The ISME Journal (2014) 8, 1534–1543

www.nature.com/ismej

ORIGINAL ARTICLE

Crenarchaeal heterotrophy in salt marsh sediments

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Mesophilic Crenarchaeota (also known as Thaumarchaeota) are ubiquitous and abundant in marine habitats. However, very little is known about their metabolic function *in situ*. In this study, salt marsh sediments from New Jersey were screened via stable isotope probing (SIP) for heterotrophy by amending with a single ^{13}C-labeled compound (acetate, glycine or urea) or a complex ^{13}C-biopolymer (lipids, proteins or growth medium (ISOGRO)). SIP incubations were done at two substrate concentrations (30–150 μM; 2–10 mg ml^{-1}), and ^{13}C-labeled DNA was analyzed by terminal restriction fragment length polymorphism (TRFLP) analysis of 16S rRNA genes. To test for autotrophy, an amendment with ^{13}C-bicarbonate was also performed. Our SIP analyses indicate salt marsh crenarchaea are heterotrophic, double within 2–3 days and often compete with heterotrophic bacteria for the same organic substrates. A clone library of ^{13}C-amplicons was screened to find matches to the ^{13}C-TRFLP peaks, with seven members of the Miscellaneous Crenarchaeal Group and seven members from the Marine Group 1.a Crenarchaeota being discerned. Some of these crenarchaea displayed a preference for particular carbon sources, whereas others incorporated nearly every ^{13}C-substrate provided. The data suggest salt marshes may be an excellent model system for studying crenarchaeal metabolic capabilities and can provide information on the competition between crenarchaea and other microbial groups to improve our understanding of microbial ecology.
The ISME Journal (2014) **8,** 1534–1543; doi:10.1038/ismej.2014.15; published online 20 February 2014
Subject Category: Microbial ecology and functional diversity of natural habitats
Keywords: Crenarchaea; stable isotope probing; Thaumarchaea; TRFLP; 16S rRNA gene profiling

Introduction

For many years it was assumed that archaea were either methanogens or extremophiles. However, in the last few decades, small ribosomal RNA (rRNA) subunit and 16S rRNA gene analysis of environmental samples revealed the presence of archaea in aerobic, marine environments at moderate temperatures (DeLong, 1992; Fuhrman *et al.*, 1992). These archaea (mostly belonging to the subdomain Crenarchaeota) have been found in soils (Birtrim *et al.*, 1997), polar seas (Murray *et al.*, 1998), estuaries (Abreu *et al.*, 2001), caves (Gonzalez *et al.*, 2006), a wide variety of oceanic settings (DeLong *et al.*, 1994; Stein and Simon, 1996; Massana *et al.*, 1997; Massana *et al.*, 2000; Karner *et al.*, 2001), deep-sea sediments (Vetriani *et al.*, 1999; Teske *et al.*, 2002; Søresnson and Teske, 2006; Biddle *et al.*, 2008) and salt marshes (Nelson *et al.*, 2009). Although the crenarchaeota are globally ubiquitous and frequently abundant in various environments, relatively little is known about their metabolic capabilities.

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Received 22 October 2013; revised 21 December 2013; accepted 6 January 2014; published online 20 February 2014

Recent evidence suggests some crenarchaea possess the ammonium monooxygenase gene and may have a role in ammonia oxidation (Venter *et al.*, 2004; Francis *et al.*, 2005; Schleper *et al.*, 2005; Treusch *et al.*, 2005). Therefore, many studies on their metabolism have focused on uptake of inorganic carbon. For example, ^{13}C-bicarbonate incorporation into crenarchaeal lipids was observed in waters of the North Sea, the deep waters of the North Atlantic and the Pacific Gyre (Wuchter *et al.*, 2003; Herndl *et al.*, 2005; Ingalls *et al.*, 2006). In addition, genomic sequencing results indicate an uncultivated marine crenarchaeote contained genes associated with a modified 3-hydroxypropionate cycle for autotrophic carbon assimilation (Hallam *et al.*, 2006). In contrast, there is also evidence of archaeal heterotrophy as described by Ouverney and Fuhrman (2000), where up to 60% of the crenarchaeota in the deep Mediterranean and Pacific accumulate amino acids as measured by STARFISH (MICRO-FISH) methods. Likewise, a study by Biddle *et al.* (2006) determined that deep-sea sedimentary crenarchaeota are mostly heterotrophic based on stable isotope signatures of carbon in archaeal membrane lipids. Finally, metagenomic research on open ocean Group IA crenarchaeaota found genes for 3-hydroxypropionate carbon fixation and oligopeptide transport, suggesting amino acid uptake in addition to fixing inorganic carbon

for cellular needs (Martin-Cuadrado *et al.*, 2008). Unfortunately, these bulk approaches or metagenomic studies cannot elucidate the particular substrates used by specific crenarchaea under a given condition, nor can they provide information on the doubling time of crenarchaea *in situ*. A method is needed, such as stable isotope probing (SIP; Radajewski *et al.*, 2000), which directly links carbon utilization with specific members of the mesothermal crenarchaea community.

In this report, SIP experiments are presented showing ^{13}C-incorporation of various organic substrates by salt marsh sediment-associated members of the Miscellaneous Crenarchaeota Group (Inagaki *et al.*, 2003) and Marine Group I (Vetriani *et al.*, 2003). Our results demonstrate salt marsh crenarchaeota are capable of assimilating a wide array of organic carbon substrates, which are also utilized by bacterial populations *in situ*, suggesting competition between the two domains. In addition, there is evidence of resource partitioning for urea and whole proteins. Interestingly, a minimum incubation of 5 days was required to obtain unambiguous SIP signal for the crenarchaea, suggesting a relatively long (2–2.5 day) doubling time compared with the bacterial community. These findings suggest both top-down and bottom-up mechanisms are allowing for the stable coexistence of crenarchaea and bacteria in salt marsh settings.

Materials and methods

Surficial, salt marsh sediment was collected using a sterile syringe from the tidal levee at Hooks Creek in Cheesequake State Park, NJ, USA. Approximately 10 g of sediment was placed in 170 ml glass serum vials, filled to the top with sterile-filtered (0.2 μm Supor, Pall, Port Washington, NY, USA) site water as in Kerkhof *et al.* (2011). To determine heterotrophic activity, the SIP microcosms were amended with a single ^{13}C-labeled compound (acetate, glycine or urea) or a single ^{13}C-labeled biopolymer (methanol extract of algal lipids/pigments, extract of algal proteins) or complex growth medium (ISOGRO, Sigma-Aldrich, St Louis, MO, USA). Each organic substrate was added at two concentrations: 30 or 150 μM for the simple organics and 2 or 10 mg ml^{-1} for the biopolymers/growth medium. The vials were sealed, mixed, incubated for 3–14 days in the dark and turned once per day to ensure exposure of ^{13}C-substrate to the sediment. SIP controls included no amendment and ^{12}C-urea amendments. For assessing autotrophic activity, the experiment was repeated using 5 mM ^{13}C-bicarbonate and a 5-day incubation, with ^{12}C-labeled substrates or no-amendment microcosms as controls. This concentration of bicarbonate (2.5 times that of seawater) raised the pH of the site water from 7.6 to 8.3, which is close to the range normally observed in seawater (7.6–8.2; Emerson and Hedges, 2008).

After each SIP incubation, the sediment was collected by centrifugation at 16 000 *g* for 1 min to remove any liquid, then immediately frozen in liquid nitrogen. DNA was extracted by phenol–chloroform methods and fractionated by isopycnic cesium chloride gradient ultracentrifugation at 200 000 *g* for 36 h, with ^{13}C-labeled *Halobacterium salinarum* as a carrier when detecting bacterial DNA or ^{13}C-*Escherichia coli* DNA when targeting archaeal DNA (Gallagher *et al.*, 2005). After ultracentrifugation, the ^{12}C- and ^{13}C-bands were collected by pipette and amplified by PCR using 5′-fluorescently labeled, archaea-specific (21F, 5′-TTCCGGTTGATCC YGCCGGA-3′/958R, 5′-YCCGGCGTTGAMTCCAAT T-3′) and crenarchaeota-specific (7F, 5′-TTCCGGTT GATCCYGCCGGACC-3′/518R, 5′-GCTGGTWTTACC GCGGCGGCTGA-3′) or bacteria-specific forward/reverse primers (27F, 5′-AGAGTTTGATCCTGGCT CAG-3′/1100R, 5′-AGGGTTGCGCTCGTTG-3′) targeting the 16S rRNA gene. Seven nanograms of amplicon were digested with *Mnl I* in 20 μl volumes for 6 h at 37 °C, ethanol precipitated and resuspended in deionized formamide with ROX 500 size standard (Applied Biosystems, Foster City, CA, USA) as in McGuinness *et al.* (2006). Terminal restriction fragment length polymorphism (TRFLP) fingerprinting (Avaniss-Aghajani *et al.*, 1994) was carried out on an ABI 310 genetic analyzer (Applied Biosystems) using Genescan software (Applied Biosystems) with a peak detection of 25 arbitrary fluorescent units.

To determine the phylogenetic affiliation of the various ^{13}C-crenarchaeal peaks, a crenarchaeal ^{13}C-amplicon clonal library was constructed using the Topo TA cloning kit, as per the manufacturer's instruction (Invitrogen, Carlsbad, CA, USA). One hundred recombinant clones were screened in a multiplex format as in Babcock *et al.* (2007), to find clones (500 bp) matching the ^{13}C-SIP peaks. Twenty four clones were identified in this way and sequenced via Sanger methods using M13 primers (Genewiz Inc., South Plainfield, NJ, USA), producing 14 unique sequences (<99% similarity). These crenarchaeal sequences were initially screened by BLAST to find nearest matches, and a maximum likelihood phylogenetic tree was reconstructed using 433 unambiguously aligned bases among 44 taxa with Geneious analysis software (Guindon and Gascuel, 2003; Drummond *et al.*, 2009).

Results

SIP is predicated on detecting a PCR signal in the ^{13}C-carrier band when ^{13}C-substrates are added, and not detecting a PCR signal in the ^{13}C-carrier when no substrate or ^{12}C-substrates are added, as shown in Figure 1a. In this incubation, crenarchaeal amplicons are only detected when ^{13}C-lipids/pigments (lanes 1a-6 and 7) or ^{13}C-ISOGRO at the higher concentration (lane 1a-11) had been added to the

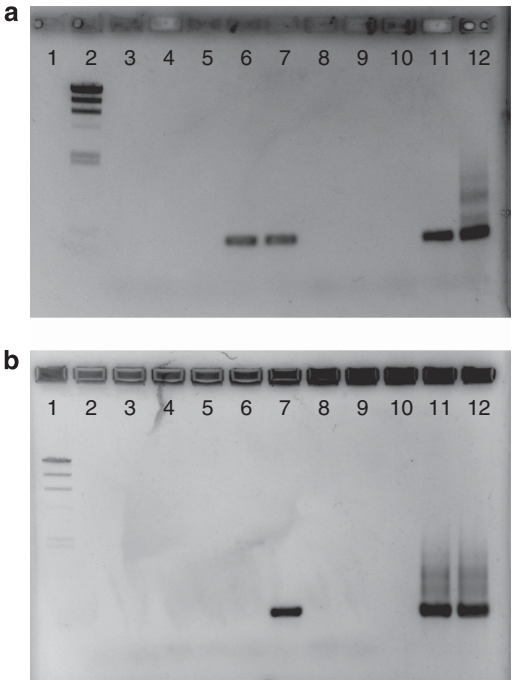

Figure 1 Agarose gel of crenarchaeal 16S rRNA gene amplicons from ^{13}C-bands: a1) empty; a2) lambda DNA; a3) negative; a4) carrier; a5) no substrate; a6) ^{13}C-lipid (2 mg ml^{-1}); a7) ^{13}C-lipid (10 mg ml^{-1}); a8) ^{13}C-protein (2 mg ml^{-1}); a9) ^{13}C-protein (10 mg ml^{-1}); a10) ^{13}C-ISOGRO (2 mg ml^{-1}); a11) ^{13}C-ISOGRO (10 mg ml^{-1}); a12) positive control; b1) lambda DNA; b2) negative; b3) ^{13}C-carrier; b4) T_0 (time = 0; sample taken before amendment); b5) no substrate; b6) ^{12}C-urea; b7) ^{13}C-urea; b8) ^{12}C-bicarbonate; b9) ^{13}C-bicarbonate; b10) empty; b11) inhibitor test (positive plus sample); b12) positive control.

incubations. With carrier DNA alone (lane 1a-4), when no substrate has been added (lane 1a-5), or with ^{13}C-whole proteins in the incubations (lanes 1a-8 and 9), no crenarchaeal PCR signal was observed. For all SIP incubations, a minimum incubation time of 5 days was required to observe this unambiguous ^{13}C-uptake by crenarchaea. The ^{13}C-genome replication was detected at both high and low concentrations of ^{13}C-acetate, glycine, urea and the algal lipid/pigment extract. ^{13}C-DNA synthesis was only detected at higher concentrations for ^{13}C-ISOGRO. A summary of crenarchaeal ^{13}C-incorporation is presented in Table 1.

Bacterial ^{13}C-incorporation could also be detected in the SIP experiments. In 83% of the microcosms, bacterial ^{13}C-DNA synthesis was observed, except for the low concentrations of urea and ISOGRO (Table 2). The relatively long SIP incubation time (5 days) most likely allowed for extensive cross-feeding between the bacteria (Gallagher *et al.*, 2005) and the generation of ^{13}C-CO_2 (data not shown). To test whether the crenarchaea were actually taking up $^{13}CO_2$ rather than the ^{13}C-labeled substrates during our SIP experiment, a ^{13}C-bicarbonate incubation was also conducted. The results are presented in Figure 1b. The ^{13}C-bicarbonate treatment did not yield any crenarchaeal amplicon in the ^{13}C-band (lane 1b-9). A test for PCR inhibitors by spiking a positive DNA sample with ^{13}C-DNA from the bicarbonate incubation indicated no PCR suppression from this extract (lane 1b-11). These results demonstrate the SIP crenarchaeal signal resulted from the incorporation of ^{13}C-organics, not ^{13}C-labeled bicarbonate produced by bacterial respiration.

To ascertain which specific members of the crenarchaeal community were actively synthesizing DNA from the ^{13}C-carbon sources, TRFLP analysis of the 16S rRNA gene amplicons was performed (see example in Figure 2). A small number of crenarchaeal peaks (5–10) was found to dominate the overall community profiles for both the ^{12}C- and ^{13}C-bands (Supplementary Figures 1–4). For lipids, TRFs 66, 124, 220, 223 and 253 bp were present in the ^{12}C-fraction, but were not detected in the ^{13}C-fraction. Other TRFs (101, 116, 158, 251 bp) were detected in both ^{12}C- and ^{13}C-fractions, whereas the TRF 89 bp was mainly observed only in the ^{13}C-fraction. A compilation of all crenarchaeal fingerprints from our SIP experiments is provided in Figure 3. It can be seen that many of the ^{13}C-TRFs (89, 93, 116 and 158 bp) are detected in nearly all SIP experiments, with the exception of the 30 μM glycine, urea and acetate treatments (Figure 3a). There was a general pattern of lower peak area for the lower concentrations of ^{13}C-substrates and higher peak area for the higher concentration amendments for these particular TRFs. In contrast, other crenarchaeal TRFs were only detected in the ^{13}C-labeled community under specific treatments. For example, TRFs (124, 223, 253 bp) were found in the 30 μM acetate-amended microcosms, but did not appear to be active in any other treatment. Overall, the crenarchaeota present in the salt marsh sediment appear to be much less diverse (in terms of operational taxonomic units) than the bacteria. The average number of peaks at the lower TRFLP peak detection settings in the archaeal ^{13}C-community was 23 ± 5, whereas the bacterial ^{13}C-community profiles averaged 42 ± 14 (data not shown).

Screening of a clone library from the ^{13}C-bands yielded seven of the observed TRFLP peaks within our SIP profiles and have been highlighted in gray (Figure 2). Specifically, three clones matched the 101 bp peak (two unique sequences), five clones matched the 116 bp peak (three unique sequences), four clones matched the 124 bp peak (two unique sequences), one clone matched the 159 bp peak, four clones matched the 223 bp peak (two unique sequences), six clones matched the 251 bp peak (three unique sequences) and one clone matched the 253 bp peak. Three major peaks (66, 89 and 93 bp) were not detected in our clonal library. The cloned crenarchaeal 16S rRNA genes were sequenced, aligned and a phylogenetic tree was reconstructed using maximum likelihood methods (Figure 4). Seven of these sequenced clones grouped with uncultured crenarchaeota from marine environments, belonging to the Miscellaneous

Table 1 Summary of crenarchaeal TRFs representing at least 2% of the total community profile on the various ^{13}C-carbon amendments

TRF	*Acetate*		*Glycine*		*Urea*		*Lipids*		*Proteins*		*ISOGRO*		*Bicarbonate*
Length (bp)	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*	
58	+				+								
66	+				+								
76	+				+ + +								
78		+ +			+								
84			+		+								
89	+	+ +		+ + +	+	+ + +		+				+ + +	
91		+		+ +			+ + +	+ + +					
93					+	+	+	+ +				+ +	
101*	+	+ + +	+	+	+ + +	+	+					+	
112							+	+					
115*		+ + +	+	+ + +		+ + +	+ + +	+ + +				+ + +	
123*	+ +					+							
126	+		+										
136			+										
152			+										
158*		+ + +		+		+	+	+				+	
164					+								
166	+		+										
173								+					
178	+		+										
182	+		+										
188													
192	+		+										
207													
213			+										
217		+	+										
223*	+ +												
244	+		+										
250*			+ + +	+		+	+ +						
252*	+ + +											+	

Abbreviation: TRF, terminal restriction fragment.
The plus signs indicate: (+) 250–5000 arbitrary fluorescence units (a.f.u.); (+ +) 5000–10000 a.f.u.; (+ + +) >10 000 a.f.u. The asterisks (*) are TRFs represented in the clonal library.

Crenarcheotal Group (MCG-6 and MCG-8; Kubo *et al.*, 2012). The other seven clones detected in our clone library grouped with members of the Marine Group 1.a Crenarchaeota. Several of the peaks in our TRFLP profiles yielded more than one clone, suggesting that archaeal diversity may be more extensive than our profiles indicate, and additional enzyme digests are required for higher TRFLP resolution.

Discussion

SIP methodology has only recently been used to elucidate the metabolic potential of archaea. Most of these studies have focused on autotrophy and ammonia oxidation in various environments such as rice paddies (Lu and Conrad, 2005), soils (Adair and Schwartz, 2011; Pratscher *et al.*, 2011; Lu and Jia, 2013) and in freshwater sediment (Wu *et al.*, 2013). However, there are also reports of heterotrophic activity from an acidic fen (Hamberger *et al.*, 2008) and an estuarine setting in the UK (Webster *et al.*, 2010). The fen study utilized soil samples suspended in a minimal salt media, pre-incubated for 8 days and supplemented with ^{13}C-xylose or ^{13}C-glucose. After 13 days, most archaeal clones associated with the ^{13}C-fraction were methanogens, whereas 25% of the colonies screened ($n=16$) were identified as being crenarchaea. Likewise, in the Severn Estuary SIP study, various sediments were added to a minimal salt media and amended with ^{13}C-glucose, ^{13}C-acetate and $^{13}CO_2$ for up to 14 days (Webster *et al.*, 2010). There was no archaeal CO_2 uptake observed in sediment taken from the methanogenic zone, and after ^{13}C-glucose amendment the aerobic zones were highly similar in the ^{12}C- and the ^{13}C-fractions. However, in sediments from the sulfate-reducing zone amended with ^{13}C-acetate, active members of the Miscellaneous Crenarchaeal Group were detected, which were clearly enriched when compared with the ^{12}C-fraction.

In this report, SIP microcosms were made using filter-sterilized site water and the incubations were concluded at 5 days. The crenarchaeota of the Cheesequake salt marsh were found to be heterotrophic, with no ^{13}C-uptake from bicarbonate over the time frame of the experiment. Certain crenarchaeal TRFs were detected in the ^{13}C-fraction from nearly every microcosm amended with organic

Table 2 Summary of bacterial TRFs representing at least 2% of the total community profile on the various ^{13}C-carbon amendments

TRF	*Acetate*		*Glycine*		*Urea*		*Lipids*		*Proteins*		*ISOGRO*	
Length (bp)	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*	*Low*	*High*
63								+	+			
66							+					
89				+ +								
94							+					
101				+								
103				+						+		
106								+ +				
108				+								
113										+		
117							+					
119	+						+		+ +			+
126	+	+ +		+			+					
128												+
130	+			+						+		
134	+			+			+	+	+			
138								+				
142									+			
145							+					
148				+			+			+		+
150	+						+	+				
153										+		+
162	+			+								
164	+		+ + +			+ +		+	+ + +			
167			+			+ +		+		+		
169	+							+				
172							+					
177		+										
180	+	+		+			+					
182		+		+			+					+
186	+	+		+				+ +		+		
189							+	+ +				
192			+ + +	+								+ + +
198			+ + +				+					
200		+ + +		+				+				
204		+ + +	+			+			+			
206	+								+			
208								+ +		+		+
210	+			+			+			+		+
213	+			+			+			+		+
215								+				
216	+					+	+	+		+		+
219	+									+		
225				+						+		+
227								+				
235						+ +						
237												+
240	+		+	+				+	+			+
246						+ + +						
248	+							+				
250	+			+ +			+		+ + +	+ +		+ + +

Abbreviation: TRF, terminal restriction fragment.
The plus signs indicate: (+) 250–5000 arbitrary fluorescence units (a.f.u.); (+ +) 5000–10 000 a.f.u.; (+ + +) >10 000 a.f.u.

^{13}C-carbon, regardless of the type of substrate provided. This suggests these particular crenarchaea are able to assimilate a wide variety of organic substrates: simple metabolic intermediates (acetate), amino acids (glycine), organic nitrogen compounds (urea), lipids and pigments, and complex mixtures of biopolymers (ISOGRO). Other crenarchaeal TRFs, however, were only detected in the ^{13}C-bands from microcosms amended with a specific substrate (acetate or urea) and may have more restricted metabolic capabilities.

Interestingly, bacterial and crenarchaeal populations seem to be competing for the same resources in our SIP incubations. Members from both domains were active on acetate, glycine, lipids, and high levels of urea and ISOGRO (Tables 1–2). The only resource partitioning for active microbes was observed in microcosms amended with whole

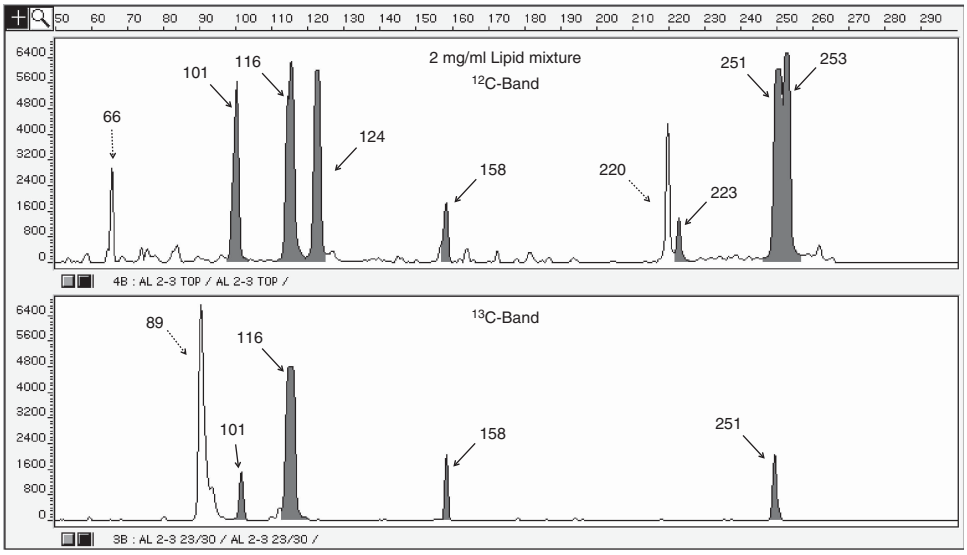

Figure 2 Example of TRFLP profiles of crenarchaeal 16S rRNA genes in ^{12}C- and ^{13}C-bands from lipid amendment. Highlighted peaks (gray) are represented in the clonal library.

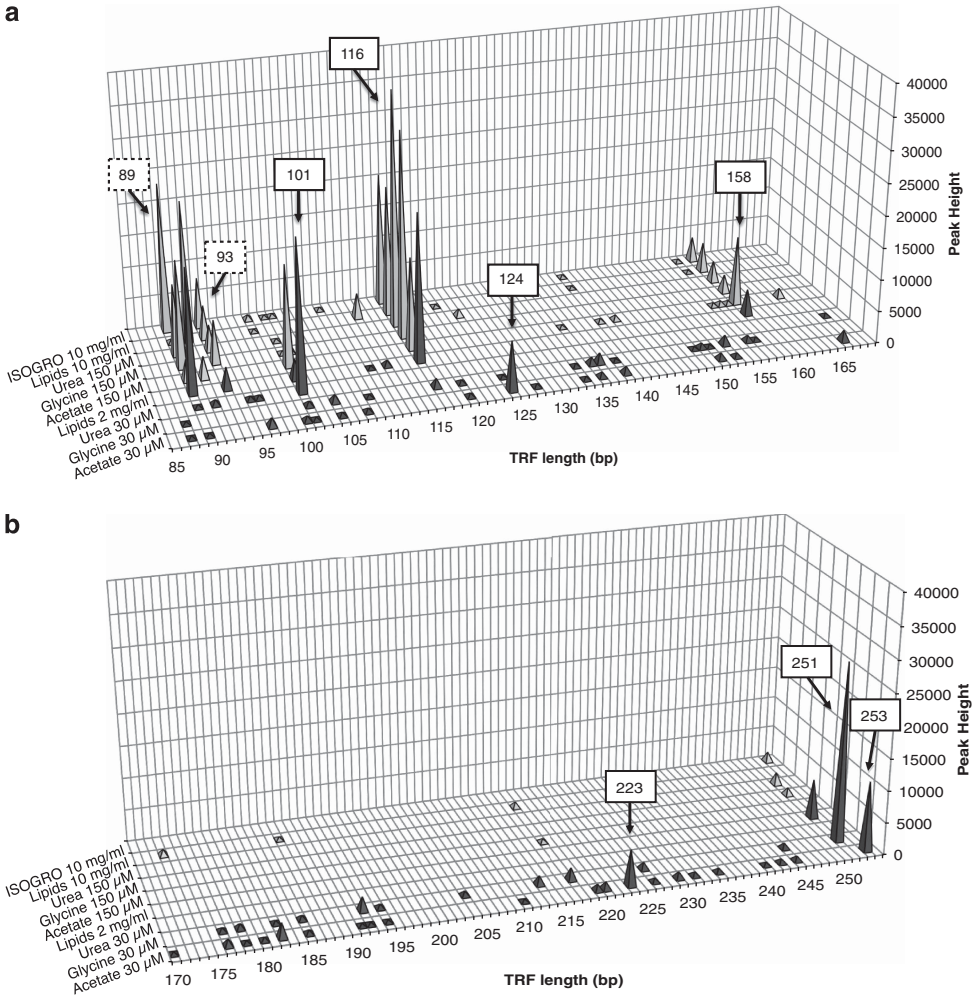

Figure 3 A compilation of crenarchaeal fingerprints with TRFs from 85 to 169 bp (**a**) and 170–254 bp (**b**) in length. Major peak sizes are indicated by boxes (solid, detected in the clone library; dashed, not detected). Light shades indicate higher concentration amendments; dark shades indicate lower concentration amendments.

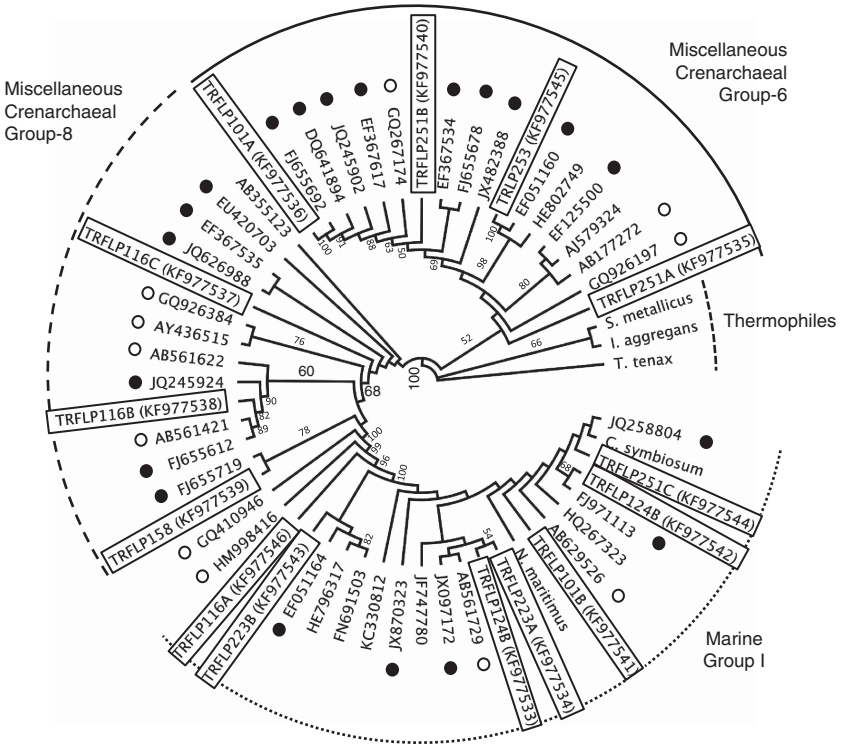

Figure 4 Phylogenetic estimation using Maximum Likelihood (PhyML) tree with support from 100 bootstrap runs indicated. The clones in this study are indicated with TRF size and GenBank accession numbers. Black dots represent sequences found in coastal or estuarine sediments. Open dots signify sequences from the marine deep biosphere.

proteins (used exclusively by bacteria) and at low concentrations of urea (used exclusively by crenarchaea). The former result was surprising, considering recent single-cell genome sequencing of a member of the Miscellaneous Crenarchaeal Group (MCG) from marine sediment discovered several genes for extracellular cysteine peptidases (Lloyd *et al.*, 2013). This finding underscores the need for direct incubations (such as SIP) to determine metabolic capabilities, in addition to assessing genetic potential by sequence analysis at the genome level. Likewise, it was surprising that bacteria did not take up any carbon from urea at low concentrations, whereas the crenarchaea did. There is ample evidence of bacteria possessing urease genes (Collier *et al.*, 2009; Solomon *et al.*, 2010) and reports of *Campylobacter nitrofigilis* isolated from *Spartina* with urease activity along the United States eastern seaboard (McClung *et al.*, 1983). While no bacterial activity was detected at 30 μM on urea, there was bacterial activity at 150 μM urea. This result implies the crenarchaea are taking up virtually all of the urea at the lower concentration, preventing the bacteria from accessing this substrate for heterotrophic (or autotrophic) growth.

While the type of ^{13}C-substrate influenced the crenarchae replicating their DNA, the concentration of ^{13}C-substrate also had a marked effect on the profiles of the active crenarchaeal community. Specifically, low concentrations of substrate produced more active TRFs compared with microcosms amended with high concentrations (Figure 5). Higher concentrations of acetate (150 μM) produced communities that were dominated by three TRFs (101, 116 and 158 bp) comprising 68% of the community profile. High concentrations of glycine (150 μM) yielded an active community in which two

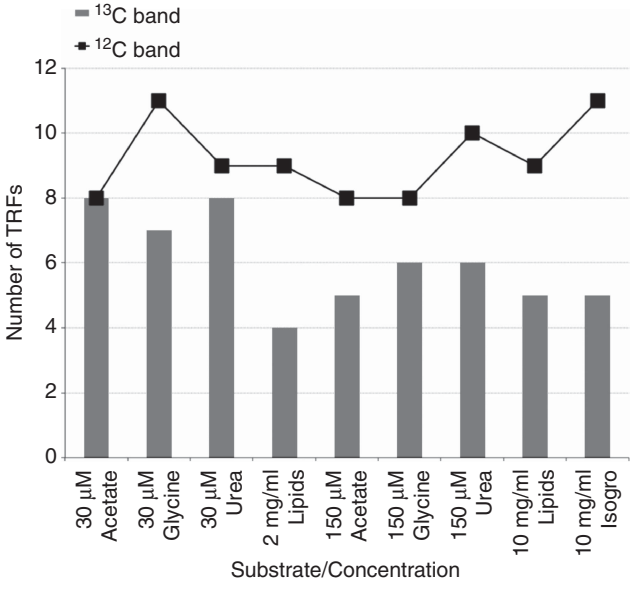

Figure 5 Total number of TRFs in the ^{12}C-band (line) and in the ^{13}C-band (gray bars) for peaks representing more than 2% of the community profile.

of the same TRFs (116 and 158 bp) accounted for 80% of the community profile, whereas 150 μM urea yielded 89 and 116 bp peaks, which also dominated the community profile (80%). There are several possible explanations for this result. One is that these particular crenarchaea are very successful at competing for substrates at high concentration and their transport systems internally mobilize virtually all of the substrate. This prevents other microorganisms from accessing the ^{13}C-organic carbon and synthesizing DNA. If this is the case, there must be a tipping point between low concentrations (tens of micromolar) and higher concentrations (hundreds of micromolar), which triggers a hoarding response by certain crenarchaea. Another possibility is that minor TRFs in the ^{13}C-carrier band are having their signal suppressed by the dominant TRF in the high concentration SIP experiment. Although our fingerprinting methods are highly reproducible (McGuinness *et al.*, 2006; Tuorto *et al.*, 2013), there is a suppression of smaller peaks in the TRFLP profile when screening clone libraries (Babcock *et al.*, 2007) or samples where one microorganism is >10% of the community. This same mechanism may be inhibiting the detection of other active crenarchaea that are not synthesizing large amounts of DNA in our SIP incubations. Another explanation is that low concentrations of some carbon compounds, such as urea, may not be bioavailable to the bacteria. Our low concentration ISOGRO additions did not produce any ^{13}C-archaeal or ^{13}C-bacterial signal. However, we know that 2 mg ml^{-1} of ISOGRO in liquid culture will allow for bacterial growth. It is possible that the salt marsh sediments are preventing the bioremineralization of proteins and other biomolecules at low ISOGRO concentrations, as has been reported by Keil *et al.* (1994). Alternatively, uptake of ^{13}C-substrates at low concentrations may be observed given a longer incubation.

From the incubation time of our SIP experiments, it is also possible to estimate crenarchaeal growth parameters *in situ*. Assuming two doubling events are required for complete ^{13}C-labeling of DNA and detection in our 100%-labeled ^{13}C-carrier band, this suggests salt marsh crenarchaea have a doubling time on the order of 2–2.5 days. Prior studies have measured the growth rate of cultured ammonia-oxidizing crenarchaea (Könneke *et al.*, 2005) and/or inferred doubling times by determining copy numbers of 16S rRNA (Park *et al.*, 2010). These reports indicate a maximum growth rate of ammonia oxidizing archaea (AOA) in liquid medium at 25–28 °C between 0.57 and 0.78 per day (or a doubling rate between 1.28 and 1.75 days). In contrast, our findings are consistent with prior observations of slightly slower growth rates in sediment (0.2 per day, Mosier *et al.* (2012)). However, these results may be an overestimate of the potential growth rate, as SIP requires that all precursor pools for DNA synthesis become uniformly labeled with ^{13}C for detection.

In conclusion, many mesothermal Crenarchaea (Thaumarchaea) in salt marsh sediments were found to assimilate a wide variety of organic ^{13}C-substrates to replicate their genomes. Other crenarchaea were much more selective in the ^{13}C-carbon sources used for growth. The SIP approach demonstrates salt marshes can be used for studying crenarchaeal metabolic capabilities and may help in determining laboratory conditions for isolating these heterotrophic microorganisms. Once in pure culture, traditional microbiological techniques can be utilized to improve our understanding of crenarchaeal physiological capabilities. Finally, experiments that directly link carbon-utilization patterns with specific members of the microbial community can provide insight into the competitive relationships between crenarchaea and bacteria and improve our understanding of microbial ecology.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

The authors wish to thank Dr S.K. Rhee for his preliminary work on culturing Crenarchaeota in our lab and the Cheesequake State Park rangers for their cooperation during this project. This research was partially supported by funds from Rutgers University and from the National Science Foundation (1131022) in Ocean Technology and Interdisciplinary Coordination to LJK and Dr Jingang Yi.

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