Dynamics of Autotrophic Marine Planktonic 
Thaumarchaeota in the East China Sea

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Abstract

The ubiquitous and abundant distribution of ammonia-oxidizing Thaumarchaeota in marine environments is now well documented, and their crucial role in the global nitrogen cycle has been highlighted. However, the potential contribution of Thaumarchaeota in the carbon cycle remains poorly understood. Here we present for the first time a seasonal investigation on the shelf region (bathymetry≈200 m) of the East China Sea (ECS) involving analysis of both thaumarchaeal 16S rRNA and autotrophy-related genes (acetyl-CoA carboxylase gene, accA). Quantitative PCR results clearly showed a higher abundance of thaumarchaeal 16S and accA genes in late-autumn (November) than summer (August), whereas the diversity and community structure of autotrophic Thaumarchaeota showed no statistically significant difference between different seasons as revealed by thaumarchaeal accA gene clone libraries. Phylogenetic analysis indicated that shallow ecotypes dominated the autotrophic Thaumarchaeota in the ECS shelf (86.3% of total sequences), while a novel non-marine thaumarchaeal accA lineage was identified in the Changjiang estuary in summer (when freshwater plumes become larger) but not in autumn, implying that Changjiang freshwater discharge played a certain role in transporting terrestrial microorganisms to the ECS. Multivariate statistical analysis indicated that the biogeography of the autotrophic Thaumarchaeota in the shelf water of the ECS was influenced by complex hydrographic conditions. However, an in silico comparative analysis suggested that the diversity and abundance of the autotrophic Thaumarchaeota might be biased by the ‘universal’ thaumarchaeal accA gene primers Cren529F/Cren981R since this primer set is likely to miss some members within particular phylogenetic groups. Collectively, this study improved our understanding of the biogeographic patterns of the autotrophic Thaumarchaeota in temperate coastal waters, and suggested that new accA primers with improved coverage and sensitivity across phylogenetic groups are needed to gain a more thorough understanding of the role of the autotrophic Thaumarchaeota in the global carbon cycle.

Citation: Hu A, Yang Z, Yu C-P, Jiao N (2013) Dynamics of Autotrophic Marine Planktonic Thaumarchaeota in the East China Sea. PLoS ONE 8(4): e61087. doi:10.1371/journal.pone.0061087

Editor: David L. Kirchman, University of Delaware, United States of America

Received November 30, 2012; Accepted March 5, 2013; Published April 2, 2013

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Funding: This work was supported by the NSFC projects (41106096 and 41191021), the SOA project (201105021) and the Science and Technology Planning Project of Xiamen (3502220102017), China. AH was also partially supported by the MEL Young Scientist Visiting Fellowship (MELS1026) from the State Key Laboratory of Marine Environmental Science at Xiamen University. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The Archaea, one of the three domain systems, was introduced in 1977 by Carl Woese based on genes encoding the small ribosomal subunit (namely, 16S rRNA gene) [1]. Members of the Archaea were originally thought to be restricted to extreme environments, but are now known to be very common in marine environments and elsewhere [2]. The marine planktonic Archaea consist of both mesophilic Euryarchaeota and Crenarchaeota, the latter dominating planktonic archaean communities [3,4], and recently proposed as a novel archaeal phylum [5,6]. There is growing evidence that members of the Thaumarchaeota play an important role in carbon and nitrogen biogeochemical cycling in both marine and terrestrial environments [7–10].

Previous metagenomic investigations of marine and soil microbial communities have revealed that the Thaumarchaeota may be involved in ammonia oxidation, an essential microbially mediated process for converting reduced N into oxygenated N [11,12]. Further studies on the enrichment and isolation of several thaumarchaeal cultures provide strong evidence that the Thaumarchaeota have the capability to oxidize ammonia to nitrite [13–15]. Quantitative analyses of amoA genes showed that ammonia-oxidizing Archaea (AOA) are ubiquitous in natural and man-made environments, and outnumber their bacterial counterparts in many environments, suggesting that the Thaumarchaeota play an essential role in global nitrification [16]. On the other hand, the autotrophic carbon metabolism of the Thaumarchaeota has received increasing attention with recognition of a their significant role in marine carbon cycling as revealed by both genomic and physiological evidence [3,17–18]. The 3-hydroxypropionate/4-hydroxybutyrate (3-HP/4-HB) CO2 fixation pathway was first found in a thermophilic crenarchaeon Metallosphaera sedula by Berg et al. (2007) [19] but it is now known as a unique CO2 fixation pathway for the Thaumarchaeota and found in all available thaumarchaeal genomes [17,18,20–23]. The accA gene, encoding the 3-subunit of acetyl-CoA carboxylase, one of the reversed enzymes in this 3-HP/4-HB pathway proves to be a useful molecular marker for the...
autotrophic Thaumarchaeota in the environment [7,24,25]. Recent open ocean studies show that the ratios of thaumarchaeal accA genes to 16S rRNA or amoA genes are lower than 1:1 in the euphotic zone but increase with depth, implying that the marine planktonic Thaumarchaeota may grow heterotrophically in the upper water of the open ocean [7,26]. However, until now no investigation has examined the distribution of the autotrophic Thaumarchaeota or how this functional group responds to environmental gradients in the coastal ocean.

The East China Sea (ECS), located in the North West Pacific, is the largest continental shelf sea in the temperate zone. The hydrological conditions in the ECS are extremely complicated and dynamic, due to the interactions between the nutrient enriched freshwater from the Changjiang (Yangtze River) and the oligotrophic oceanic water of the Kuroshio Current [27,28]. It is thus an ideal ecosystem for ecological studies of microbial dynamics along both temporal and spatial dimensions. A study using clone libraries of 16S rRNA genes shows that the marine Group 1.1a Thaumarchaeota (Group 1.1a) is prevalent in the estuarine region of the ECS [29], while another work suggests that the community structure of sediment AOA in the Changjiang estuary and the adjacent ECS shifts along hydrological gradients [30]. In a recent investigation, we report clear niche partitioning of Group 1.1a within the water column of the open region of the ECS [26]. However, the temporal and spatial variations of the marine planktonic Thaumarchaeota in the ECS shelf are still poorly understood, especially in the case of the autotrophic functional group. In the present study, we compared the abundance, distribution and community structure of the autotrophic Thaumarchaeota in the shelf waters (bathymetry ≤200 m) of the ECS between summer and autumn using clone libraries and quantitative PCR (qPCR) of thaumarchaeal 16S rRNA and accA genes.

Materials and Methods

Sampling

Sampling was conducted in the late autumn (3rd and 24th November 2007) and summer (7th and 18th August 2008) on board the R/V ‘Dongfanghong #2’ at 20 and 29 stations, respectively, along four cross-shelf transects (transect A, B, C and D) from the Changjiang estuary to the Kuroshio area (Fig. 1). No specific permits were required for these field studies in that: a) no specific permission was required for these locations/activities; b) the locations were not privately-owned or protected in any way; and c) the field studies did not involve endangered or protected species. Water samples were collected with 12 L Niskin bottles mounted in a SeaBird CTD (conductivity-temperature-depth) system. Water samples (0.5–2L) were pre-filtered through 20 μm mesh (Millipore, Billerica, MA, USA) and subsequently filtered onto 0.2-μm pore-size polycarbonate filters (Millipore) at a pressure of ~<0.03 MP. The filters were immediately frozen and stored at ~−80°C until further analysis.

Nucleic acid extraction

The microbial community genomic DNA was extracted using the UltraClean Soil DNA kit (MoBio, San Diego, CA, USA), as processed in reference 7. DNA integrity and size was checked in a 0.8% agarose gel stained with SYBR Green I (Molecular Probes, Eugene, OR, USA), and the concentrations were quantified in duplicate using FlexStation® 3 (Molecular Devices, Sunnyvale, CA, USA) with a Quant-iT™ dsDNA HS Assay Kit (Molecular
Quantitative PCR analysis

The abundance of thaumarchaeal 16S rRNA and accA genes were quantified in triplicate with an ABI PRISM 7500 system (Applied Biosystems, Foster City, CA, USA) using the SYBR Green based method. Thaumarchaeal 16S rRNA gene copy numbers were determined using primers GL_751F and GL_956R [31] and the following reaction chemistry was used: a 20 μL reaction mixture consisting of 10 μL of SYBR GreenER™ qPCR SuperMix Universal (Molecular Probes), 50 nM ROX dye, 5 μg BSA, plus 0.4 μM of each primer and 1 μL of template (1–10 ng) was used. For quantification of the abundance of thaumarchaeal accA genes, the following reaction mixture was used: 10 μL of SYBR® Premix Ex Taq™ (TakaRa, Dalian, China), 50 nM ROX dye, 5 μg BSA, 0.4 μM primers (Cren529F/Cren981R) [24] and 1 μL template DNA of 1–10 g in a final volume of 20 μL. The thermal cycling conditions were the same as those used in our previous study [7]. The specificity of qPCR reactions was confirmed using melting curve analysis and agarose gel electrophoresis after amplification.

The plasmids used as standards in qPCR were constructed previously [7]. The concentrations of plasmid DNAs were determined using a Quant-iT™ dsDNA BR Assay Kit (Molecular Probes). Ten-fold serial dilutions of a known number of plasmids were subjected to qPCR assay in triplicate to generate an external standard curve. The assay efficiency of the thaumarchaeal 16S rRNA gene was 102–108% with R² values more than 0.997, while the corresponding values for the thaumarchaeal accA gene were 90–96% and 0.998.

PCR amplification of thaumarchaeal accA genes and clone library analyses

The thaumarchaeal accA gene was amplified with primers Cren529F/Cren981R [24], but the nested PCR strategy was employed when few positive PCR products were obtained from the summer samples. The PCR mixture (30 μL) contained 15 μL FailSafe Premix F (Epicentre Biotechnologies, Madison, WI, U.S.A.), 0.5 μM of each primer, 1 U of Ex Taq DNA polymerase (TakaRa), 6 μg BSA and 1 μL (c. 3–20 ng DNA) of template. The PCRs were run for 35 cycles, following the PCR conditions described in the literature listed previously. Three independent PCR products were pooled and purified with an Agarose Gel DNA Purification kit (TakaRa), ligated into the pMD18-T vector (TakaRa) and then transformed into competent Escherichia coli DH5α (TakaRa). Positive clones were screened using PCR re-amplification with vector primers M-13F/M-13R and randomly selected for sequencing with an ABI 3730 XL sequencer (Applied Biosystems).

Phylogenetic analysis

The thaumarchaeal accA gene sequences, along with their closest relatives retrieved from GenBank, were imported into ARB [32]. The sequences were first translated and aligned using Clustal W in ARB, and then the nucleotides were realigned according to their protein alignment. Ambiguously and incorrectly aligned positions were corrected manually using the ARB-edit tool. A sequence bases frequency filter was created to remove ambiguous positions and columns containing gaps. The maximum likelihood (ML) tree was generated using RAxML 7.2.8 [33] at the CIPRES website (http://www.phylo.org), and a general time-reversible model plus gamma distribution plus the invariant sites model of
molecular evolution. Tree topologies were evaluated based on 1,000 bootstrap replicates.

Diversity indices and statistical analyses

The thaumarchaeal accA gene sequences were grouped into operational taxonomic units (OTUs) based on a 5% sequences divergence cutoff using MOTHUR v1.22.0 with the furthest neighbor algorithm [34]. Diversity indices including the nonparametric richness estimator Chao1, the Shannon diversity ($H'$) and the sampling Coverage index ($C$) were also calculated using MOTHUR.

Community classification of different thaumarchaeal accA gene clone libraries was compared using non-phylogenetic (OTU-based) clustering and phylogenetic (weighted UniFrac) clustering analyses [35]. Analysis of similarities (ANOSIM) was used to test the significance of the autotrophic thaumarchaeal community structures between seasons. A Mantel test was performed to evaluate whether there was a correlation between autotrophic thaumarchaeal communities and environmental variables or geographic distance. Correlations between thaumarchaeal gene abundances and environmental variables were calculated using non-parametric Spearman’s correlation since normality of distribution of the individual data sets was not always met. Analyses were conducted with the PAST v1.92 program [36].

Nucleotide sequence accession number

The non-redundant sequences reported in this study have been deposited in the GenBank database under accession numbers JQ952676 to JQ952734.

Results

Environmental parameters

The Kuroshio Current along the ECS shelf edge originates from the West Pacific Warm Pool, and is characterized by high temperature and high salinity. Conversely, low salinity and low temperature content are identified from the coastal water [27,37]. Because of the influences of different water masses, hydrographic conditions in the ECS are very different in autumn and summer (Figs 2 and 3). As seen in Fig. 2 (which was derived from in situ measurements of temperature and salinity), the cold freshwater plume from the Changjiang and the shoreward intrusion of the warm water were less extensive in autumn, while in summer greater shoreward intrusion of the offshore warm water could be observed. Also, the summer freshwater discharges from the Changjiang were more intense, and the majority of discharges intruded into transect B, although several high salinity water tongues were found in this transect due to the interactions among the Changjiang Diluted Water (CDW), oceanic water from the Kuroshio Current and Taiwan Warm Current (Fig. 2).

Two autumn and seven summer stations were chosen (as indicated in Fig. 1) to investigate the depth distribution of autotrophic Thaumarchaeota, and vertical profiles of the temperature

![Figure 3. Depth profiles of temperature, salinity, and the abundance of thaumarchaeal 16S rRNA and accA genes in two autumn stations (B3w and B8w) and seven summer stations (B1s, B4s, B8s, A1s, A4s, A5s and A8s). Bars denote one standard deviation of the triplicate qPCR determination. doi:10.1371/journal.pone.0061087.g003](http://www.plosone.org/figure3.jpg)
and salinity at these stations are shown in Fig. 3. The salinity had constant values in the autumn stations since the CDW was weaker in autumn than in summer, but the thermocline was deeper in autumn than in summer (Fig. 3).

**Abundance of 16S rRNA and accA genes of the planktonic Thaumarchaeota**

qPCR results demonstrated that both thaumarchaeal 16S rRNA and accA genes were significantly more abundant during the autumn than the summer (Mann-Whitney, $P<0.01$) (Figs 3 and 4). Vertically, both thaumarchaeal 16S rRNA and accA genes were more abundant in subsurface water than those in surface water, with clear increases in gene abundance with depth (Fig. 3).

In surface water, thaumarchaeal 16S rRNA gene abundance ranged from $6.50 \times 10^4$ to $5.66 \times 10^7$ copies L$^{-1}$ in autumn, whereas only 10 of 19 summer samples could be detected with thaumarchaeal 16S rRNA genes, and their abundance varied from $1.46 \times 10^3$ to $5.99 \times 10^5$ copies L$^{-1}$ (Fig. 4). The abundance of thaumarchaeal accA genes exhibited a similar trend to that of thaumarchaeal 16S rRNA gene abundance, ranging from below detection limit to $1.72 \times 10^7$ copies L$^{-1}$ in the ECS surface water (Fig. 4).

A linear regression analysis indicated that the thaumarchaeal 16S rRNA and accA genes were significantly correlated with each other ($R^2 = 0.87; P<0.001$). However, the accA genes were almost always less abundant than the thaumarchaeal 16S rRNA genes,

![Figure 4. Surface distribution of the abundance of thaumarchaeal 16S rRNA and accA genes in the ECS. Bars denote one standard deviation of the triplicate qPCR determination. Asterisks indicate that abundance of thaumarchaeal 16S rRNA gene was not determined in stations D3 and D4 since there was not enough environmental DNA. Surface water samples were only collected in autumn of 2007 (grey shaded area). doi:10.1371/journal.pone.0061087.g004](image-url)
with an averaged relative proportion of 16.3% ± 2.6% of thaumarchaeal 16S rRNA genes. Correlation relationships between environmental parameters and thaumarchaeal 16S rRNA or accA gene abundances were also calculated. Collectively, only temperature showed a negative correlation with thaumarchaeal 16S rRNA (Spearman rank correlation, \( r = -0.65, P < 0.001 \)) and accA (\( r = -0.44, P < 0.001 \)) gene abundances. Within each season, thaumarchaeal 16S rRNA gene abundance exhibited significant correlation with temperature (autumn: \( r = -0.62, P < 0.001 \); summer: \( r = -0.74, P < 0.001 \)) and salinity (autumn: \( r = -0.36, P < 0.05 \); summer: \( r = -0.45, P < 0.05 \)). However, thaumarchaeal accA gene abundance was found to be strongly correlated with salinity in autumn (\( r = -0.64, P < 0.01 \)) but with temperature in summer (\( r = -0.60, P < 0.001 \)).

### Genetic diversity of thaumarchaeal accA genes

To explore the diversity and structure of the autotrophic thaumarchaeal community in the ECS, nine and 10 thaumarchaeal accA gene clone libraries were analyzed from autumn and summer samples, respectively (Table 1). A total of 620 thaumarchaeal accA gene sequences were obtained, and could be grouped into 59 OTUs at a 5% divergence cutoff value at the nucleic acid identity, 94–99% amino acid identity. Indeed, these sequences were grouped phylogenetically with sequences from meso- or bathypelagic waters including the SCS [7], North Pacific Gyre [40] and Mediterranean Sea [24].

| Table 1. Diversity indices of thaumarchaeal accA clone libraries from the ECS. |
|---|---|---|---|---|
| Season | Samples | n | No. of OTUs | C (%) | H’ | Chao1 |
| Autumn | A1w_0 | 25 | 8 | 84.0 | 1.75 | 14 |
| | A4w_0 | 26 | 8 | 88.5 | 1.62 | 9 |
| | A8w_0 | 25 | 10 | 78.3 | 2.05 | 15 |
| | A11w_0 | 34 | 6 | 97.1 | 1.50 | 6 |
| | A12w_0 | 35 | 7 | 94.3 | 1.29 | 8 |
| | B3w_0 | 33 | 6 | 91.0 | 1.38 | 9 |
| | B3w_40 | 31 | 6 | 93.6 | 1.44 | 7 |
| | B8w_0 | 36 | 11 | 83.3 | 1.79 | 16 |
| | B8w_75 | 38 | 13 | 81.6 | 2.20 | 34 |
| | Summer | A1s_0 | 25 | 13 | 68.0 | 2.21 | 19 |
| | A1s_25 | 38 | 7 | 92.1 | 1.12 | 8 |
| | A5s_10 | 37 | 8 | 89.2 | 1.37 | 11 |
| | A5s_50 | 39 | 11 | 82.1 | 1.54 | 18 |
| | A7s_0 | 30 | 3 | 100 | 0.84 | 3 |
| | A8s_75 | 39 | 9 | 89.7 | 1.45 | 11 |
| | B4s_10 | 30 | 4 | 96.7 | 0.90 | 4 |
| | B4s_50 | 35 | 8 | 91.4 | 1.61 | 10 |
| | B8s_0 | 32 | 5 | 96.9 | 1.05 | 5 |
| | B8s_75 | 34 | 10 | 88.2 | 1.94 | 13 |

Phylogenetic analysis indicated that the thaumarchaeal accA sequences recovered in this study fell into three major lineages: Group 1.1a, Group 1.1a-associated and Estuarine group (Fig. 5). The majority of the sequences (552 out of 620, 89%) fell into Group 1.1a and could be further divided into two subclusters (‘shallow’ and ‘deep’ clusters) which were previously reported to represent depth-stratified ecotypes of pelagic *Thaumarchaeota* based on the *ammonia* [38,39] and accA genes [7]. The shallow cluster contained 535 accA gene sequences grouped with those from the open ocean waters of the ECS [26], the South China Sea (SCS) [7] and the Sargasso Sea [12] (Fig. 5). Several thaumarchaeal accA OTUs dominated this cluster. For instance, OTUs A8w_0_accA27, B8w_75_accA30 and A7s_0_accA27 accounted for 9.3% (50 out of 535), 16.4% (88 out of 535) and 51.2% (274 out of 535) of the sequences of the shallow cluster, respectively. These abundant autotrophic thaumarchaeal ‘shallow species’ were cosmopolitan in the ECS, i.e., they were found in most clone libraries (≥ 16) (Fig. 5). Consistent with the shallow depth characteristics of the sampling area, only a small fraction of all sequences (16 out of 620, 2.6%) were grouped phylogenetically with sequences from meso- or bathypelagic waters including the SCS [7], North Pacific Gyre [40] and Mediterranean Sea [24].

Most accA gene sequences from the summer estuarine sample A1s_0 (20 out of 25) formed a monophyletic lineage distantly related to Group 1.1b with no environmental sequences clustering together (Fig. 5). ML bootstrap analyses indicated that this phylogenetic node was supported by values ≥80% (Fig. 5). Therefore, we provisionally named it here as the Estuarine group, with any two sequences in this cluster sharing 83–94% nucleic acid identity and 94–99% amino acid identity. Indeed, these sequences were distantly related to reference sequences in GenBank (≤ 81% nucleic acid identity, ≤79% amino acid identity).

### Community spatial structure of thaumarchaeal accA genes

Genetic differentiation among the thaumarchaeal accA clone libraries obtained from the ECS was assessed using both OTU-based cluster and weighted UniFrac cluster analysis (Fig. 6). Both cluster analyses demonstrated almost identical patterns (Mantel test, \( r = 0.93, P < 0.001 \)), and there was no clear environmental clustering with regard to season (ANOSIM analysis, \( R = 0.07, P > 0.05 \)). Most of the clone libraries (13 out of 19) clustered together, sharing more than 60% Bray-Curtis or 85% UniFrac similarity (Fig. 6). Two continental shelf clone libraries (A7s_0 and A8w_0) were closer to the clone libraries mentioned above, whereas two surface samples from estuarine station A1 and two deep water samples from continental shelf station B8 shared lower similarity with other clone libraries (Fig. 6). This cluster pattern was also supported by analysis of the number of OTUs shared between any two clone libraries. For example, most clone libraries, either autumn or summer, exhibited significant compositional overlap (\( L_{dabla}=0.5 \)) except that A1s_0 shared almost no OTUs with other samples (\( L_{dabla}=0.06 \)), and A1w_0, B8w_75 and B8s_75 shared a relatively lower number of OTUs with several samples (\( L_{dabla}=0.05 \)) (Fig. 7).

Statistical analysis demonstrated that hydrographic conditions might have had significant effects on the community structures of...
Autotrophic *Thaumarcheota* in Coastal Waters

**Group 1.1a**

- **Shallow cluster**
  - ECS clade I
  - **Deep cluster**
    - ECS clade II (zonts)
    - Nitrospumona salinaris 6311, Fattening sediment, NZ_AEXL01000670
      - **Nitrospumona morrisoniana SC1.1**, Marine aquaculture, NZ_AHEG01000001
      - **G1-ACC2-3**, Marine sediment, NZ_AHEG01000001
      - **AE-ACC2-6**, Marine sediment, NZ_AHEG01000001
    - Nitrospumona haeckeni f.2020, Low salinity sediment, AE0001000246
    - Nitrospumona haeckeni f.1811, Low salinity sediment, NZ_CM001010
    - Nitrospumona haeckeni f.1811, Marine sediment, NZ_AHEG01000001

**Group 1.1b**

- **Estuarine group** (A1g_0, B OTUs)
  - **Conchocelis zumbiensis**, Marine sponge, DP0000238
Mantel tests indicated significant positive correlations between autotrophic thaumarchaeal communities and hydrographic parameters (temperature and salinity) (Table 2). Taken together, these results suggested that some ubiquitous lineages of autotrophic *Thaumarchaeota* were distributed in the ECS but that hydrographic factors shaped its community spatial structures.

**Discussion**

The recently described AOA, responsible for the first and rate-limiting step of nitrification (ammonia oxidation), have emerged as an important microbial functional group in the global N cycle. Thaumarcheal amoA genes are reported to be more abundant than those of their bacterial counterparts in diverse environments, especially in oligotrophic habitats [8,10,38]. Although field experiments or genome based studies demonstrate that AOA may live chemolithoautotrophically or mixotrophically [3,18,42,43], limited information is available regarding the contributions of autotrophic *Thaumarchaeota* to the global carbon cycle, or environmental factors that control their distribution and abundance. To better understand the ecology of this novel primary producer, we investigated the abundance, distribution and community structure of autotrophic *Thaumarchaeota* in the shelf area of the ECS in two seasons (autumn and summer).

Our study indicated that the abundance of thaumarchaeal 16S rRNA and *accA* genes exhibited clear seasonal variation, being higher in late autumn (November) and lower in summer, although the abundance of the thaumarchaeal *accA* gene was quite low in the surface waters of the ECS shelf investigated (Figs 3 and 4). This was consistent with the fact that periodical blooms of *Thaumarchaeota* or AOA occur in other coastal oceans during late autumn-winter, including the North Sea [10,44], Mediterranean Sea [45], Antarctic Ocean [46,47] as well as Arctic coastal waters [48]. In addition, the strong relationship (positive correlation) between the dynamics of thaumarchaeal 16S rRNA and *accA* genes implied similar responses of the total and autotrophic *Thaumarchaeota* to environmental controls. Among possible planktonic *Thaumarchaeota* regulatory factors, temperature, nutrient levels, light availability and phytoplankton abundance and composition are considered as possible candidates [44,48–50]. Previous studies propose that AOA may live chemolithoautotrophically since the 3-HP/4-HB pathway can be found in all published thaumarchaeal genomes, and ammonia oxidation of *Thaumarchaeota* is thought to provide the energy for CO₂ fixation [18,20–23,42]. However, our qPCR results indicated that only 16.3% of planktonic *Thaumarchaeota* harbored the key gene for thaumarchaeal autotrophic pathways. This result was in accordance with our previous findings in the epipelagic waters of the ECS and SCS [7,26], but appeared contrary to the metagenomic studies, which show a nearly 1:1 ratio of thaumarchaeal *accA* to 16S rRNA gene in the surface water of the Sargasso Sea [12] and Gulf of Maine [51]. This incongruity might be explained by the following two reasons. On the one hand, some thaumarchaeal subpopulations in the coastal

![Figure 5. Maximum likelihood tree of thaumarchaeal accA gene sequences. Bacteroides fragilis NCTC 9343 (CR626927) was used as the outgroup to root the tree. Sequences with ≤5% distance cutoff are represented by only one of them. Clone sequences recovered in this study are in boldface. Boxes behind the representative sequences indicate samples obtained from autumn (white) or summer (grey). The number in the box indicates the number of occurrences and the number of clones in the OTUs found in that season. For example, OTU A7s_0_accA27 (JQ952676) was found in nine autumn stations with 112 sequences and nine summer stations with 162 sequences. Bootstrap values greater than 80% and 50% were shown, respectively, by solid and open circles in the nodes. Nodes without circles were not resolved. Scale bar indicates 0.1 nucleotide substitution per site. doi:10.1371/journal.pone.0061087.g005](image)

![Figure 6. Clustering of the thaumarchaeal accA gene clone libraries based on the Bray-Curtis algorithm of OTU table (≤0.5% cutoff) (a) and the weighted UniFrac algorithm (b). Open circles (white) and close circles (grey) indicate samples obtained from autumn and summer, respectively. Scale bar indicates the Bray-Curtis (a) or the UniFrac distance (b). doi:10.1371/journal.pone.0061087.g006](image)
ECS might have lacked the accA gene or harbored alternative autotrophic CO2 fixation pathways. On the other hand, the thaumarchaeotal accA primers used in the present study might have missed certain fraction of epipelagic *Thaumarchaeota* since those primers were primarily designed based on a few sequences (six and two sequences derived from metagenomic studies and thaumarchaeal strains, respectively) [7].

To obtain a more detailed understanding of the coverage and sensitivity of primers Cren529F and Cren981R for thaumarchaeal accA genes, we retrieved 22 full- or nearly full-length thaumarchaeal accA gene sequences from GenBank and CAMERA database (http://camera.calit2.net/) (as at 30 January 2013), and an in silico comparison of primer sets Cren529F/Cren981R to these sequences was performed (Table 3). The analysis revealed that the mismatches between thaumarchaeotal accA gene primers used in this study and reference sequences were not uniformly distributed among the phylogenetic groups (Table 3). For instance, both forward and reverse accA primers had no mismatch with members of the deep cluster, whereas primers Cren529F and Cren981R had $1 \pm 0.45$ and $1.5 \pm 0.34$ mismatches per sequence, respectively, with the shallow cluster. Moreover, a relatively high frequency of mismatches between primer pair Cren529F/Cren981R and members of Group 1.1a-associated *Thaumarchaeota* was observed, especially for primer Cren981R (Table 3). Previous studies show that primer mismatches may result in a lower number of molecules being detected in qPCR assays [32,53]. As a consequence, the abundance of the shallow cluster and Group 1.1a-associated group might be underestimated relative to the deep cluster of autotrophic *Thaumarchaeota* although our clone libraries were dominated by the sequences related to the shallow cluster. Further studies based on newly designed or modified accA primers are required to obtain a comprehensive view of the diversity and abundance of autotrophic *Thaumarchaeota*.

The autotrophic thaumarchaeal diversity in the shelf water of the ECS (3–13 OTUs) revealed by accA gene sequences was comparable to previously studied open regions of the ECS [7], SCS (5–9 OTUs) [26] and Mediterranean Sea (4–6 OTUs) [24]. However, phylogenetic analysis indicated that the majority of thaumarchaeal accA gene sequences (86.3%) obtained from the coastal waters of the ECS, even from twilight zone waters (40–75 m) [54], were affiliated with the shallow cluster (Fig. 5), although the deep cluster was extensively retrieved below the euphotic zone in the open ECS [bathymetry >200 m] [26]. Vertical phylogenetic segregation of *Thaumarchaeota* in the ocean is observed in numerous studies based on analysis of 16S rRNA and different functional genes (accA, ammA, nirK, ureC and 4-hid) of marine *Thaumarchaeota* [7,25,39,55]. Several environmental parameters, such as light level, temperature and oxygen concentration, are considered as the critical factors in the development of the depth stratification phylogeny of marine planktonic *Thaumarchaeota* [56]. However, two recent studies argue that community structures of Group 1.1a are significantly influenced by nutrient status (ammonia concentrations) of the ocean rather than light gradients [57,58]. These authors note that shallow and deep ecotypes of AOA dominate marine environments with medium and low ammonia concentrations, respectively. Considering the slightly higher concentration of ammonia (0.07–0.65 \(\mu\)M) present in the ECS shelf [59], it is possible that a predominance of shallow ecotypes of Group 1.1a was detected in the current study. This result agreed well with the observation that shallow Group 1.1a dominate throughout the water column down to 300m of the coastal Arctic [58], providing further evidence that ammonia supply rates might play a pivotal role in determining the community structures of the autotrophic *Thaumarchaeota*.

Although only a few accA OTUs (three OTUs, 66.5% of total sequences) had cosmopolitan distribution in the ECS shelf, a uniform seasonal distribution pattern was found for most OTUs (Fig. 5). This pattern was confirmed by cluster and ANOSIM analysis, which indicated that community structures of the autotrophic *Thaumarchaeota* did not differ markedly between autumn and summer. However, a Mantel test demonstrated that the community structures of the autotrophic *Thaumarchaeota* in the ECS shelf were closely tied to water mass properties, i.e. temperature and salinity either within a season or between seasons (Table 2). This observation agreed with those of previous studies, which show that planktonic archaeal communities may tend to be shaped by complex hydrographic conditions in the Arctic Ocean where different water masses meet [60,61], although Massana et al. [2000] note that a few abundant OTUs of planktonic archaea exhibited a ubiquitous distribution pattern in the world’s oceans [62].

The Changjiang is the third largest river in the world and contributes huge anthropogenic inputs of nutrient and organic matter to the ECS [63,64]. Previous studies show that CDW brings terrestrial (allochthonous) source microorganisms to the Changjiang estuary and the adjacent ECS [30,65]. Consequently,
greater bacterial and archaeal diversity is observed in that area [30,65,66]. As expected, the highest diversity of autotrophic Thaumarchaeota was found in the surface water of estuarine station A1 in summer 2008 in our study (Table 1), where strong freshwater plumes were observed (Fig. 2d). Furthermore, most of the thaumarchaeal accA gene OTUs (69.2%) obtained from sample A1s_0 were exclusively related to Group 1.1b Thaumarchaeota and formed a unique monophylogenetic cluster (Fig. 5). Because this group was only observed in summer (when freshwater plumes became larger) but not in autumn (Fig. 2), it might have originated from terrestrial habitats. Although there is no universal genetic threshold for distinguishing autotrophic thaumarchaeal species (≤ 87% nucleic acid identity for thaumarchaeal amoA gene) [67], the high divergence between the Estuarine group and reference sequences (≤ 81% nucleic acid identity) indicated that a novel putative terrestrial cluster of autotrophic Thaumarchaeota was observed, highlighting the importance of freshwater runoff in the transport of terrestrial autotrophic Thaumarchaeota to marine environments.

In conclusion, the distribution, abundance and community structure of the autotrophic Thaumarchaeota were investigated for the first time along four transects in the ECS shelf during different

**Table 3. Summary of thaumarchaeal accA gene mismatches to primer pair Cren529F/Cren981R.**

| Phylogenetic groups* | Sequence or strain name | Accession number | Cren529F (5′-3′) | Cren981R (5′-3′) |
|----------------------|-------------------------|------------------|------------------|------------------|
|                      |                         |                  |                  |                  |
| Shallow cluster      | Sargasso Sea metagenome 1095460025585 AACY020784810 No mismatch |                  |                  |
|                      | Sargasso Sea metagenome 1096626735572 AACY020559552 No mismatch |                  |                  |
|                      | Sargasso Sea metagenome IBEA_CTG_2097946 AACY01016865 =A=G=C=A= |                  |                  |
|                      | Sargasso Sea metagenome IBEA_CTG_2151838 AACY01042731 =G=A=C=A= |                  |                  |
|                      | Sargasso Sea metagenome IBEA_CTYG_2033382 AACY01063159 =G=C=A= |                  |                  |
|                      | Sargasso Sea metagenome IBEA_CTYG_UAAAYB841F AACY01523534 =G=C=A= |                  |                  |
| Deep cluster         | HF4000_ANIW97P9 fosmid clone EU016593 No mismatch |                  |                  |
|                      | HE4000_APKGD03 fosmid clone EU016643 No mismatch |                  |                  |
|                      | Marine metagenome HOTS_Contig54507 ABEO10554507 No mismatch |                  |                  |
|                      | Marine metagenome HOTS_Contig48812 ABEO10548812 No mismatch |                  |                  |
| Group 1.1a-associated | ‘Gulf of Maine’ metagenome scf1108793271369 JH165397 =A=G=A=G=A= |                  |                  |
|                      | ‘Gulf of Maine’ metagenome scf1108793271546 JH165451 =A=G=A=G=A= |                  |                  |
|                      | Nitrospumilus maritimus SCM1 NZ_ABEO10000001 No mismatch |                  |                  |
|                      | Nitrospumilus salaria BD31 NZ_AEXL01000070 =A=G=A=G=A= |                  |                  |
|                      | Nitrospumilus koreensis AR1 CP003842 =A=G=A=G=A= |                  |                  |
|                      | Nitrospumilus sp. SJ AJV01000006 =A=G=A=G=A= |                  |                  |
|                      | Nitrospumilus sp. AR AJV01000016 =A=G=A=G=A= |                  |                  |
|                      | Nitrospumilus sediminis AR2 CP003843 =A=G=A=G=A= |                  |                  |
|                      | Nitrosoarchaeum limnia BG20 AHUG01000246 =A=G=A=G=A= |                  |                  |
|                      | Nitrosoarchaeum limnia SFB1 NZ_CM001158 =A=G=A=G=A= |                  |                  |
|                      | Nitrosoarchaeum koreensis MY1 NZ_AFPU01000001 =A=G=A=G=A= |                  |                  |
|                      | Cenarchaeum Cenarchaeum symbiosum DP000238 =A=G=A=G=A= |                  |                  |
|                      | Nitrosopumilus gargensis Ga9.2 CP002408 =A=G=A=G=A= |                  |                  |

Phylogenetic groups were identified based on thaumarchaeal accA genes.
doi:10.1371/journal.pone.0061087.t003
seasons. The abundance of thaumarcheal 16S rRNA and accA genes showed strong seasonal dynamics, whereas the community structures of autotrophic *Thaumarchaeota* might have been affected by the hydrographic conditions, i.e. temperature and salinity. Although caution is needed before establishing a direct link between *accA* gene abundance and autotrophic *Thaumarchaeota* activity, our results suggested that dark CO$_2$ fixation by *Thaumarchaeota* might be more important in the late-autumn ECS ecosystem, which was in agreement with the high expressions of thaumarcheal 3-HB/4-HP cycle-related genes in the Antarctic Peninsula coastal surface waters during the winter [49,60].

Acknowledgments

We thank the captain and crew of the RV ‘Dongfanghong’ #2, L. K. Hao, H. Y. Cai and Q. Zheng for their assistance in sample collection during the cruises. We also thank Dr. Yao Zhang and two anonymous referees for their constructive comments which improved this manuscript, and Professor John Hodgkiss for his assistance with the English.

Author Contributions

Conceived and designed the experiments: AH NJ. Performed the experiments: AH ZY CY. Analyzed the data: AH CY. Wrote the paper: AH CY NJ.

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