Chapter 37

Diversity of Archaea in Terrestrial Hot Springs and Role in Ammonia Oxidation

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37.1 INTRODUCTION

Archaea are one of the three domains of life (the other two domains are Bacteria and Eukarya). These organisms all lack peptidoglycan in their cell walls, contain ether-linked lipids, and have complex RNA polymerases [Madigan et al., 2008]. Based on 16S rRNA gene analysis [Woese and Fox, 1977; Woese et al., 1990; see also Chapter 15, Vol. I], Archaea were initially classified into two phyla, Crenarchaeota and Euryarchaeota. With advancements in both microbial cultivation approaches and molecular microbiology, three new phyla have been proposed in recent years, the Korarchaeota [Barns et al., 1996], the Nanoarchaeota [Huber et al., 2002], and the Thaumarchaeota [Brochier-Armanet et al., 2008]. To date, Korarchaeota have only been reported from geothermal environments, whereas Crenarchaeota and Euryarchaoeta exist in nearly every niche on the planet Earth. Thaumarchaeota is currently defined to be a group of archaea that exist in low-temperature environments [Brochier-Armanet et al., 2008].

This review summarizes research on archaea that occur in terrestrial hot spring environments with a focus on thermophilic ammonia-oxidizing archaea. (Thermophiles and hyperthermophiles have an optimal growth at temperature above 45°C and 80°C, respectively [Reysenbach and Shock, 2002].) Section 37.2 will present a brief history of the discovery and isolation of archaea in geothermal environments. One important observation is that early isolates of thermophilic archaea have diverse physiological properties, including heterotrophic and autotrophic metabolism. Among the autotrophic species, sulfur- and hydrogen-based metabolisms are widely distributed (see review by Stetter [2006]). However, ammonia oxidation was not known to be performed by thermophilic archaea until 2008 [de la Torre et al., 2008; Hatzenpichler et al., 2008]. Another surprising observation is the scarcity of reports on archaeal cultivation-independent 16S rRNA gene studies in Yellowstone, particularly those with near-full-length gene sequencing, because Yellowstone is generally thought to be the most intensively studied geothermal area in the world. It is expected that a major effort in metagenomic sequencing of thermophilic microbial communities will quickly bridge our knowledge gap in archaeal diversity and add insights into community structure in Yellowstone (e.g., Shock et al. [unpublished data] and Inskeep et al. [unpublished data]). Section 37.3 will focus on cultivation-independent analysis of thermophilic ammonia-oxidizing archaea inspired by the discovery of archaeal nitrification in low-temperature environments such as oceans and soils. Section 37.4 presents future challenges and opportunities for studying archaea in terrestrial geothermal systems.

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37.2 THERMOPHILIC/ HYPERTHERMOPHILIC ARCHAEA IN GEOTHERMAL ECOSYSTEMS

37.2.1 Cultivation-Dependent Studies

Thomas Brock and Karl Stetter are among the pioneers in the cultivation and physiology of microorganisms from both terrestrial and marine geothermal environments [Brock et al., 1972; Stetter et al., 1981]. While most species were isolated from terrestrial hot springs, subsurface oil reservoirs, or marine geothermal systems (see review by Stetter, [2006]), other high-temperature ecosystems also host thermophilic archaea. For example, self-heating coal refuse piles are the habitat for isolation of most of the *Thermoplasma* species [Madigan et al., 2008]. Among cultivated Crenarchaeota, almost all thermophilic or hyperthermophilic species are ascribed to one class, Thermoprotei, which is comprised of three orders, Thermoproteales, Desulfurococcales, and Sufolobales [Boone and Castenholz, 2001]. Isolates of Euryarchaeota are grouped into eight classes, four of which are composed of thermophilic or hyperthermophilic species, Thermoplasmata, Archaeoglobi, Thermococci, and Methanopyri [Boone and Castenholz, 2001]. Although Thermoplasmata and Archaeoglobi are commonly reported from both marine and terrestrial springs, Thermococci and Methanopyri are most commonly reported from marine geothermal environments.

Nanoarchaeota were first reported as a symbiont of the hyperthermophilic marine crenarchaeote *Ignicoccus* and were named *"Nanoarchaeum equitans"* [Huber et al., 2002]. Subsequently, 16S rRNA genes representing Nanoarchaeota were described from a variety of marine and terrestrial geothermal environments, with considerable phylogenetic depth [Hohn et al., 2002]. They were initially described as a new phylum-level group [Huber et al., 2003]; however, more recently, analysis of the *"Nanoarchaeum equitans"* genome has shown a high percentage of predicted proteins with affinity to the Euryarchaeota and phylogenetic analyses of subsets of ribosomal proteins, and other genes suggested a relationship with the Thermococcales [Brochier et al., 2005]. Studies of the *"Nanoarchaeum"* genome show the absence of a number of core metabolic pathways, which indicate that *"N. 'equitans"* lives a parasitic lifestyle [Waters et al., 2003]. Recently, Nanoarcheaota 16S rRNA genes have also been detected in hypersaline sediments, suggesting that these organisms are not restricted to high-temperature environments [Casanueva et al., 2008].

Korarchaeota is another group whose position in the phylogenetic tree is uncertain [Brochier-Armanet et al., 2008; Elkins et al., 2008]. Korarchaeota 16S rRNA genes were first discovered in Obsidian Pool, Yellowstone [Barns et al., 1994] and suggested to represent a novel phylum of archaea [Barns et al., 1996]. Subsequently, they were detected in a variety of terrestrial and marine hydrothermal environments (e.g., Auchtung et al. [2006], Reigstad et al. [2009]) and eventually cultivated and physically purified, leading to full genome sequencing [Elkins et al., 2008]. Analysis of conserved single or concatenated proteins, as well as concatenated ribosomal RNA genes, suggested that a deep branching position within the Crenarchaeota could not be ruled out. The predicted energy-conserving physiology of "*Candidatus* Korarchaeum cryptofilum" is peptide fermentation with proton reduction. Like the Nanoarchaeota, Korarchaeota are predicted to be highly dependent on other microorganisms in the microbial community since they apparently lack pathways to synthesize some essential cofactors, vitamins, and purines [Elkins et al., 2008]. Lastly, as will be described more fully in Section 37.3, Thaumarchaeota are proposed to represent low-temperature, marine, ammonia-oxidizing Crenarchaeota [Brochie-Armanet et al., 2008].

All known archaea have ether-linked lipids in their cell membrane. In particular, hyperthermophilic Crenarchaeota and the thermophilic and acidophilic Euryarchaeota, Thermoplasma, are characterized by tetraether lipids that can have up to eight cyclopentyl rings [DeRosa and Gambacorta, 1988]. Note that no cyclohexyl ring was found until the late 1990s and the early 2000s [DeLong et al., 1998; Schouten et al., 2000], after nonthermophilic archaea were discovered based on cultivation-independent studies of marine pelagic communities [DeLong, 1992; Furhman et al., 1992]. Among cultivated thermophiles, an increase in the number of cyclopentyl rings corresponds to an increase in growth temperature [Uda et al., 2001]. Hydrophobic stacking of cyclopentyl rings helps to maintain membrane stability at high temperature [Gliozzi et al., 1983; Uda et al., 2001]. Valentine [2007] discussed the possible role of lipids in archaeal evolution and adaptation. Comprehensive studies of ether-linked lipids of methanogens also revealed that lipid profiles reflect phylogenetic relationships determined using the 16S rRNA gene and can be useful for taxonomic and ecological studies [Koga et al., 1998]. As to be discussed later, the newly discovered presence of a cyclohexyl ring in Crenarchaeota has considerable ecological, climate, and evolutionary implications.

Isolates of thermophilic/hyperthermophilic archaea are known to mediate a variety of chemical reactions as part of their metabolic endeavors [Stetter, 1996, 2006; Stetter et al., 1981]. These include heterotrophy and autotrophy fueled by oxidation of reduced chemicals such as molecular hydrogen, sulfur species [H_2S and S^0], and metals [Stetter, 2006]. In particular, it has been proposed

that that H_2 oxidation is the predominant force for primary productivity for most high-temperature ecosystems [Stetter, 1996; Spear et al., 2005]. While oxygen is an electron acceptor for microaerophilic growth of some hyperthermophiles, nitrate, ferric iron, sulfur, sulfate, and carbon dioxide can be used as electron acceptors for anaerobic respiration [Stetter, 2006; Kashefi and Lovley, 2000]. Fermentative metabolisms also exist among many hyperthermophilic archaea. These metabolic features are consistent with the reduced geochemical environment of many hydrothermal systems [Reysenbach and Shock, 2002]. Note, however, that the chemolithoautotrophic oxidation of ammonia was not reported for any of the hyperthermophilic isolates even though ammonia oxidation is thermodynamically feasible in hot spring environments [Shock et al., 2005; Costa et al., 2009].

37.2.2 Cultivation-Independent Studies

In 1992, two important papers [Furman et al., 1992; DeLong, 1992] described the occurrence of Crenarchaeota in coastal and open marine water. Since then, cultivation-independent studies of archaea based on analysis of nucleic acids isolated directly from nature have steadily increased and it has been apparent that archaea, particularly Crenarchaeota, are widely dispersed, diverse, and abundant in low-temperature marine and terrestrial environments (see reviews by DeLong [1998], Dawson et al. [2000]).

The first comprehensive analysis of archaeal 16S rRNA genes in terrestrial hot springs was performed by Barns et al. [1994, 1996], who observed that natural populations of archaea from Obsidian Pool, Yellowstone, contains at least 36 unknown organisms, among which most are distantly related to cultivated species. Since then, research on archaeal diversity in terrestrial hot springs has grown steadily and most studies have come from Yellowstone [Reysenbach et al., 2000; Jackson et al., 2001; Meyer-Dombard et al., 2005; Spear et al., 2005; de la Torre et al., 2008], the Great Basin [Pearson et al., 2004; Huang et al., 2007; Costa et al., 2009; Vick et al., 2010] and Lassen volcanic field [Siering et al., 2006; Wilson et al., 2008] in the United States, Kamchatka, Russia (Perevalova et al., 2008; Kublanov et al., 2009; Zhang et al., unpublished data), New Zealand [Niederberger et al., 2008], Iceland [Marteinsson et al., 2001; Kvist et al., 2007; Perevalova et al., 2008], Italy [Kvist et al., 2005], Japan [Takai and Sako, 1999], Thailand [Kanokratana et al., 2004; Purcell et al., 2006], and China [Yim et al., 2006; Song et al., 2010]. One consistent finding is the higher diversity and abundance of Crenarchaeota over Euryarchaeota in 16S rRNA PCR-based phylogenetic surveys of terrestrial hot springs, which was initially observed by Barns et al. [1994, 1996]. This is in contrast to the higher relative abundance and diversity of Euryarchaeota in phylogenetic surveys of marine hydrothermal environments [Reysenbach and Shock, 2002; Rusch and Amend, 2004; Lebedinsky et al., 2007]. Although we are aware that 16S rRNA gene censuses of microbial communities are not inherently quantitative, we believe that Crenarchaeota do dominate over Euryarchaeota in terrestrial geothermal habitats because this pattern has been observed by essentially all studies and is also reflected in the diversity of Crenarchaeota over Euryarchaeota among cultivated organisms.

Although a large number of environmental sequences have been obtained from a variety of hot spring locations, comparison between them has been difficult because of biases in PCR, particularly when studies differ in extraction methods and in the primers used for PCR amplification and DNA sequencing, as well as in different PCR cycling conditions; see Chapter 16 and 17, Vol. I. Thus, in this chapter we compare the 16S rRNA genes of archaea from two hot spring regions in the US, Yellowstone and the Great Basin (**Fig. 37.1**), with hope of identifying possible broad-scale phylogenetic patterns that can be addressed more rigorously in future studies. The classification of the archaeal groups follows that of Meyer-Dombard et al. [2005], which defines Yellowstone sequences into the orders Desulfurococcales, Thermoproteales, Sulfolobales, and unclassified clusters (UC) I-V (Fig. 37.1). Here UC-III is omitted because that cluster includes only sequences from marine hydrothermal vents [Meyer-Dombard et al., 2005]. The recently discovered order "Nitrosocaldales," including the thermophilic ammonia-oxidizing crenarchaeon "*Candidatus* Nitrosocaldus yellowstonii", belongs to cluster UC-II in this system; however, cluster UC-II forms a well-supported monophyletic cluster with the Thaumarchaeota and all "Marine Group 1.1" phylotypes [de la Torre et al., 2008; Costa et al., 2009]. Thus, if the proposal for a separate phylum for this lineage is formally accepted, cluster UC-II should belong to that phylum.

For Figure 37.1, sequences from Yellowstone are those reported by Barns et al. [1996], Meyer-Dombard et al. [2005], and de la Torre et al. [2008]. Sequences from the Great Basin are those reported by Huang et al. [2007] and Costa et al. [2009]. The phylogenetic tree is only representative because many cultivation-independent studies of archaea in Yellowstone (e.g., Reysenbach et al. [2000] and Jackson et al. [2001]) and the Great Basin [Vick et al., 2010] are omitted for clarity. Communities of Crenarchaeota in the two geothermal regions are broadly similar, with a more limited diversity in Great Basin springs. The lower diversity of Crenarchaeota in Great Basin springs may partly be an artifact of less

intense sampling; alternatively, this may be a natural reflection of the more limited geochemical diversity of springs in the Great Basin. For example, the Sulfolobales, which are thermoacidophiles, have thus far not been detected in Great Basin springs, likely due to the absence of acidic hot springs in the Great Basin [Huang et al., 2007; Costa et al., 2009]. Sulfolobales were also not detected in three studies of circumneutral Yellowstone springs included in Figure 37.1. Desulfurococcales and Thermoproteales are each present in both environments. Within the Desulfurococcales, members of the family Desulfurococcaceae are present in springs in Yellowstone and the Great Basin, as well as some 16S rRNA gene sequences that branch within the Desulfurococcales but are not associated with any currently described family. Among Desulfurococcaceae, phylotypes related to *Stetteria, Thermosphaera*, and *Desulfurococcus* exist in both geothermal systems. However, *Acidilobus* and *Caldisphaera*, which make up significant populations in some acidic hot springs in Yellowstone [Boyd et al., 2007], have not been detected in the Great Basin, presumably due to the absence of acidic springs. In contrast, *Ignisphaera* has been detected in Great Basin springs but not in Yellowstone. The more deeply branching family, Pyrodictiaceae, has not been described from continental hot springs either through cultivation-dependent or -independent approaches. Within the Thermoproteales, both families, Thermoproteaceae and Thermofilaceae, are present in both regions, though only the genus *Pyrobaculum* is closely related to phylotypes from Great Basin springs.

In both Yellowstone and the Great Basin, most studies reveal that Crenarchaeota distantly related to the Thermoproteales are dominant in 16S rRNA gene clone libraries, though we discourage interpretation of these data quantitatively, particularly because most

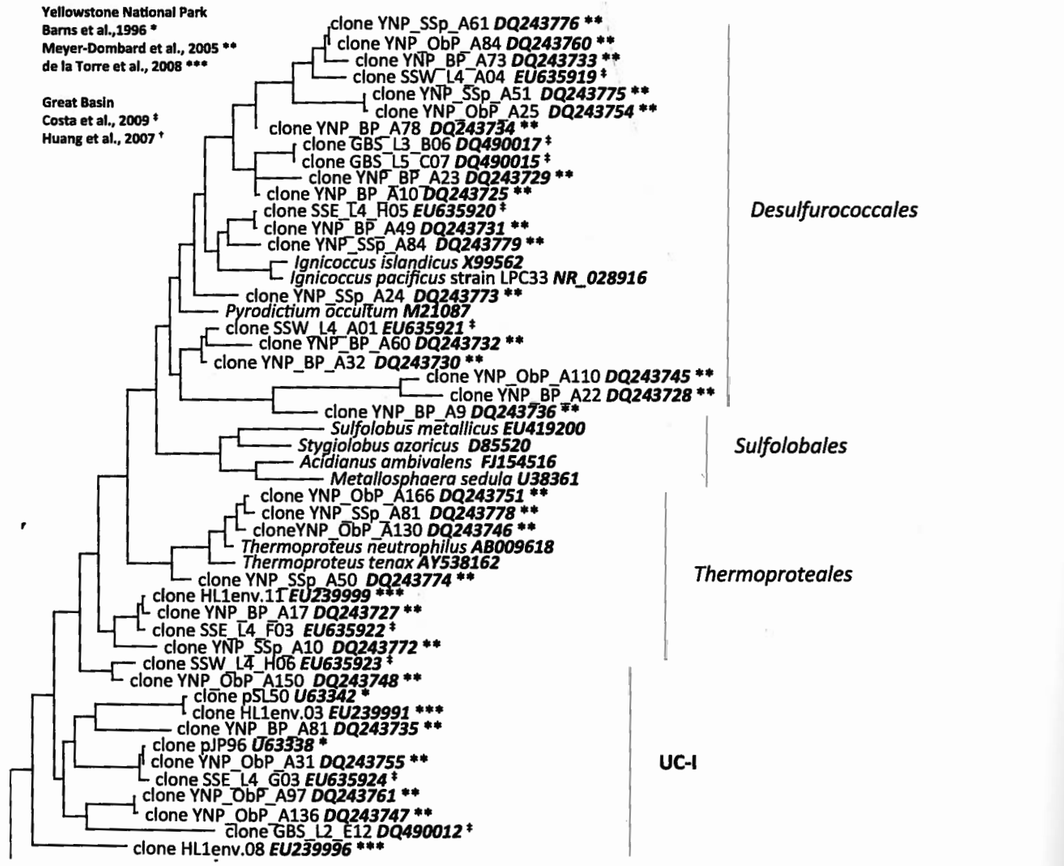

Figure 37.1 Phylogenetic tree of selected environmental sequences of the archaeal 16S rRNA genes from Yellowstone and the Great Basin.

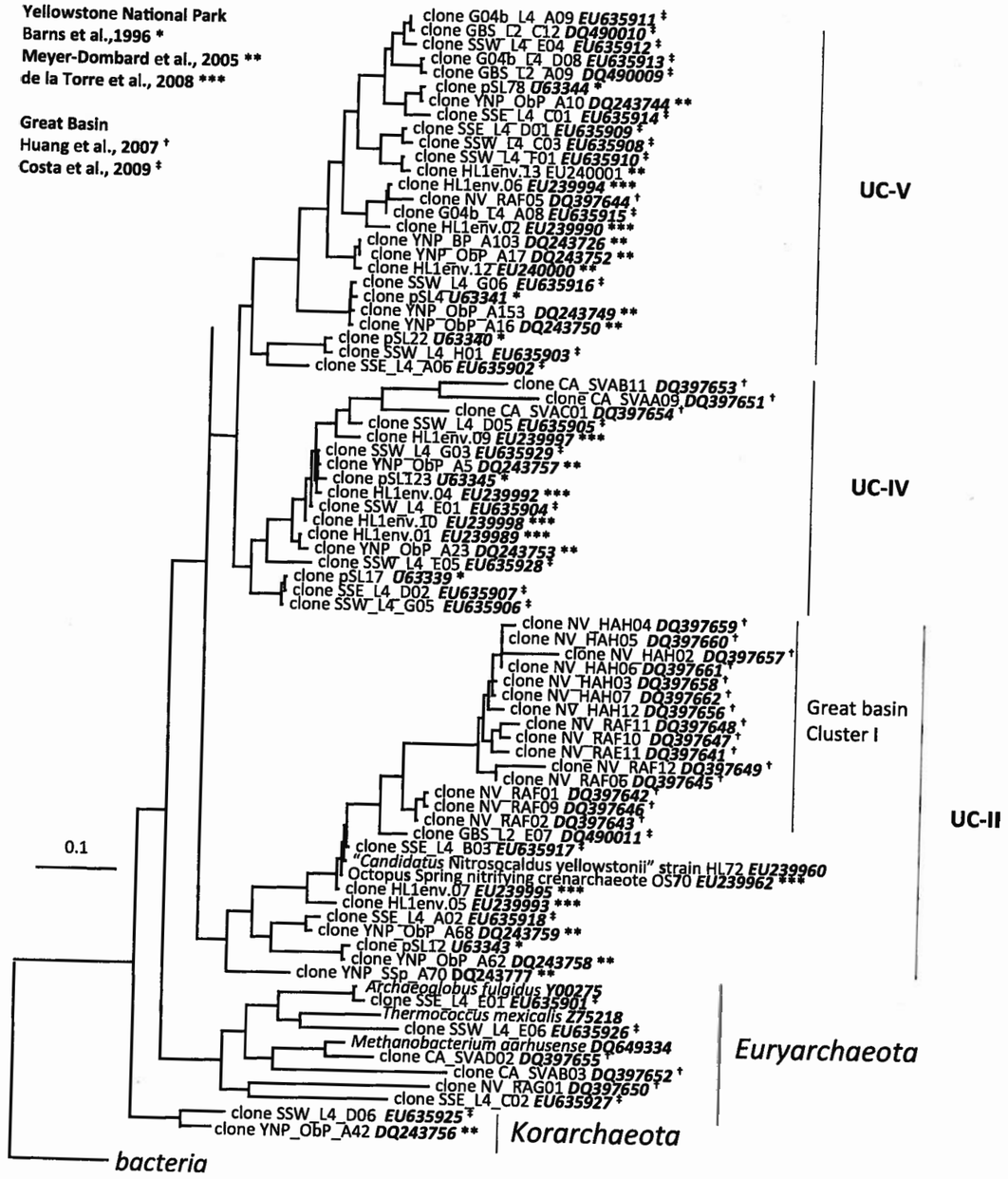

Figure 37.1 (*Continued*)

isolates in the Thermoproteales contain large introns in their 16S rRNA genes (e.g., Itoh et al. [2003]). These introns may lead sequences from Thermoproteales to be outcompeted by other sequences during PCR, particularly when DNA extension times are short. Clusters UC II, UC-IV, and UC-V are present in both Yellowstone and Great Basin hot springs. Group UC-II, which includes the thermophilic ammonia-oxidizing crenarchaeon "*Candidatus* Nitrosocaldus yellowstonii", includes basally branching phylotypes from high-temperature hot springs in Yellowstone and the Great Basin (70–86.6°C) [de la Torre et al., 2008; Costa et al., 2009; Vick et al., 2010] and a more shallow branch that includes phylotypes from cooler Great Basin springs (49–67°C), referred to as "Great Basin Hot Spring Crenarchaeota Cluster I" (GBHSCI) [Huang et al., 2007]. Although we urge caution in interpreting clone library data quantitatively, 60 of 61 archaeal phylotypes from the cooler Great Basin springs grouped within GBHSCI, and this group is so far only known from moderate-temperature Great Basin hot springs. These springs also host high concentrations of crenarchaeol and a high diversity of archaeal *amoA* genes, so it is tempting to speculate on whether this clade is composed entirely of crenarchaeol-containing ammonia oxidizers. However, this hypothesis awaits more conclusive experiments.

Clusters UC-IV and UC-V appear to be widely distributed in both geothermal regions since both have been detected in all studies used to generate Figure 37.1, as well as others. Similar to UC-II, the branching point and branch lengths within UC-IV are related to the temperature of the hot springs sampled, with low-temperature springs in the Great Basin forming the longest, shallowest branches. The deeper, shorter branches are comprised of phylotypes from higher temperature hot springs, with close relatives being detected in springs in both Yellowstone and the Great Basin. Cluster UC-V may be exclusive to higher temperature springs since only one phylotype was recovered from the study of the cooler-temperature Great Basin springs [Huang et al., 2007]. Again, as with Cluster UC-IV, closely related phylotypes have been recovered from the two different regions.

As previously noted [Meyer-Dombard et al., 2005], the phylogenetic structure of UC-I is poorly resolved and it appears that the group is not monophyletic. However, the sequences designated to this group also may be exclusive of higher temperature springs and are widely distributed in both the Great Basin and Yellowstone.

Although attempts have been made to delineate the underlying mechanisms that drive the distribution of archaea in different geothermal systems, little is known about the relative importance of environmental variables or geographical location in controlling archaeal diversity [Meyer-Dombard et al., 2005; Huang et al., 2007; Costa et al., 2009]. We are hopeful that advancements in high-throughput DNA sequencing technologies (see Chapter 18, Vol. I) and improvements in universal primer sets for microbial censuses (see Chapter 16–17, Vol. I) will enhance the capacity of the research community to discern, and begin to understand, local, regional, and global patterns of archaeal biodiversity within a physicochemical and geographical context.

37.3 ARCHAEAL AMMONIA OXIDATION IN TERRESTRIAL HOT SPRINGS

37.3.1 Overview of Archaeal Ammonia Oxidation in the Natural Environment

Autotrophic ammonia oxidation plays a central role in global nitrogen cycle because it is the first step in nitrification [Kowalchuk and Stephen, 2001]. Until recently, ammonia-oxidizing bacteria were thought to be exclusively responsible for ammonia oxidation, including members of the Betaproteobacteria and Gammaproteobacteria [Purkhold et al., 2000; Kowalchuk and Stephen, 2001; Prosser and Embley, 2002]. However, recently it has become well known that some archaea are capable of chemolithotrophic ammonia oxidation. Originally, hints that nonthermophilic marine and soil Crenarchaeota might be involved in chemolithotrophic ammonia oxidation were made through the discovery of homologues of bacterial ammonia monooxygenase subunits, ammonia permease, urease, and urea transporters in association with Crenarchaeota phylogenetic anchors [Venter et al., 2004; Schleper et al., 2005; Treusch et al., 2005; Hallam et al., 2006]. This hypothesis was subsequently solidified through the isolation and characterization of the pure culture "*Candidatus* Nitrosopumilus maritimus" [Könneke et al., 2005]. Since that discovery, numerous studies have focused on the distribution, diversity, and abundance of putative ammonia-oxidizing archaea, mostly by using PCR for the gene of the large subunit of the ammonia monooxygenase, *amoA*, as a biomarker (see Chapter 5, Vol. I). Collectively, these studies revealed that putative archaeal *amoA* genes are globally distributed and diverse and suggested that nonthermophilic Crenarchaeota may be the dominant ammonia-oxidizing microorganisms globally, particularly in habitats with low ammonia concentrations [Francis et al., 2005; Könneke et al., 2005; Treusch et al., 2005; Leininger et al., 2006; Nicol and Schleper, 2006; Wuchter et al., 2006; De Corte et al., 2009; Martens-Habbena et al., 2009]. The dominance of putative ammonia-oxidizing archaea over bacteria in ammonia-poor habitats may be due to their extremely high affinity for ammonia, which allows them to transport and oxidize ammonia at concentrations of $\leq$10 nM, as was recently shown with pure cultures of "*Candidatus* Nitrosopumilus maritimus" [Martens-Habbena et al., 2009].

Studies of the distribution and diversity of *amoA* genes also provided a phylogenetic framework for studies of other habitats [Francis et al., 2005; Beman and Francis, 2006]. Broadly, *amoA* alleles were found to segregate into two major phylogenetic lineages, one of which is predominantly found in the marine water column and sediments (Cluster A) and the other lineage is predominantly found in soils and sediments (Cluster B) [Beman and Francis, 2006]. Although some studies show that ammonia-oxidizing archaea are strict autotrophs that are sensitive to low concentrations of organic compounds [Könneke et al., 2005; Hallam et al., 2006; de la Torre et al., 2008], other molecular and geochemical studies show that some take up amino acids and other forms of organic carbon in certain environments, indicating that some of the ammonia-oxidizing archaea are heterotrophs or mixotrophs [Ouverney and Fuhrman, 1999, 2000; Herndl et al., 2005; Ingalls et al., 2006; Teira et al., 2006].

37.3.2 Ammonia Oxidation in Hot Springs

Nitrogen metabolism and cycling is poorly understood in geothermal environments (e.g., Nicol and Schleper [2006] and Francis et al. [2007]). Although ammonia/ammonium is often an abundant chemical species in hot springs, several decades of microbial cultivation studies failed to isolate any thermophilic ammonia-oxidizing organisms. Some successes were reported from studies of hot springs in Russia [Golovacheva, 1976; Lebedeva et al., 2005]. In Golovacheva [1976], thermophilic ammonia-oxidizing bacteria capable of growth at 55°C were enriched from geothermal springs in Kamchatka. However, the cultures were unstable and the organisms, though morphologically similar to ammonia-oxidizing bacteria in the genus *Nitrosomonas*, were not identified. In Lebedeva et al. [2005], enrichment cultures were obtained from Garga spring in the Baikal rift zone, which is characterized by slightly alkaline water (pH 7.9) and an outlet temperature of 75°C. The enrichment cultures of the ammonia oxidizers grew at temperature ranges of 27–55°C (optimal 50°C), suggesting that they are moderate thermophiles, and the organisms were identified as *Nitrosomonas* by immunofluorescence using genus-specific polyclonal antibodies against the ammonia monooxygenase. Again, no pure cultures of ammonia-oxidizing microorganisms were obtained.

The first hint of thermophilic ammonia-oxidizing archaea in geothermal environments was uncovered by Pearson et al. [2004], who reported the presence of the lipid biomarker "crenarchaeol" from several hot springs in the Great Basin (40–85°C). Crenarchaeol was originally thought to be unique to nonthermophilic, planktonic marine Crenarchaeota [Schouten et al., 2000; Damsté et al., 2002] and is present in *"Cenarchaeum symbiosium"* [Damste et al., 2002] and "*Candidatus* Nitrosopumilus maritimus" [Schouten et al., 2008]. Now we know that crenarchaeol occurs widely in terrestrial hot springs, particularly at moderate temperatures [Zhang et al., 2006; Pearson et al., 2008], and is actively produced by thermophilic archaea [de la Torre et al., 2008; Pitcher et al., 2009]. It has been observed that the abundance of crenarchaeol correlates with the abundance of the *amoA* gene in soil [Leininger et al., 2006] and hot spring [Pitcher et al., 2009] environments; thus, crenarchaeol may serve as a biomarker for archaeal ammonia oxidation [de la Torre et al., 2008]. This hypothesis, however, needs to be further tested. Historically speaking, it is important to point out that when crenarchaeol was initially discovered in hot springs, the functions of marine crenarchaeol-containing archaea (e.g., ammonia oxidation) were only beginning to be uncovered [Venter et al., 2004]. Thus, tying this discovery to ammonia oxidation awaited a firm connection between marine crenarchaeol and the capability to oxidize ammonia. However, once this link was made, and armed with the knowledge that crenarchaeol is also abundant in some hot springs, studies on the oxidative nitrogen cycle at high temperature began to intensify.

The presence of putative archaeal *amoA* genes in geothermal environments was first published by Weidler et al. [2007] and Spear et al. [2007] and was reported at an international meeting [Schleper, 2007 and Zhang et al., 2007; American Society for Microbiology Annual Meeting, Toronto]. Weidler et al. [2007] reported evidence of ammonia-oxidizing Crenarchaeota in a subsurface radioactive thermal spring (temperature 42.1°C, pH 8.1) in the Austrian central Alps, and Spear et al. [2007] reported putative *amoA* genes in a geothermal (50°C) mine adit near Glenwood Springs, Colorado.

While Weidler et al. [2007] and Spear et al. [2007] performed their research in the subsurface environment, these studies were quickly followed by several reports of archaeal *amoA* genes from surface hot springs from around the globe [de la Torre et al., 2008; Hatzenpichler et al., 2008; Reigstad et al., 2008; Zhang et al., 2008] (**Table 37.1**). So far, a large number of *amoA* gene sequences have been obtained from hot springs in Austria, China, Iceland, Russia, and the United States (Table 37.1). The majority of sequences are grouped into the two clusters that were previously defined [Francis

Table 37.1 Summary of Subsurface and Surface Hot Springs that Have Been Studied for the Archaeal *amoA* Genes

Location	Type of Material	pH	T(°C)	Total Sequences	Reference
Austrian Central Alps subsurface spring	Biofilm, filtered water, glass slide	8.1	42.3	15	Weidler et al. [2007]
Austrian Central Alps subsurface spring	Biofilm, filtered water, glass slide	7.9	45.6	17	Weidler et al. [2007]
Glenwood Springs mine adit, USA	Mat-like biofabrics in speleotherm	6.4	49.6	82	Spear et al. [2007]
Great Basin hot springs, USA	Mat or sediment	6.0–9.0	41.0–86.0	281	Zhang et al. [2008]
Great Basin hot springs, USA	Mat or sediment	9.0	41.0	34[a]	Zhang et al. [2008]
Iceland hot springs, Iceland	Mat or mud	3.0–7.0	38–97	32	Reigstad et al. [2008]
Kamchatka hot springs, Russia	Mat or sediment	6.0–8.0	50.5–72.6	97	Zhang et al. [2008]
Kamchatka hot springs, Russia	Mat/mud/filaments	3.0–6.5	49–94	5	Reigstad et al. [2008]
Kamchatka hot springs, Russia	Mat or sediment	5.5–7.0	42.0–86.8	323	Zhao et al. [2010]
Siberian Garga hot spring, Russia	Enrichment culture	7.4	46.0	19	Hatzenpitchler et al. [2008]
Yellowstone hot springs, USA	Mat or sediment	3.4–8.4	41.0–85.0	239	Zhang et al. [2008]
Yellowstone hot springs, USA	Mat or sediment	3.0–9.0	54–95	N/A[b]	de la Torre et al. [2008]
Yunnan hot springs, China	Mat or sediment	7.5–8.2	43.6–77.0	195	Zhang et al. [2008]
Yunnan hot springs, China	Mat or sediment	2.4–9.0	44.5–94.0	337[a]	Jiang et al. [in review]

[a]cDNA.

[b]Not available.

et al., 2005; Zhang et al., 2008] (**Fig. 37.2**). A third cluster, herein designated Cluster C, also exists, and is so far only composed of *amoA* genes from "*Canidatus Nitrosocaldus yellowstonii*", discussed below, and closely related sequences from Yellowstone (Fig. 37.2) [de la Torre et al., 2008]. However, it should be noted that the forward PCR primer commonly used for PCR amplification of *amoA* alleles, Arch-amoAF [Francis et al., 2005; Reigstad et al., 2008; Zhang et al., 2008], contains two mismatches near the 3′ end with Cluster C sequences. Thus, Cluster C *amoA* genes may be more widely distributed and more diverse than currently known. The forward primer of de la Torre et al. [2008], Arch_amoA_F, includes degenerate positions to allow for amplification of Cluster C sequences. However, it is noteworthy that that primer failed to reveal Cluster A and B sequences in several Yellowstone springs. Thus, evaluation and improvement of PCR primers for amplification of archaeal *amoA* gene homologues are clearly an area for further research. The following sections describe characteristics of *amoA* genes from different geothermal environments.

In Reigstad et al. [2008], 22 hot springs in Iceland and Kamchatka were surveyed for the archaeal *amoA* genes, among which 14 showed positive results. Most of these *amoA*-positive hot springs had temperatures between 82°C and 97°C and had pH values between 2.5 and 7.0. Phylogenetically, the authors grouped these *amoA* genes into three independent lineages within the known sequence clusters of marine and soil origin [Reigstad et al., 2008]. In Figure 37.2, we separated sequences from Reigstad et al. [2008] into Iceland and Kamchatka; the Kamchatka sequences are grouped with those reported in Zhang et al. [2008] from the same location. The majority of *amoA* genes from Iceland also occur within Group A1.1 and A1.2, with the other sequences distributed within Cluster B (Fig. 37.2). The combined Kamchatka sequences from Reigstad et al. [2008] and Zhang et al. [2008] occur predominantly in Cluster A, and they are distributed among all groups except group A1.1 (Fig. 37.2).

Reigstad et al. [2008] also performed in situ gross nitrification rates at about 85°C in two of the Icelandic hot springs by the $^{15}NO_3^-$ pool dilution technique, which yielded rates of nitrate production at 12.8–21.1 μmol nitrate L^{-1} per day at in situ temperatures. Furthermore, nitrification rate was enhanced more than twofold by addition of ammonium to the hot spring samples before incubation, indicating that nitrification in these hot springs was limited by ammonia supply.

In Zhang et al. [2008], microbial mats and surface sediments were analyzed for the archaeal *amoA* genes in 21 hot spring samples (pH 3.4–9.0; temperature, 41–86°C) from the United States (Yellowstone and Great Basin), China (Tengchong, Yunnan Province), and Russia (Uzon Caldera of Kamchatka). A total of 846 sequences were obtained, which represent 41 *amoA* operational taxonomic units (OTUs) at 98% nucleotide sequence identity. The *amoA* gene sequences were highly diverse among Great Basin, Yellowstone, Kamchatka, and China and were grouped within Cluster A and Cluster B

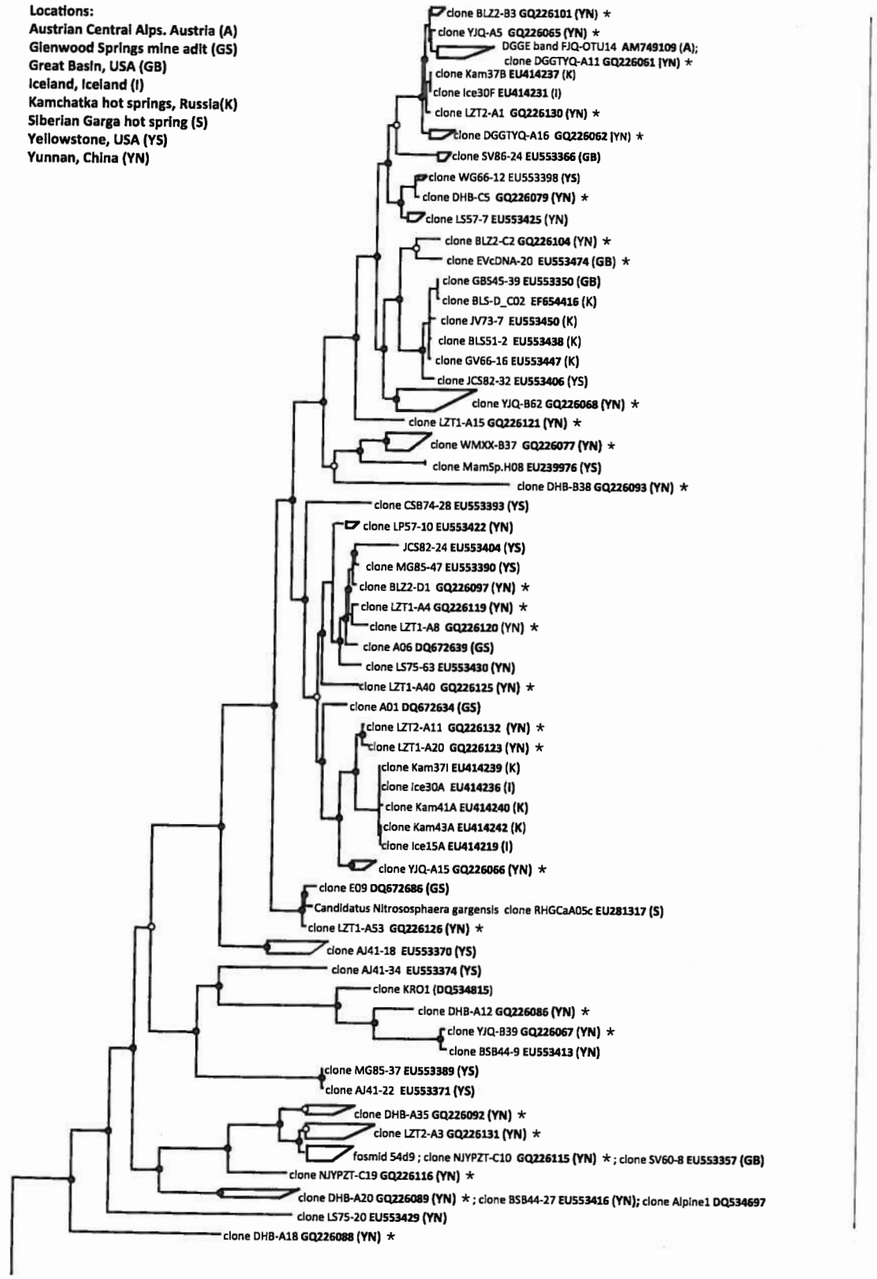

Figure 37.2 Phylogenetic analysis of archaeal *amoA* genes from terrestrial hot springs.

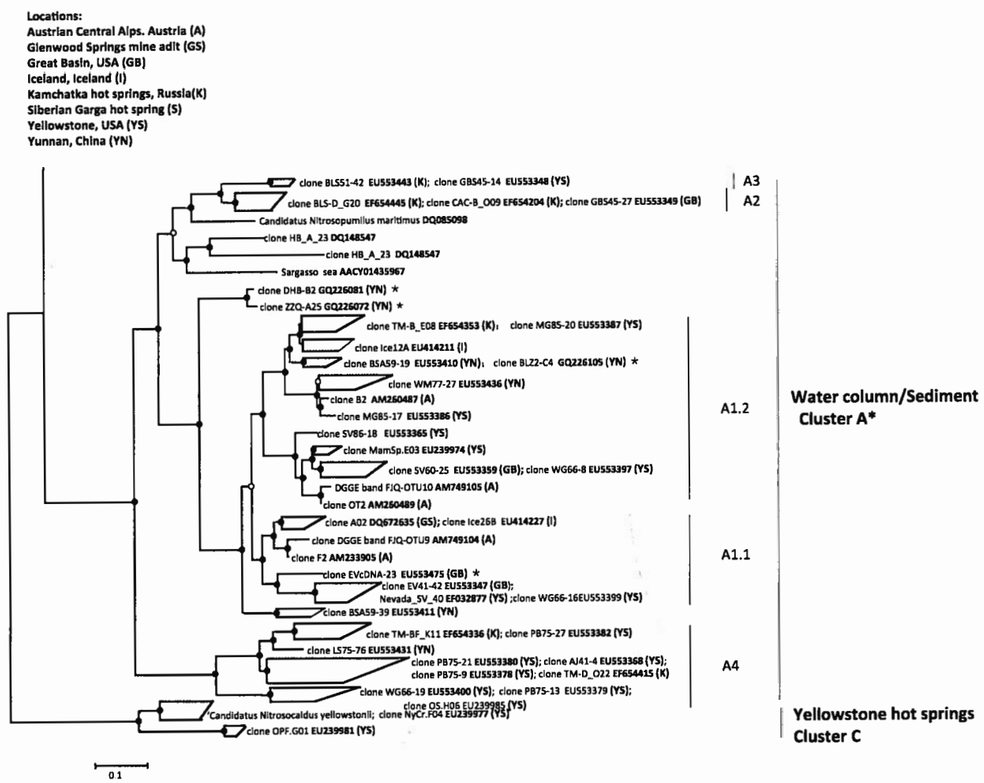

Figure 37.2 (*Continued*)

(Fig. 37.2). The relative distribution of *amoA* genes between these clusters were summarized in Zhang et al. [2008] (**Table 37.2**), which shows the predominance of Cluster A (71–97% of total clones) over Cluster B (3–29%) among the four locations they compared (Table 37.2). One important observation is that Cluster A *amoA* genes formed geographic groups, regardless of temperature or bulk water pH [Zhang et al., 2008], suggesting that geography may play a role in structuring communities of putative ammonia-oxidizing archaea within the Cluster A. Zhang et al. [2008] also showed the distinctness of hot spring sequences from those of sympatric soils, which shared less than 85% nucleotide sequence identity.

These studies demonstrate the global occurrence of putative archaeal *amoA* genes in a wide variety of terrestrial hot springs and suggest that geography may play an important role in selecting different assemblages of ammonia-oxidizing archaea. Zhang et al. [2008] also showed that several *amoA* genes are transcribed in situ

Table 37.2 Relative Abundance of *amoA* Gene Clusters in Clone Libraries from Hot Springs of Different Geographic Regions Reported in Zhang et al. [2008]

	Distribution of *amoA* Gene Groups (%)			
amoA Group	Great Basin	Yellowstone	Tengchong	Kamchatka
A1	81.5	7.0	76.5	0.0
A2	13.5	0.0	0.0	72.0
A3	2.0	0.0	0.0	13.5
A4	0.0	64.0	0.5	0.0
B	3.0	29.0	23.0	14.5
Total	100.0	100.0	100.0	100.0

in a 42°C hot spring in the Great Basin by reverse transcriptase PCR, suggesting the potential activity of these organisms under in situ conditions. Jiang et al. [2010] expanded the RNA-based studies and determined the abundance and diversity of potentially active

ammonia-oxidizing archaea in hot springs (temperature: 44.5–94.0°C; pH 2.35–9.0) of Yunnan Province, China. Cloning and phylogenetic characterization of the transcripts showed that archaeal *amoA* genes of Cluster B of Zhang et al. [2008] were expressed and dominant in the studied hot springs. However, these springs are different from those in Zhang et al. [2008], so it's possible that these springs are dominated by cluster B types. Clearly, there's a need for both DNA and RNA studies done together at the same time and place, as was done in Zhang et al. [2008].

While Reigstad et al. [2008] and Zhang et al. [2008] focused on natural occurrence of putative archaeal *amoA* genes from a variety of hot springs with varying temperature and pH values, de la Torre et al. [2008] and Hatzenpichler et al. [2008] provided much-needed definitive evidence linking crenarchaeol-containing thermophiles to chemolithotrophic ammonia oxidation in hot springs.

Isolation of ammonia-oxidizing archaea proves to be notoriously difficult and so far only the nonthermophilic crenarcheon "*Candidatus* Nitrosopumilus maritimus" is in pure culture [Könneke et al., 2005]. In Hatzenpichler et al. [2008], a single OTU of Crenarchaeota was detected in an enrichment culture obtained from Garga Hot Spring, which had been maintained at 46°C for over six years. The authors ascribed the *amoA* and *amoB* sequences to the single archaeal 16S rRNA phylotype, which belongs to the widely distributed group I.1b (soil group) of the Crenarchaeota. The enriched ammonia-oxidizing archaeon is provisionally classified as "*Candidatus* Nitrososphaera gargensis", which is characterized by high ammonia oxidation activity at relatively low ammonia concentrations (0.14 and 0.79 mM) at moderately high temperature (46°C).

In de la Torre et al. [2008], a simplified mixed culture (containing 90% of archaeal cells and 10% of bacterial cells) was obtained from a higher temperature (>70°C) spring in Yellowstone (Heart Lake 1). The single archaeal population grew autotrophically by aerobic ammonia oxidation at temperatures up to 74°C and was provisionally assigned as "*Candidatus* Nitrosocaldus yellowstonii". Phylogenetically, it is grouped with Crenarchaeota 16S rRNA gene sequences from cultivation-independent studies of geothermal environments but is only distantly related to "*Candidatus* Nitrososphaera gargensis" (see below). "*Candidatus* Nitrosocaldus yellowstonii" contains abundant crenarchaeol, which supports its natural occurrence in the hot spring from which this organism was isolated [de la Torre et al., 2008]. On the other hand, Costa et al. [2009] reported that "*Candidatus* Nitrosocaldus yellowstonii" dominated the archaeal 16S rDNA clone libraries in some Nevada hot springs at temperatures above 70°C. This observation suggests that crenarchaeol in Great Basin hot springs may be produced by "*Candidatus* Nitrosocaldus yellowstonii"-like organisms.

37.4 SUMMARY AND FUTURE RESEARCH CHALLENGES

Mounting evidence indicates that ammonia-oxidizing archaea are widespread over a large range of temperatures and pH in terrestrial hot springs. The distribution of *amoA* genes seems to be controlled by geography and to some extent by local environmental variables. Yet, further study is required to disentangle the effects of physichochemistry and geography in shaping communities of ammonia-oxidizing archaea. The potential activities of some of these organisms have been demonstrated by the recovery of *amoA* transcripts and through bulk rate measurements of in situ nitrification, which are supported by physiological studies of highly purified cultures of Crenarchaeota under laboratory conditions. These results support the notion that thermophilic ammonia-oxidizing archaea play an important role in nitrogen cycle in the geothermal ecosystem.

However, the study of nitrification in hot springs is still in its infancy, and several challenges are ahead that must be met in order to advance the research to the next level. The first challenge is to intensify efforts to cultivate and study themophilic ammonia-oxidizing archaea in the laboratory. Only with the availability of pure cultures can detailed and precise biochemical studies be performed for a better understanding of the pathways and kinetics of ammonia oxidation used by these thermophilic species. Work with pure isolates will also help us to understand the roles of these organisms in the carbon cycle (i.e., autotrophy or mixotrophy) and to characterize the pathways of CO_2 fixation coupled to ammonia oxidation performed by chemolithoautotrophic species. Pure and highly simplified cultures of thermophilic ammonia-oxidizing archaea will also provide anchor genomes for interpreting large-scale metagenomic efforts that are currently underway in variety of geothermal ecosystems (Inskeep et al. and Meyer-Dombard et al., Yellowstone; Hedlund et al., Great Basin). Since metagenomic efforts are not subject to the same biases as the PCR-based studies, focus on archaeal metagenomic contigs will help to determine whether patterns in 16S rRNA and *amoA* genes that have been discussed in this chapter are valid or artifactual. Metagenomic data will also be useful in the evaluation and refinement of PCR primers 16S rRNA and *amoA* genes for future PCR-based inquiries. Throughout cultivation and metagenomic approaches focused on thermophilic archaea, particularly those with putative *amoA* genes, efforts must be made to cover all

major phylogenetic groups. For example, with regard to *amoA* alleles, efforts need to be made to cultivate and study organisms with Cluster A and Cluster B *amoA* genes to dissect the functions and ecological niches of each group.

Along with the cultivation effort, careful and more thorough measurements of the activities of these organisms in nature must be conducted along with cultivation-independent studies of the resident microorganisms and careful characterization of the physicochemical habitat. Studies of gross nitrification should be coupled with measurements of other major processes in the nitrogen cycle, such as N_2 fixation, nitrate respiration (particularly with regard to different products), anaerobic ammonia oxidation, nitrite oxidation, ammonification, and assimilatory metabolisms. These types of integrated analyses are needed to begin to understand how temperature affects the nitrogen cycle. Efforts are also needed to better link geochemical processes with distribution, community structure, and activity of ammonia-oxidizing archaea in the natural environments. In particular, the production of crenarchaeol as a proxy for archaeal ammonia oxidation needs to be thoroughly evaluated. Also, the relative importance of autotrophy and heterotrophy among thermophilic ammonia oxidizers in situ must be addressed, for example, through stable isotope probing of DNA/RNA and membrane lipids or by studying the natural carbon isotopic values of natural lipids. Fortunately, we can face these challenges with advanced technologies that are emerging rapidly. For example, metagenomics or single-cell genomics using 454 pyrosequencing and other high-throughput sequencing platforms are powerful tools for addressing the community structure and ecological function. As research becomes more and more interdisciplinary and globalized, and with continuing advancements and democratization of DNA sequencing technology, the next 10 years will be an exciting decade of new discoveries in the fast-evolving field of archaeal nitrification.

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