong *et al*., 2006).

Q-PCR analyses of environmental DNA templates

All Q-PCR experiments were run using an MJ Research DNA Engine Opticon1 thermocycler equipped with Opticon

Monitor software version 1.08. Q-PCR conditions in general were as follows: 12.5 μl QuantiTect SYBR Green PCR Master Mix (Qiagen, Chatsworth, CA), 0.5 μM final concentration of each primer, 2 μl template (corresponding to 2 ng of environmental DNA total) and water were added to a final 25 μl volume. All reactions were performed in 96 well, white Q-PCR plates (Bio-Rad, Hercules, CA) with optical ultra clear cap strips (ABgene, Epsom, UK). Specificity for Q-PCR reactions were tested by agarose gel electrophoresis, and melting curve analyses. Overall, efficiencies for all quantification reactions ranged from 87% to 105% with R^2 values ranging from 0.890 to 0.999. No inhibitory effects of purified environmental DNA samples were observed using a method previously described (Horz *et al*., 2004).

Crenarchaeal *amoA* genes were detected using the primer set CrenAmoAQ-F (5′-GCARGTMGGWAARTTCTAYAA) CrenAmoAModR (5′-AAGCGGCCATCCATCTGTA) with the linearized fosmid clone HF500_37A10 in 10-fold dilutions ranging from 2×10^7 to 200 copies per reaction, used as a quantification standard. Thermocycling was performed as follows: initial denaturation 94.0°C for 15 min; amplification 50 cycles, denature 94.0°C for 15 s, primer annealing 52.0°C for 30 s, extension 72.0°C for 30 s, 78°C for 1 s to assure stringent product detection, with a plate read between each cycle; melting curve, 50.0–95.0°C with a read every 1.0°C held for 1 s between reads.

Bacterial *amoA* Q-PCR detection was carried out using the previously described primer set AmoA1-F and AmoA2-R (Rotthauwe *et al*., 1997) with the following reaction and cycling parameters modified from above: primer annealing 58.0°C for 30 s, extension 72.0°C for 1.5 min. The standard used for quantification was genomic DNA prepared from *N. europaea* ATCC 19718, in a dilution series ranging from 2×10^7 to 200 copies of *amoA*, taking into account that *N. europaea* has two copies of *amoA* per genome of 2.81 Mbp.

Marine Group I archaeal SSU rRNA genes were quantified using the primers GI_751F (5′-GTCTACCAGAACAYGTTC) and GI_956R (5′-HGGCGTTGACTCCAATTG) with the following parameters modified from above: primer annealing 58°C for 30 s, incubation at 78°C for 1 s before each plate read to ensure stringent product quantification. An amplified DNA fragment was generated using the primers Ar20F and 1390R and the fosmid clone 4B7 (Béjà *et al*., 2002) as a template. The product was quantified and 10-fold dilutions ranging from 2×10^7 to 200 copies were used as a quantification standard.

pSL12-like SSU rRNA genes were quantified using the primers pSL12_750F (5′-GGTCCRCCAGAACGCGC) and pSL12_876R (5′-GTACTCCCCAGGCGGCAA) with the following modifications from above: primer annealing 65°C for 30 s, incubation at 80°C for 1 s before each plate read to ensure stringent product quantification. An amplified DNA fragment was generated using the primers Ar20F and 1390R, from the template fosmid HF770_041I11 as a standard (DeLong *et al*., 2006). The product was quantified and 10-fold dilutions ranging from 2×10^7 to 200 copies were used as a quantification standard. Negative controls were performed with Group I archaeal clone DNA samples to confirm that pSL12-related gene copy numbers were faithfully represented.

Nitrospina-related SSU rRNA genes were quantified using the primers NitSSU_130F (5′-GGGTGAGTAACACGTG AATAA) and NitSSU_282R (5′-TCAGGCCGGCTAAMCA) with the following conditions modified from above: primer annealing 57.5°C for 15 s, incubation at 77°C for 1 s before each plate read to ensure stringent product quantification. The linearized BAC clone EB080L20_F04 in 10-fold dilutions ranging from 5×10^7 to 50 copies was used as a quantification standard.

Crenarchaeal and euryarchaeal cell counts in Monterey Bay

Cell counts were obtained using a polyribonucleotide *in situ* hybridization technique (polyFISH) method as previously described (DeLong *et al*., 1999). Briefly, seawater samples were collected in bulk and singleton formalin-fixed aliquots of 3–10 ml were filtered onto 0.02 μm polycarbonate filters under vacuum. The filters were incubated 1 min in 1 ml of 2% (wt/vol) NaCl – 50% (v/v) ethanol and filtered to dryness. The immobilized samples were then stored at –20°C and quantified using polyribonucleotide probes at a later date. Time-series water samples were collected from 1 October, 1997 to 11 May, 1999, as indicated in the environmental DNA sampling method detailed above. Contour plots were drawn using the Surfer 8 software (Scientific Software Group, Sandy, UT). X, Y, Z arrayed data sets of date, depth, and measured value (respectively) were then gridded using the Kriging method with the linear variogram and default parameters. Filled contour plots were then generated using the smoothing option and default settings.

DNA sequencing and annotation

PCR amplicons were generated using primers 27F and 907R (5′-CCGTCAATTCMTTTRAGTTT) for *Nitrospina* SSU rRNA containing fosmids, with ABI PRISM BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA).

The EB080L20_F04 BAC sequence was annotated using the FGENESB pipeline for automated annotation of bacterial genomes from Softberry (http://www.softberry.com/berry.phtml) using the following parameters: open reading frame size = 100 amino acids, expectation = 1×10^{-10}. Predicted ORFs were queried against COG and GenBank non-redundant (NR) databases (updated 4/18/2006). The BLASTN searches for clone termini alignments and rRNA operon identification were run with the following changes from default settings: $x = 150$ (drop-off value for gapped alignment), $q = -1$ (penalty for nucleotide mismatch), and $F = \mathrm{F}$ (filter for repeated sequences) (Konstantinidis and Tiedje, 2005). Automated FGENESB annotation was manually refined using the annotation tool Artemis (http://www.sanger.ac.uk/Software/Artemis) (Rutherford *et al*., 2000).

Phylogenetic analyses

Comparisons for *amo* subunits *A*, *B* and *C* found in this study and similar sequences available in GenBank and the Sargasso Sea whole genome shotgun data set (Venter *et al*.,

2004) were analysed based on 187, 180 and 134 parsimony informative residues respectively, and phylogenetic trees were generated using distance and parsimony methods implemented in PAUP version 4.0b10 (Swofford, 2000) and rooted with sequences belonging to its corresponding particulate methane monooxygenase. Replicates of 1000 bootstraps for distance and parsimony methods were performed using step-wise addition and full heuristic methods respectively.

GenBank accession numbers

All sequences generated for use in this study were deposited in the GenBank public database with the following accession numbers DQ989186–DQ989205, EF106792–EF106952, EF106972 and EI010972–EI011009.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Cartoon representing alignment of fosmid end-sequences to the fully sequenced BAC clone EB080L20_F04, *c.* 64 kb in length. Coloured blocks at fosmid termini indicate nucleotide pairwise similarity as indicated. In total, termini sequences from 11 of 19 fosmids showed significant similarity to the BAC clone, see Fig. 5 in main text.

Fig. S2. A–C. Phylogenetic comparison of crenarchaeal ammonia monooxygenase subunit A (S2A), subunit B (S2B) and subunit C (S2C) identified from macroarray probing of fosmid library clones (see *Experimental procedures*).

Table S1. Q-PCR gene copy number data of betaproteobacterial related *amoA* genes for Monterey Bay and Hawaii, Station ALOHA.

Table S2. List of ORFs deduced from a completely sequenced BAC clone EB080L20_F04 from Monterey library, 80 m depth. Nearest neighbour descriptions were determined using BLASTX (except for rRNA operon which was determined using BLASTN) against the GenBank NR database as of 4-18-06 and env_nt database (using TBLASTX) as of 7-06-06, see *Experimental procedures*.

Table S3. Fosmid insert termini sequences of clones determined to contain *Nitrospina*-related SSU rRNA gene sequences from a quantitative macroarray probing experiment. Fosmid names denoted with an asterisk (*) indicate that sequence has a significant alignment to the BAC EB080L20_F04 sequence by BLASTN analysis, see *Experimental procedures*.

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