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Microbial Community Analysis of a Coastal Hot Spring in Kagoshima, Japan, Using Molecular- and Culture-based Approaches

Minako Nishiyama, Shuichi Yamamoto, and Norio Kurosawa*

Department of Environmental Engineering for Symbiosis, Faculty of Engineering, Soka University, Tokyo, 192-8577, Japan

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Ibusuki hot spring is located on the coastline of Kagoshima Bay, Japan. The hot spring water is characterized by high salinity, high temperature, and neutral pH. The hot spring is covered by the sea during high tide, which leads to severe fluctuations in several environmental variables. A combination of molecular- and culture-based techniques was used to determine the bacterial and archaeal diversity of the hot spring. A total of 48 thermophilic bacterial strains were isolated from two sites (Site 1: 55.6°C; Site 2: 83.1°C) and they were categorized into six groups based on their 16S rRNA gene sequence similarity. Two groups (including 32 isolates) demonstrated low sequence similarity with published species, suggesting that they might represent novel taxa. The 148 clones from the Site 1 bacterial library included 76 operational taxonomy units (OTUs; 97% threshold), while 132 clones from the Site 2 bacterial library included 31 OTUs. Proteobacteria, Bacteroidetes, and Firmicutes were frequently detected in both clone libraries. The clones were related to thermophilic, mesophilic and psychrophilic bacteria. Approximately half of the sequences in bacterial clone libraries shared <92% sequence similarity with their closest sequences in a public database, suggesting that the Ibusuki hot spring may harbor a unique and novel bacterial community. By contrast, 77 clones from the Site 2 archaeal library contained only three OTUs, most of which were affiliated with Thaumarchaeota.

Keywords: bacteria, archaea, diversity, coastal hot spring

Introduction

It has been noted that only a small fraction (<1%) of the microorganisms found in nature can be cultured using traditional cultivation methods (Amann *et al*., 1995; Pace, 1997). Thus, the rapid development of culture-independent molecular techniques has revolutionized our perspective on microbial diversity and ecology over recent decades (Pace, 1997; Hugenholtz *et al*., 1998; Rappe and Giovannoni, 2003). The construction of clone libraries of 16S rRNA phylogenetic markers, based on the direct amplification of genes in environmental samples using the polymerase chain reaction (PCR), is one of the most widely used methodologies for investigating microbial diversity (Amann *et al*., 1995; Hugenholtz *et al*., 1998; Benlloch *et al*., 2001). This new approach has led to the discovery of numerous novel microbial taxa in bacterial and archaeal communities, especially those in geothermal areas (Delong and Pace, 2001; Rappe and Giovannoni, 2003; Schleper *et al*., 2005).

Ibusuki hot spring is located on the coastline of Kagoshima Bay within the Ata caldera in southwest Japan. The geothermal system is related to Quaternary volcanism, which formed a sequence of craters and cones in the old Ata caldera (Hatae *et al*., 1965). Hot, saline water emerges from below the ground near the shore in this area. There are approximately 800 hot spring outlets in Ibusuki city alone (Sakamoto *et al*., 1993) and the total volume discharged is approximately 30,000 kL/day (Tsuyuki, 1976). Previous geochemical studies have suggested that the hot spring water is not a simple mixture of local meteoric water and seawater. The origin of the water is up to several hundred meters deep underground (Akaku *et al*., 1991) and the major chemical components are derived from the interaction between seawater and heated rocks in the thermal systems (Matsubaya *et al*., 1973; Ikemi and Chiba, 1993; Oi *et al*., 1996). Thus, the hot spring water is generally deficient in Na^{+}, Mg^{2+}, and SO_4^{2-}, but enriched with K^{+} and Ca^{2+} compared with seawater. The physicochemical properties of the hot spring water is relatively stable at each site, but the water temperature and the concentrations of the major dissolved components differ significantly among sites (Matsubaya *et al*., 1973; Oi *et al*., 1996).

Another interesting characteristic of the hot spring is that some of the outlets are located in the intertidal zone, which is covered by the sea during high tide. Therefore, microorganisms present in the hot spring may be subjected to severe fluctuations in the levels of several environmental variables, such as temperature, light intensity, oxygen concentration, and salinity. All these unique characteristics suggest that the Ibusuki hot spring may contain microbial communities different from those described previously in other thermal systems. Indeed, some novel bacterial species have been isolated from the region (Nakagawa *et al*., 2004; Iizuka *et al*., 2006). No archaeal species have yet been isolated from the Ibusuki hot spring, but other studies suggest that ammonia-oxidizing archaea (AOA) are abundant in intermediate salinity environments, such as an estuary (Beman and Francis, 2006; Dang *et al*., 2008; Bernhard *et al*., 2010). AOA have also been frequently detected from many terrestrial and marine

*For correspondence. E-mail: kurosawa@soka.ac.jp; Tel. & Fax: +81-42- 691-8175

hot springs around the world (Zhang *et al.*, 2008; Wang *et al.*, 2009). Nevertheless, the microbial diversity of this unique environment has remained unexplored.

This study was conducted to determine the bacterial and archaeal diversity of the Ibusuki hot spring using a combination of molecular- (16S rRNA gene clone library analysis) and culture-based approaches. This combination of both methods may lead to a more comprehensive characterization of the microbial diversity present in the environment and identify novel microbial species.

Materials and Methods

Study site and sampling procedure

Sampling was conducted at two sites (Site 1 and Site 2) on Yunohama beach (31°13′32″N, 130°39′18″E), Ibusuki, Kagoshima, Japan, during the low tide in March, 2011 (Fig. 1). The sampling sites were submerged beneath the sea during the high tide period and they reappeared on the beach for only a few hours twice each day. Both sampling sites were approximately 1 m from the shore at the time of sampling, but were not covered by the waves. The distance between the two sites was approximately 10 m. At both sites, hot spring water emerged continuously from underground, creating small hot water pools and channels toward the sea. Water samples and sediment water samples were collected aseptically at a depth of 10 cm in the hot water pool at each site. The water temperature was measured *in situ*. The samples were transported to the laboratory at ambient temperature and part of the water samples was immediately filtered through a 0.2-μm membrane filter (Corning International, USA), followed by measurement of the pH and salinity. The water temperature was higher at Site 2, while the pH and salinity were similar at both sites: Site 1: 55.6°C, pH 6.9, and salinity 1.5%; Site 2: 83.1°C, pH 6.9, and salinity 1.3%. The sediment water samples were stored at 4°C until further analysis.

Isolation and characterization of microbes

Isolation and purification were conducted using modified Brock's basal salts (MBS) medium containing the following constituents per L of deionized water: 1.30 g $(NH_4)_2SO_4$, 0.20 g KH_2PO_4, 0.25 g $MgSO_4{\cdot}7H_2O$, 0.07 g $CaCl_2{\cdot}2H_2O$, 2.00 mg $FeCl_3{\cdot}6H_2O$, 1.80 mg $MnCl_2{\cdot}4H_2O$, 4.50 mg $Na_2B_4O_7{\cdot}10H_2O$, 0.22 mg $ZnSO_4{\cdot}7H_2O$, 0.05 mg $CuCl_2{\cdot}2H_2O$, 0.03 mg $VOSO_4{\cdot}2H_2O$, and 0.01 mg $CoSO_4{\cdot}7H_2O$, which was supplemented with 0.1% (w/v) yeast extract and 1.5% (w/v) sea salts. The pH of the MBS medium was adjusted to 6.9 with NaOH. For plate cultivation, 1.2% (w/v) gellan gum (Wako, Japan) was added to the MBS medium. A 100-μl aliquot of the hot spring water from two sediment water samples was spread on to plates. The plates were incubated at 60°C for Site 1 sample and at 75°C for Site 2 sample for up to 3 days. Single colonies were picked and purified by repeated streaking on plates. Some colonies isolated from Site 1 sample did not grow fast at 60°C, so they were incubated at 50°C in a further experiment.

The enzymatic activities of the isolates were examined using MBS medium gellan gum plate containing 1% (w/v) microcrystalline cellulose (MCC) (cellulose microcrystalline; Merck, Germany), 0.5% (w/v) colloidal chitin, 2% (w/v) xylan (xylan from oat spelt; Sigma, USA), 1% (w/v) starch (starch, soluble; Wako), or 1% (w/v) skim milk (skim milk powder, Wako). Cells were inoculated onto plates and incubated at the same temperature used for isolation/purification (50, 60, or 75°C) for up to 7 days. The substrate degrading activities were detected based on halo formation.

Cells grown in MBS medium were resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing Triton X-100 (0.2%, w/v) and heated at 70°C for 5 min, followed by DNA extraction using a DNA extraction machine (GC12, Precision System Science, Japan). The 16S rRNA gene was amplified by PCR using the primers B27F (5′-AGAGTTTGATCMTGGCTCAG) and U1492R (5′-GGYTACCTTGTTACGACTT). The 50-μl reaction mixtures contained 0.5 μM of each primer, 25 μl of Premix Ex *Taq* DNA polymerase (TaKaRa Bio, Japan), and 2 μl of genomic DNA. The thermal cycle was performed using an iCycler (Bio-Rad, USA) and the PCR cycle consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 1 min 40 sec, and a final extension step at 72°C for 2 min. The sequences obtained were aligned automatically using the CLUSTALW program in GENETYX Version 12.1.0 (Genetyx, Japan), and manually checked or edited when necessary (Thompson *et al.*, 1994). The sequences were compared to the closest relatives in the DNA Data Bank of Japan (DDBJ) using the nucleotide Basic Local Alignment Search Tool (BLASTN) program (Altschlul *et al.*, 1990). The sequences were categorized into groups using a 98.7% similarity threshold (Stackebrandt and Ebers, 2006).

Community DNA extraction and purification

Community DNA was extracted from the sediment water

Fig. 1. Sampling site. Ibusuki hot spring in Kagoshima, Japan. The hot spring water emerges from underground near the shore, creating a small hot spring pool and channels (circle).

samples using a DNA extraction machine or a DNA extraction kit (UltraClean Soil DNA Mega Prep Kit, MO BIO Laboratories, USA). The sample tube was shaken vigorously to remove microbial cells from the sediment in the water samples. The supernatant water was centrifuged at 15,000 rpm for 3 min at 4°C. A 5-ml sample of filtered seawater was added to the tube and the same procedure was repeated five times. The cells were resuspended in 100 µl of TE buffer containing Triton X-100 and heated at 70°C for 5 min, followed by DNA extraction using the DNA extraction machine. The extracted DNA was used for bacterial community analysis. For the archaeal community analysis, sufficient DNA for PCR could not be extracted using the procedure described above. Therefore, community DNA was extracted directly from the sediments (1.0 g) using the DNA extraction kit, according to the manufacturer's instructions.

The extracted DNA was purified using GFX PCR DNA and a Gel Band Purification Kit (GE Healthcare, USA), according to the manufacturer's instructions, and used for PCR amplification of the 16S rRNA gene.

PCR amplification and construction of 16S rRNA gene clone libraries

The bacterial 16S rRNA gene was amplified using the primers B27F and U1492R, while the archaeal 16S rRNA gene was amplified using the primers A21F (5′-TTCCGGTTGA TCCYGCCGGA) and U1492R. The 25-µl reaction mixtures contained 0.5 µM of each primer, 12.5 µl of Premix Ex *Taq* DNA polymerase, and 1 µl of community DNA. The thermal cycle was performed using an iCycler and the PCR cycle for primer pair B27F-U1492R consisted of initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 72°C for 1 min 40 sec, and a final extension step at 72°C for 2 min. The following conditions were used for primer set A21F-1492R: initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 2 min, with a final extension at 72°C for 5 min. The amplified DNA was purified using the GFX Kit mentioned above.

The 16S rRNA gene fragments obtained by PCR were cloned into the pT7 Blue T-vector (Novagen, Germany) and the recombinant plasmids produced were transformed into *Escherichia coli* DH5α (TaKaRa Bio). The transformants were plated on LB plates containing 100 µg/ml ampicillin (Wako), 40 µg/ml X-gal (TaKaRa Bio), and 0.5 mM IPTG (TaKaRa Bio). Blue/white selection was conducted where individual white colonies were picked randomly and subcultured in 100 µl of LB medium containing 100 µg/ml ampicilin using a 96-well plate at 37°C overnight. The inserted 16S rRNA gene in the plasmid was amplified by PCR using 1 µl of the culture as the template with the primers T7P-F (5′-TAATACGACTCACTATAGGG) and T7U-R (5′-GTT TTCCCAGTCACGA CGT) for bacteria and A21F and U1492R for archaea. The PCR cycle for the primer pair T7P-F-T7U-R consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 51°C for 30 sec, and extension at 72°C for 2 min, with a final extension step at 72°C for 5 min.

Phylogenetic and statistical analysis of the 16S rRNA gene sequences

The sequences obtained were aligned automatically using the CLUSTALW program in GENETYX Version 12.1.0, and manually checked or edited when necessary. The sequences were grouped into operational taxonomic units (OTUs) using a 97% similarity threshold (Rohwer *et al*., 2002; Shaw *et al*., 2008). A representative 16S rRNA gene sequence from each OTU was used for further analysis. The sequences were screened manually for potential chimeric artifacts by comparing the phylogenetic affiliations of their 5′ and 3′ ends. Nonchimeric sequences (in the range of 461–533 bp for bacterial clones and 471–491 bp for archaeal clones) were compared to their closest relatives in the DDBJ database using BLASTN. Phylogenetic analyses were conducted using GENETYX Version 12.1.0 and Molecular Evolutionary Genetics Analysis (MEGA) version 5.0 (Tamura *et al*., 2011). About 470 bp were used for analysis both for bacteria and archaea. Phylogenetic trees were constructed using the neighbor-joining method with the Kimura-2 model. The bootstrap values were calculated based on 1,000 replicates.

The Shannon-Wiener diversity index (Magurran, 2004), Chao1 nonparametric richness estimator (Chao, 1987), and abundance-based coverage estimator of species richness (ACE; Chao and Lee, 1992) were calculated, and rarefaction curves were constructed using EstimateS Version 7.5 (Colwell, 2009). The coverage of the clone libraries was calculated as $C = 1 - (n/N)$, where n is the number of unique sequences in the sample, and N is the total number of sequences (Good, 1953; Singleton *et al*., 2001).

Gene accession numbers

The sequences were deposited in the DDBJ database as follows: isolated strains (AB761387–AB761392), Bacteria-related clones (AB703469–AB703575), and Archaea-related clones (AB703576–AB703578).

Results and Discussion

Isolation and characterization of microbes

A total of 48 strains were isolated as pure cultures from the hot spring samples. Starch- and casein-degrading activities were detected in 31 and six strains, respectively. No MCC-, colloidal chitin-, nor xylan-degrading activities were detected within 7 days. The 48 isolates were categorized into six groups designated as Groups A–F based on their 16S rRNA gene sequence similarity (Table 1). They were all associated with bacterial divisions, and no archaeal species were isolated in this study.

The majority of the isolates (34 strains in Groups D and E) were closely related (>97% sequence similarity) to *Rhodothermus marinus*, a thermophilic, halophilic, heterotrophic bacterium that has been isolated from marine hot springs around the world (Alfredsson *et al*., 1988; Nunes *et al*., 1992; Moreira *et al*., 1996; Sako *et al*., 1996). However, many of them (30 strains in Group D) had a 16S rRNA gene sequence similarity below the species threshold value (98.7%; Stackebrandt and Ebers, 2006), suggesting that they might repre-

Table 1. Summary of the isolates from the Ibusuki hot spring. Amylase and caseinase activities were detected by the halo formation on plates and are described as a percentage or - (negative).

Sampling point	Group	Rep. strain (Accession No.)	Number of strains	Amylase	Caseinase	Closest species (Accession No.)	Sequence similarity (%)
Site 1	A	IBS-6001 (AB761387)	3	-	-	*Albidovulum inexpectatum* (AF465833)	100
	B	IBS-6004 (AB761388)	2	-	100%	*Bacillus firmus* (D16268)	94.8
	C	IBS-6005 (AB761389)	1	-	100%	*Thermoactinomyces vulgaris* (AF138739)	100
Site 2	D	IBS-7004 (AB761391)	30	93%	-	*Rhodothermus marinus* (AF217494)	97.4–97.8
	E	IBS-7010 (AB761392)	4	75%	-	*Rhodothermus marinus* (AF217494)	99.5
	F	IBS-7002 (AB761390)	8	-	38%	*Thermus thermophilus* (X07998)	99.6

sent novel species. Two isolates in Group B also had a distant phylogenetic relationship (<95%; genus threshold; Ludwig *et al.*, 1998; Kamke *et al.*, 2010) to the nearest published species *Bacillus firmus*, which is a mesophilic, alkaline-tolerant bacterium (Guffanti *et al.*, 1980), suggesting that they may represent novel taxa, although further experiments are required to confirm their taxonomic positions. Other isolates, i.e., three in Group A, one in Group C, and eight in Group F, were closely related to *Albidovulum inexpectatum*, *Thermoactinomyces vulgaris*, and *Thermus thermophilus*, respectively (Table 1). *A. inexpectatum* is a slightly thermophilic, non-photosynthetic bacterium previously isolated from marine hot springs (Albuquerque *et al.*, 2002). *T. vulgaris* has been isolated from various environments such as soil, sediments, and composts (Flockton and Cross, 1975; Shoreit, 1992). Their thermostable enzymes have been investigated intensively, including amylases and proteases (Hausdorf *et al.*, 1980; Ohtaki *et al.*, 2006; Yang *et al.*, 2009). However, only casein-degrading activities were detected in this study. *T. thermophilus* is a widely distributed thermophilic bacterium that was previously isolated from inland hot springs, shallow marine hot springs, and even from a deep sea hydrothermal vent (Oshima and Imahori, 1974; Marteinsson *et al.*, 1999). The isolation of these species from the Ibusuki hot spring indicates that they may be geographically widely distributed in hot and saline environments. Our enzymatic activity test showed that 5 of the 6 groups of isolated strains had either amylases or caseinases that were stable at high temperature. These physiological characteristics of the isolates indicate that heterotrophic aerobic bacteria are important components that degrade polysaccharides in shallow hydrothermal systems, as previously reported in the Mediterranean Sea (Lucila *et al.*, 1996).

Diversity and novelty of the microbial communities

Bacterial 16S rRNA gene clone libraries were successfully constructed from both sampling sites. A total of 148 clones from the Site 1 bacterial library contained 76 OTUs (97% threshold), while 132 clones from the Site 2 bacterial library contained 31 OTUs. An archaeal 16S rRNA gene clone library was constructed only from Site 2 and the 77 clones contained three OTUs. Good's coverage and rarefaction curves indicated that the natural archaeal communities were sufficiently covered by our sequencing effort, although the true diversity of bacterial communities may be higher than that measured in this study (Table 2 and Fig. 2). Statistical analyses using the Shannon-Wiener diversity index, Chao 1 richness estimator, and ACE suggested that Site 1 had a higher bacterial diversity than Site 2 (Table 2). This was in agreement with previous findings, which state that the bacterial diversity decreases with increasing temperature (Miller *et al.*, 2009). Archaeal communities were less diverse than the bacterial ones (Table 2). Although careful interpretation is needed to compare the diversity index with other studies that have used different methodologies, the bacterial diversity in Ibusuki hot spring (Shannon-Weiner index: 2.3–3.9) appeared to be much higher than that (0.2–1.9) in other saline geothermal systems (Jing *et al.*, 2006; Tobler and Benning, 2011) and it was closer to that (3.0–3.8) in terrestrial hot springs (Elshahed *et al.*, 2003).

All archaeal clones shared >98% sequence similarity with the nearest 16S rRNA gene sequences deposited in the DDBJ database. However, many of the bacterial clones had only distant sequence similarity with DDBJ entries. Approximately 41% (Site 1) and 52% (Site 2) of the bacterial clones in our libraries shared <92% similarity with previously characterized sequences. This level of novelty was similar to that in the coral-associated bacterial communities described by Rohwer *et al.* and much higher than that observed in other marine environments (Rohwer *et al.*, 2002). Furthermore, 7% (Site 1) and as many as 19% (Site 2) of the sequences were distantly (<78% sequence similarity) related to any described bacterial species. This suggests that some members of the bacterial communities in the Ibusuki hot spring are highly novel and they might represent new bacterial divisions. The unique environmental conditions found in the Ibusuki hot spring may explain the novelty and diversity of the bacterial communities in the area.

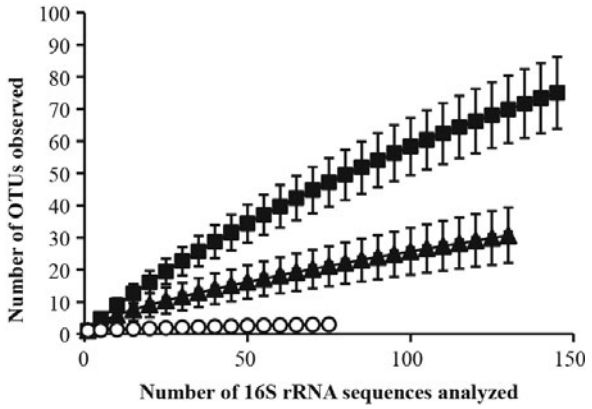

Fig. 2. Rarefaction curves for Site 1 bacterial (■), Site 2 bacterial (▲), and Site 2 archaeal (○) OTUs in 16S rRNA gene clone libraries. The lines represent 95% confidence intervals.

Table 2. Coverage and diversity indices for bacterial and archaeal 16S rRNA gene clone libraries from Site 1 (55.6°C) and Site 2 (83.1°C). Archaeal 16S rRNA genes were not detected from Site 1. OTU was determined by 97% sequence similarity cut-off.

Domain	Sample	No. of clones	No. of OTUs	Coverage (%)	Shannon	Chao 1	ACE
Bacteria	Site 1	148	76	67.6	3.87	132	146
	Site 2	132	31	84.1	2.31	73	115
Archaea	Site 2	77	3	98.7	0.19	3	4

In this study, different DNA extraction methods were used for the bacterial and archaeal analyses because sufficient DNA was not obtained for PCR amplification of the archaeal 16S rRNA gene using the bacterial analysis method. Furthermore, an archaeal clone library was constructed only from Site 2, which had a very low diversity community compared with the bacterial clone libraries. These results indicate that archaea may not be the dominant microorganisms in the Ibusuki hot spring, or that the PCR condition used in this study may not be suitable for detecting the archaeal communities present in the hot spring.

Bacterial community composition and phylogenetic analysis

Despite the big differences in water temperature (~30°C) and the estimated diversity (Table 2), the two bacterial 16S rRNA gene clone libraries had similar community structures (Fig. 3). This might be explained by the similarities of other environmental variable, such as the pH, salinity, and the chemical composition of the water, which originated from a common geographical source.

Proteobacteria constituted the most abundant phylum (Site 1: 58%; Site 2: 42% of the sequences). In this group, the alpha-, gamma-, and delta-proteobacteria subclasses were detected frequently in both clone libraries (Fig. 3). Alpha- and gamma-proteobacteria are the major bacterial components found in coastal seawater (Na *et al*., 2011), where they play important roles in organic decomposition. The predominance of these phyla has been reported in other studies of saline geothermal waters (Petursdottir *et al*., 2009; Tobler and Benning, 2011). Furthermore, Petursdottir *et al*. (2009) reported that the Rhodobactereaceae family, which is a major bacterial lineage, especially in coastal waters, was predominant in a saline geothermal lagoon in Iceland. This was also true for the Ibusuki hot spring (Fig. 4). These results suggests that the microbial communities found in coastal geothermal ecosystems, including the Ibusuki hot spring, are composed primarily of members with a marine origin. Over half of the alpha-proteobacterial 16S rRNA genes were closely related to cultured species (>97% sequence similarity) that were previously isolated from marine and terrestrial environments, such as sea water, marine biofilms, and soil (Fig. 4). Surprisingly, the described growth temperatures of these species, with the exception of *Albidovulum inexpectatum*, were lower than 45°C (the temperatures of both sampling sites were above 55°C) and one species (*Octadecabacter antarcticus*: U14583) was a psychrophile that was originally isolated from Antarctic sea ice (Gosink *et al*., 1997; Pukall *et al*., 1999; Yoon and Oh, 2005; Kwon *et al*., 2007; Yoon *et al*., 2009; Vandecandelaere *et al*., 2009a, 2009b). It is possible that contaminated DNA from the adjacent seawater was amplified by PCR and that it comprised a proportion of the clone libraries. However, a previous investigation of the microbial ecology of intertidal hot springs based on *in situ* enrichments clone analysis indicated that mesophilic and pyschrophilic marine-related microorganisms might develop a tolerance to heat (Hobel *et al*., 2005). Thus, there is a possibility that those found in the Ibusuki hot spring had also developed resistance to heat and were naturally present in the hot spring. One sequence (IBS1-009) was closely related (97.3% sequence similarity) to *Albidovulum inexpectatum*, which was isolated using the culture-based approach in this study. The gamma-proteobacterial genes were related to marine environmental clones or only distantly (<92% sequence similarity) related to previously characterized species. One beta-proteobacteria-related sequence (IBS2-001) was affiliated with *Ralstonia pickettii*, a typical pathogen that causes respiratory tract infections in patients with cystic fibrosis (Coenye *et al*., 2002). Most of the delta-proteobacterial genes were related to uncultured sequences that were previously retrieved from marine environments. Many unclassified Proteobacteria-related genes were also detected. They were related to marine, hydrothermal, or terrestrial hot spring clones.

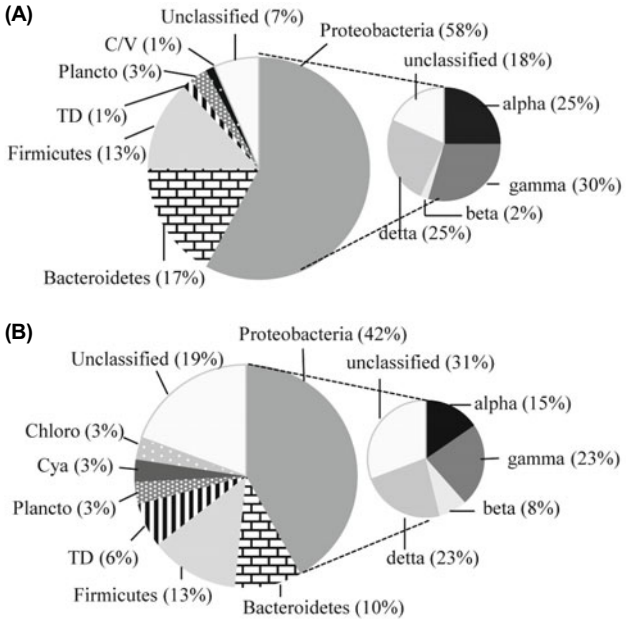

Fig. 3. Community structure of the two clone libraries. (A) Site 1 bacterial library. (B) Site 2 bacterial library. The larger circle represents the phylotype composition at the phylum level, while the smaller inset circle represents the phylotype composition of Proteobacteria subdivisions. The proportion of phylotypes is described as a percentage. TD, Thermodesulfobacteria; Plancto, Planctomycetes; C/V, Chlamydiae/ Verrucomicrobia group; Chloro, Chloroflexi; Cya, Cyanobacteria.

Bacteroidetes and Firmicutes were the second and third

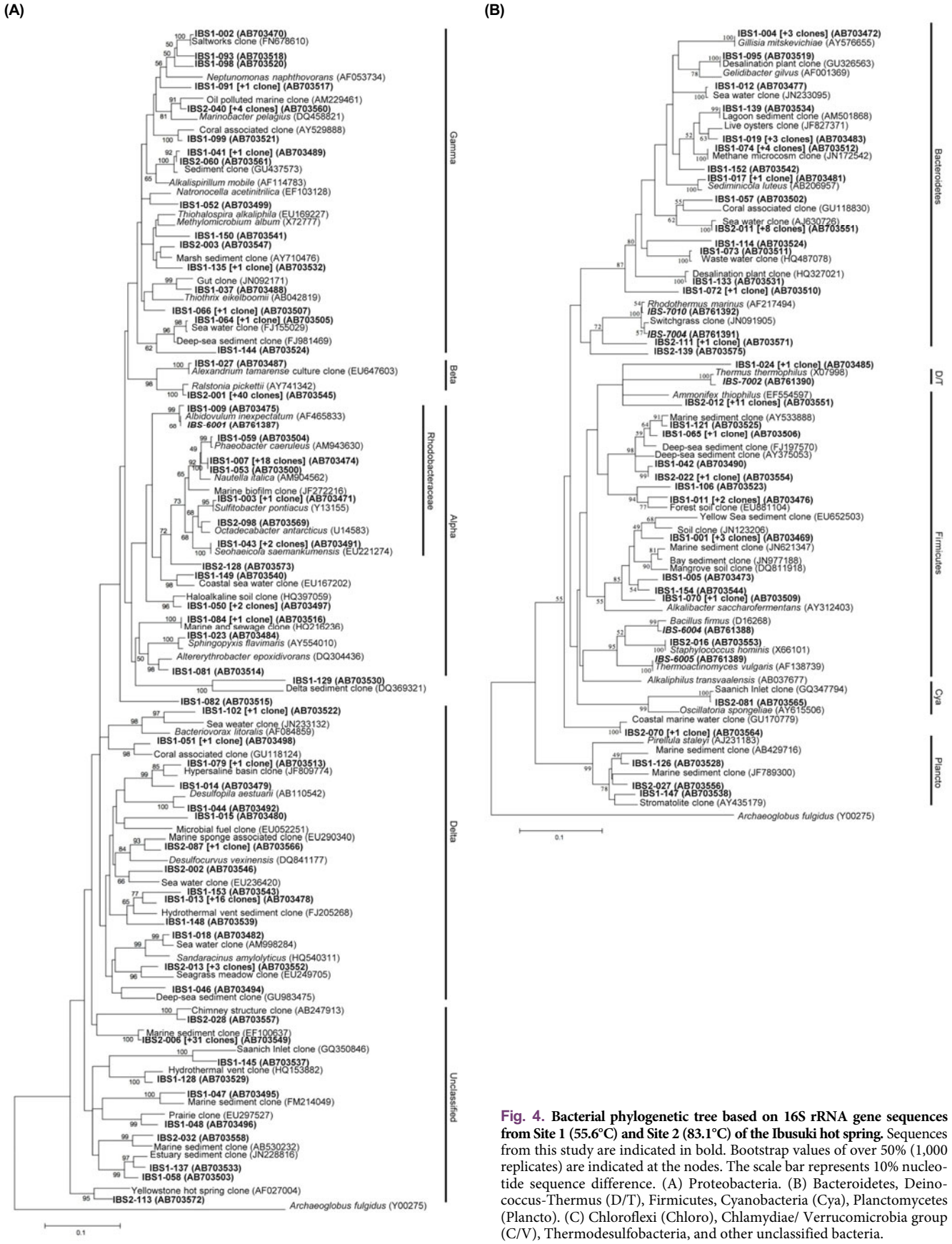

Fig. 4. Bacterial phylogenetic tree based on 16S rRNA gene sequences from Site 1 (55.6°C) and Site 2 (83.1°C) of the Ibusuki hot spring. Sequences from this study are indicated in bold. Bootstrap values of over 50% (1,000 replicates) are indicated at the nodes. The scale bar represents 10% nucleotide sequence difference. (A) Proteobacteria. (B) Bacteroidetes, Deinococcus-Thermus (D/T), Firmicutes, Cyanobacteria (Cya), Planctomycetes (Plancto). (C) Chloroflexi (Chloro), Chlamydiae/ Verrucomicrobia group (C/V), Thermodesulfobacteria, and other unclassified bacteria.

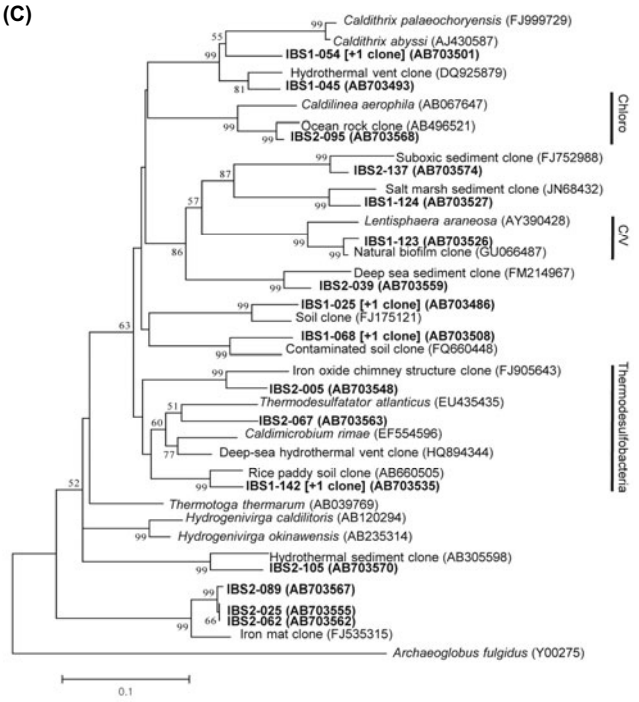

Fig. 4. Continued

most abundant phyla in the 16S rRNA gene clone libraries (Site 1, 17% and 13%; Site 2, 10% and 13% of the sequences, respectively) (Fig. 3). Many of the genes were related to uncultured sequences that were previously detected in saline environments such as seawater, marine sediments, and desalination plants (Fig. 4). Two Bacteroidetes-related sequences from Site 1 (IBS1-004 and IBS1-017) and one Firmicutes-related sequence from Site 2 (IBS2-016) were affiliated with cultured species, but they were also mesophilic bacteria (Kloos and Schleifer, 1975; Nedashkovskaya *et al.*, 2005; Khan *et al.*, 2006). One sequence from Site 2 (IBS2-111) was distantly related (91% sequence similarity) to *Rhodothermus marinus*, which was the major isolate in this study. Bacteroidetes is the most dominant heterotroph in marine ecosystems after Proteobacteria (Stevens *et al.*, 2005). The predominance of heterotrophs in the Ibusuki hot spring suggests that the microbial community in the hot spring plays a significant role in the mineralization of organic compounds in the hot intertidal zone.

The sulfate reducer Thermodesulfobacteria was detected in both clone libraries, although it was more abundant in the Site 2 bacterial library (Fig. 3). Thus, chemoautotrophic or chemoorganotrophic sulfur reduction might occur in the Ibusuki hot spring. The three genes detected (IBS1-142, IBS2-005, and IBS2-067) were distantly related to known DDBJ sequences (<94% sequence similarity), suggesting that they may represent new genera within Thermodesulfobacteria (Fig. 4).

Planctomycetes, a budding, peptidoglycan-lacking bacterial group that is widely distributed in aquatic and terrestrial habitats (Wagner and Horn, 2006), was also detected in both clone libraries (Fig. 3). Some members of the Planctomycetes were recently recognized as anaerobic ammonia oxidation (anammox) bacteria that contribute significantly to the oceanic nitrogen cycle (Strous *et al.*, 1999). However, the clones IBS1-126, IBS1-147, and IBS2-027 detected in the Ibusuki hot spring may not be involved with anaerobic ammonia oxidation, because they were related to marine aggregate clones rather than anammox clones.

There was no significant differences between the two bacterial community structures, but some of the minor phylotypes were unique in each library. One Chlamydiae/Verrucomicrobia group-related gene (IBS1-123) was detected

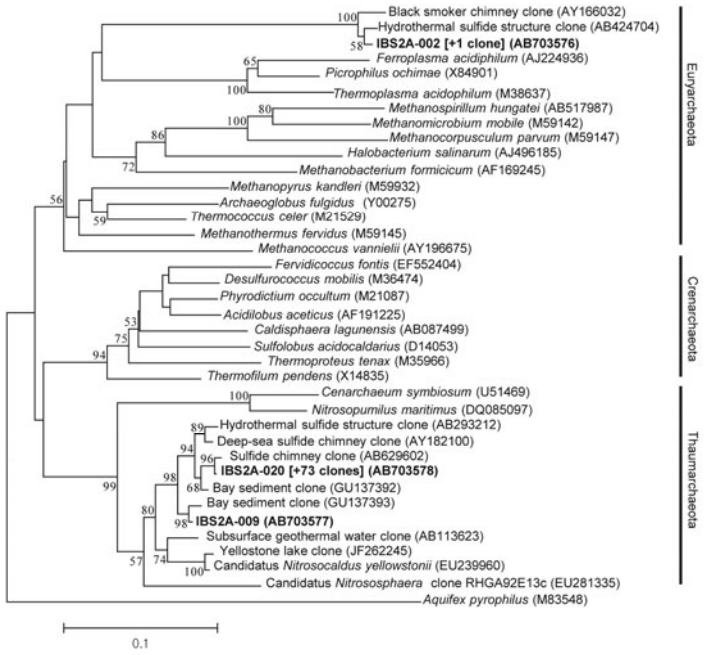

Fig. 5. Archaeal phylogenetic tree based on 16S rRNA gene sequences from Site 2 (83.1°C) of the Ibusuki hot spring. Sequences from this study are indicated in bold. Bootstrap values of over 50% (1,000 replicates) are indicated at the nodes. The scale bar represents 10% nucleotide sequence difference.

only in the Site 1 bacterial library (Fig. 3). IBS1-123 was distantly (90% sequence similarity) related to the transparent exopolymer-producing bacterium *Lentisphaera araneosa*, which was isolated from coastal seawater using the dilution to extinction method (Cho *et al.*, 2004). By contrast, one Chloroflexi-related gene (IBS2-095) and one Cyanobacteria-related gene (IBS2-139) were detected only in the Site 2 bacterial library (Fig. 3). Positive correlations have been detected between green non-sulfur bacteria, Chloroflexi, and filamentous Cyanobacteria in thermal systems in many studies (Brock, 1978; Ward *et al.*, 1987, 1998). Chloroflexi- and Cyanobacteria-related genes were also detected in intertidal hot springs in Iceland (Hobel *et al.*, 2005). The Chloroflexi-related clone IBS2-095 was distantly related (91% sequence similarity) to *Caldilinea aerophila*, a thermophilic chemoorganotrophic bacterium (Sekiguchi *et al.*, 2001). The Cyanobacteria-related clone IBS2-139 was very distantly related (<88% sequence similarity) to *Oscillatoria spongeliae*, a marine sponge-associated filamentous Cyanobacteria (Hinde *et al.*, 1994). Jing *et al.* (2006) found that *Oscillatoria*-related sequences were abundant in intertidal geothermal vents and they might have been adapted to fluctuating environmental variables in the tidal gradient. However, the water temperature in that hot spring was only 37–54°C, whereas that in this study (Site 2: 83.1°C at the time of sampling) was even beyond the upper temperature limit for photosynthetic activities (Reysenbach *et al.*, 1994; Kubo *et al.*, 2011). IBS2-139 may be a thermotorelant species that can grow phytosynthetically only during the high tide period.

Despite the existence of Deinococcus-Thermus (*T. thermophilus*) in the Ibusuki hot spring, no Deinococcus-Thermus-related genes were detected in the clone libraries constructed from the same sample. This is not surprising because only <1% of the microorganisms present in nature can be cultured using conventional methods (Amann *et al.*, 1995; Pace, 1997). With the exception of *Albidovulum inexpectatum*, it is possible that the isolated species may not be dominant species in the hot spring, and instead they may be fast-growing bacteria that were adapted to the growth conditions used in this study.

Archaeal community composition and phylogenetic analysis

The 77 Archaeal 16S rRNA gene clones contained only three OTUs and they were distributed in the two major Archaeal domains: Euryarchaeota and the recently proposed Thaumarchaeota, which is a marine mesophilic archaeal group (Brochier-Armanet *et al.*, 2008) (Fig. 5). The Euryarchaeota-related gene (IBS2A-002) was closely related (99% sequence similarity) to an environmental clone previously isolated from a hydrothermal sulfide structure, although it is very distantly related (<75% sequence similarity) to any cultured species. The two Thaumarchaeota-related genes (IBS2A-009 and IBS2A-020) were closely related (98–99% sequence similarity) to a sulfide chimney clone and a bay sediment clone. Interestingly, they were also related (92–94% sequence similarity) to Candidatus *Nitrosocaldus yellowstonii*, a thermophilic ammonia-oxidizing archaea (AOA) that was isolated from Yellowstone National Park via enrichment cultivation (Torre *et al.*, 2008). Recently, members of the Thaumarchaeota have been recognized as important participants in global geochemical cycles (Brochier-Armanet *et al.*, 2008). The dominance of AOA in intermediate salinity environments has also been suggested (Beman and Francis, 2006; Dang *et al.*, 2008; Bernhard *et al.*, 2010). These results suggest that the archaeal species present in the Ibusuki hot spring may be involved with sulfur and nitrogen cycling. Further experiments based on functional gene analysis are needed to support this hypothesis.

Conclusion

This study elucidated the bacterial and archaeal diversity in the Ibusuki hot spring using a combination of molecular- and culture-based approaches. Six distinct bacterial linages were isolated, including two possibly novel taxa. Phylogenetic and statistical analysis of the 16S rRNA genes showed that microbial communities present in the Ibusuki hot spring were composed primarily of heterotrophic Proteobacteria. Most of the genes were related to thermophilic, mesophilic, and psychrophilic bacteria with marine and coastal origins. Chemoorganotrophic and photoautotrophic bacterial genes were also detected. These structural features have also been reported in other saline geothermal systems. However, the Ibusuki hot spring had higher bacterial diversity and greater novelty. By contrast, the archaeal community was less diverse and possibly less abundant in the hot spring, although they may have important roles in biogeochemical cycling.

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References

Akaku, K., Reed, M.H., Yagi, M., Kai, K., and Yasuda, Y. 1991. Chemical and physical processes occurring in the Fushime geothermal system, Kyusyu, Japan. *J. Geochem.* **25**, 315–333.

Albuquerque, L., Santos, J., Travassos, P., Nobre, M.F., Rainey, F.A., Wait, R., Empadinhas, N., Silva, M.T., and Costa, M.S. 2002. *Albidovulum inexpectatum* gen. nov., sp. nov., a nonphotosynthetic and slightly thermophilic bacterium from a marine hot spring that is very closely related to members of the photosynthetic genus *Rhodovulum*. *Appl. Environ. Microbiol.* **68**, 4266–4273.

Alfredsson, G.A., Kristjansson, J.K., Hjörleifsdottir, S., and Stetter, K.O. 1988. *Rhodothermus marinus*, gen. nov., sp. nov., a thermophilic, halophilic bacterium from submarine hot springs in Iceland. *J. Gen. Microbiol.* **134**, 299–306.

Altschlul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. 1990. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410.

Amann, R.I., Ludwig, W., and Schleifer, K.H. 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**, 143–169.

Beman, J.M. and Francis, C.A. 2006. Diversity of ammonia-oxidizing archaea and bacteria in the sediments of a hypernutrified

subtropical estuary: Bhía del tóbari, Mexico. *Appl. Environ. Microbiol.* **72**, 7767–7777.

Benlloch, S., Acinas, S.G., Antón, J., López-López, A., Luz, S.P., and RodríguezValera, F. 2001. Archaeal biodiversity in crystallizer ponds from a solar saltern: culture versus PCR. *Microb. Ecol.* **41**, 12–19.

Bernhard, A.E., Landry, Z.C., Blevins, A., Torre, J.R., Giblin, A.E., and Stahl, D.A. 2010. Abundance of ammonia-oxidizing archaea and bacteria along an estuarine salinity gradient in relation to potential nitrification rates. *Appl. Environ. Microbiol.* **76**, 1285–1289.

Brochier-Armanet, C., Boussau, B., Gribaldo, S., and Forterre, P. 2008. Mesophilic crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. *Nature* **6**, 245–252.

Brock, T.D. 1978. Thermophilic microorganisms and life at high temperatures, pp. 181–216. Springer-Verlag, Now York, N.Y., USA.

Chao, A. 1987. Estimating the population size for capture-recapture data with unequal catchability. *Biometrics* **43**, 783–791.

Chao, A. and Lee, S.M. 1992. Estimating the number of classes via sample coverage. *J. Amer. Stat. Assoc.* **87**, 210–217.

Cho, J.C., Vergin, K.L., Morris, R.M., and Giovannoni, S.J. 2004. *Lentisphaera araneosa* gen. nov., sp. nov., a transparent exopolymer producing marine bacterium, and the description of a novel bacterial phylum, *Lentisphaerae*. *Environ. Microbiol.* **6**, 611–621.

Coenye, T., Vandamme, P., and LiPuma, J.J. 2002. Infection by *Ralstonia* species in cystic fibrosis patients: identification of *R. pickettii* and *R. mannitolilytica* by polymerase chain reaction. *Emerg. Infect. Dis.* **8**, 692–696.

Colwell, R.K. 2009. EstimateS: Statistical estimation of species richness and shared species from samples. Version 8.2. User's Guide and application published at: http://purl.oclc.org/estimates.

Dang, H., Zhang, X., Sun, J., Li, T., Zhang, Z., and Yang, G. 2008. Diversity and spatial distribution of sediment ammonia-oxidizing crenarchaeota in response to estuarine and environmental gradients in the Changjiang Estuary and East China Sea. *Microbiology* **154**, 2084–2095.

Delong, E.F. and Pace, N.R. 2001. Environmental diversity of bacteria and archaea. *Syst. Biol.* **50**, 470–478.

Elshahed, M.S., Senko, J.M., Najar, F.Z., Kenton, S.M., Roe, B.A., Dewers, T.A., Spear, J.R., and Krumholz, L.R. 2003. Bacterial diversity and sulfur cycling in a mesophilic sulfide-rich spring. *Appl. Environ. Microbiol.* **69**, 5609–5621.

Flockton, H.I. and Cross, T. 1975. Variability in *Thermoactinomyces vulgaris*. *J. Appl. Bacteriol.* **38**, 309–313.

Good, I.L. 1953. The population frequencies of species and the estimation of population parameters. *Biometrika* **40**, 237–264.

Gosink, J.J., Hervig, R.P., and Staley, J.T. 1997. *Octadecabacter arcticus* gen. nov., sp. nov., and *O. antarcticus*, sp. nov., nonpigmented, psychrophilic gas vacuolate bacteria from polar sea ice and water. *System. Appl. Microbiol.* **20**, 356–365.

Guffanti, A.A., Blanco, R., Benenson, R.A., and Krulwich, T.A. 1980. Bioenergetic properties of alkaline-tolerant and alkalophilic strains of *Bacillus firmus*. *J. Gen. Microbiol.* **119**, 79–86.

Hatae, N., Kurokawa, T., Kamada, M., Tsuyuki, T., and Osako, Y. 1965. Hot springs in Kagoshima Prefecture: Part I hot springs in the Ibusuki area. A publication of Kagoshima Prefecture (in Japanese). Kagoshima, Japan.

Hausdorf, G., Krüger, K., and Höhne, W.E. 1980. Thermitase, a thermostable serine protease from *Thermoactinomyces vulgaris*: Classification as a subtilisin-type protease. *Int. J. Peptide Protein Res.* **15**, 420–429.

Hinde, R., Pironet, F., and Borowitzka, M.A. 1994. Isolation of *Oscillatoria spongeliae*, the filamentous cyanobacterial symbiont of the marine sponge *Dysidea herbacea*. *Marine Biol.* **119**, 99–104.

Hobel, C.F.V., Marteinsson, V.T., Hreggvidsson, G.Ó., and Kristjánsson, J.K. 2005. Investigation of the microbial ecology of intertidal hot springs by using diversity analysis of 16S rRNA and chitinase genes. *Appl. Environ. Microbiol.* **71**, 2771–2776.

Hugenholtz, P., Goevel, B.M., and Pace, N.R. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *J. Bacteriol.* **180**, 4765–4774.

Iizuka, T., Tokura, M., Jojima, Y., Hiraishi, A., Yamanak, S., and Fudou, R. 2006. Enrichment and phylogenetic analysis of moderately thermophilic myxobacteria from hot springs in Japan. *Microbes Environ.* **21**, 189–199.

Ikemi, H. and Chiba, H. 1993. Effect of meteoric water-seawater-rock interaction for the chemistry of hydrothermal fluids in the Satsunan area. Abstr. A-26 (in Japanese). Abstr. Lec. Meet. Geotherm. Soc. Jpn.

Jing, H., Lacap, D.C., Lau, C.Y., and Pointing, S.B. 2006. Community phylogenetic diversity of cyanobacterial mats associated with geothermal springs along a tropical intertidal gradient. *Extremophiles* **10**, 159–163.

Kamke, J., Taylor, M.W., and Schmitt, S. 2010. Activity profiles for marine sponge-associated bacteria obtained by 16S rRNA vs 16S rRNA gene comparisons. *ISME J.* **4**, 498–508.

Khan, S.T., Nakagawa, Y., and Harayama, S. 2006. *Sediminicola luteus* gen. nov., sp. nov., a novel member of the family *Flavobacteriaceae*. *Int. J. Syst. Evol. Microbiol.* **56**, 841–845.

Kloos, W.E. and Schleifer, K.H. 1975. Isolation and characterization of *Staphylococci* from human skin. *Int. J. Syst. Evol. Microbiol.* **25**, 62–79.

Kubo, K., Knittel, K., Amann, R., Fukui, M., and Matsuura, K. 2011. Sulfur-metabolizing bacterial populations in microbial mats of the Nakabusa hot spring, Japan. *Syst. Appl. Microbiol.* **34**, 293–302.

Kwon, K.K., Woo, J.H., Yang, S.H., Kang, J.H., Kang, S.G., Kim, S.J., Sato, T., and Kato, C. 2007. *Altererythrobacter epoxidivorans* gen. nov., sp. nov., an eposide hydrolase-active, mesophilic marine bacterium isolated from cold-seep sediment, and reclassification of *Erythrobacter luteolus* Yoon *et al.* 2005 as *Altererythrobacter luteolus* comb. nov. *Int. J. Syst. Evol. Microbiol.* **57**, 2207–2211.

Lucila, M., Pomar, C.A., and Giuffrè, G. 1996. Pico-, nano- and microplankton communities in hydrothermal marine coastal environments of the Eolian Islands (Panarea and Vulcano) in the Mediterranean Sea. *J. Plankton Res.* **18**, 715–730.

Ludwig, W., Strunk, O., Klugbauer, S., Weizenegger, M., Neumaier, J., Bachleitner, M., and Schleifer, K.H. 1998. Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**, 554–568.

Magurran, A.E. 2004. Measuring biological diversity, p. 238. Blackwell Publishing, Oxford, UK.

Marteinsson, V.T., Birrien, J.L., Raguénès, G., Costa, M.S., and Prieur, D. 1999. Isolation and characterization of *Thermus thermophiles* Gy1211 from a deep-sea hydrothermal vent. *Extremophiles* **3**, 247–251.

Matsubaya, O., Sakai, H., Kusachi, I., and Satake, H. 1973. Hydrogen and oxygen isotopic ratios and major element chemistry of Japanese thermal water systems. *J. Geochem.* **7**, 123–151.

Miller, S.R., Strong, A.L., Jones, K.L., and Ungerer, M.C. 2009. Bar-coded pyrosequencing reveals shared bacterial community properties along the temperature gradients of two alkaline hot springs in yellowstone national park. *Appl. Environ. Micorbiol.* **75**, 4565–4572.

Moreira, L., Nobre, M.F., Sa-Correia, I., and Da Costa, M.S. 1996. Genomic typing and fatty acid composition of *Rhodothermus marinus*. *Syst. Appl. Microbiol.* **19**, 83–90.

Na, H., Kim, O.S., Yoon, S.H., Kim, Y., and Chun, J. 2011. Comparative approach to capture bacterial diversity of coastal waters. *J. Microbiol.* **49**, 729–740.

Nakagawa, S., Nakamura, S., Inagaki, F., Takai, K., Shirai, N., and Sako, Y. 2004. *Hydrogenivirga caldilitoris* gen. nov., sp. nov., a novel extremely thermophilic, hydrogen- and sulfur-oxidizing bacterium from a coastal hydrothermal field. *Int. J. Syst. Evol. Microbiol.* **54**, 2079–2084.

Nedashkovskaya, O.I., Kim, S.B., Lee, K.H., Mikhailov, V.V., and Bae, K.S. 2005. *Gillisia mitskevichiae* sp. nov., a novel bacterium of the family *Flavobacteriaceae*, isolated from sea water. *Int. J. Syst. Evol. Microbiol.* **55**, 321–323.

Nunes, O.C., Donato, M.M., and Da Costa, M.S. 1992. Isolation and characterization of *Rhodothermus marinus*. *Syst. Appl. Microbiol.* **19**, 83–90.

Ohtaki, A., Mizuno, M., Yoshida, H., Tonozuka, T., Sakano, Y., and Kamitori, S. 2006. Structure of a complex of *Thermoactinomyces vulgaris* R-47 α-amylase 2 with maltohexaose demonstrates the important role of aromatic residues at the reducing end of the substrate binding cleft. *Carbohydrate Res.* **341**, 1041–1046.

Oi, T., Ikeda, K., Nakano, M., Ossaka, T., and Ossaka, J. 1996. Boron isotope geochemistry of hot spring waters in Ibusuki and adjacent areas, Kagoshima, Japan. *J. Geochem.* **30**, 273–287.

Oshima, T. and Imahori, K. 1974. Description of *Thermus thermophiles* (Yoshida and Oshima) comb. nov., a nonsporulating thermophilic bacterium from a Japanese thermal spa. *Int. J. Syst. Bacteriol.* **24**, 102–112.

Pace, N.R. 1997. A molecular view of microbial diversity and the biosphere. *Science* **276**, 734–739.

Petursdottir, S.K., Bjornsdottir, S.H., Hreggvidsson, G.O., Hjorleifsdottir, S., and Kristjansson, J.K. 2009. Analysis of the unique geothermal microbial ecosystem of the Blue Lagoon. *FEMS Microbiol. Ecol.* **70**, 425–432.

Pukall, R., Buntefub, D., Frühling, A., Rohde, M., Kroppenstedt, R.M., Burghardt, J., Lebaron, P., Bernard, L., and Stackebrandt, E. 1999. *Sulfitobacter mediterraneus* sp. nov., a new sulfite-oxidizing member of the α-Proteobacteria. *Int. J. Syst. Evol. Microbiol.* **49**, 513–519.

Rappe, M.S. and Giovannoni, S.J. 2003. The uncultured microbial majority. *Annu. Rev. Microbiol.* **57**, 369–394.

Reysenbach, A.L., Whickham, C.R., and Pace, N.R. 1994. Phylogenetic analysis of the hyperthermophilic pink filament community in Octopus Spring, Yellowstone National Park. *Appl. Environ. Microbiol.* **60**, 2113–2119.

Rohwer, F., Seguritan, V., Azam, F., and Knowlton, N. 2002. Diversity and distribution of coral-associated bacteria. *Mar. Ecol. Prog. Ser.* **243**, 1–10.

Sakamoto, H., Matsumoto, A., Tomiyasu, T., and Yonehara, N. 1993. Chemical compositions of hot springs in Ibusuki city and its ambient area. Abstr. p. 260 (in Japanese). Abstr. Lec. Meet. Geotherm. Soc. Jpn.

Sako, Y., Takai, K., Ishida, Y., Uchida, A., and Katayama, Y. 1996. *Rhodothermus obamensis* sp. nov., a modern lineage of extremely thermophilic marine bacteria. *Int. J. Syst. Bacteriol.* **46**, 1099–1104.

Schleper, C., Jurgens, G., and Jonuscheit, M. 2005. Genomic studies of uncultivated archaea. *Nature* **3**, 479–488.

Sekiguchi, Y., Yamada, T., Hanada, S., Ohashi, A., Harada, H., and Kamagata, Y. 2001. *Anaerolinea thermophila* gen. nov., sp. nov. and filamentous thermophiles that represent a previously uncultured lineage of the domain *Bacteria* at the subphlum level. *Int. J. Syst. Evol. Microbiol.* **53**, 1843–1851.

Shaw, A.K., Halpern, A.L., Beeson, K., Tran, B., Venter, J.C., and Martiny, J.B.H. 2008. It's all relative: ranking the diversity of aquatic bacterial communities. *Environ. Microbiol.* **10**, 2200–2210.

Shoreit, A.A.M. 1992. Isolation, identification and germination of spores of Thermoactinomyces from Qatari soils (Arabian Gulf). *J. Basic Microbiol.* **32**, 113–118.

Singleton, D.R., Furlong, M.A., Rathbun, S.L., and Whitman, W.B. 2001. Quantitative comparisons of 16S rRNA gene sequence libraries from environmental samples. *Appl. Envion. Microbiol.* **67**, 4374–4376.

Stackebrandt, E. and Ebers, J. 2006. Taxonomic parameters revisited: tarnished gold standards. *Microbiol. Today* **33**, 152–155.

Stevens, H., Stübner, M., Simon, M., and Brinkhoff, T. 2005. Phylogeny of *Proteobacteria* and *Bacteroidetes* from oxic habitats of a tidal flat ecosystem. *FEMS Microbiol. Ecol.* **54**, 351–365.

Strous, M., Fuerst, J.A., Kramer, E.H.M., Logemann, S., Muyzer, G., Pas-Schoonen, K.T., Webb, R., Kuenen, J.G., and Jetten, M.S.M. 1999. Missing lithotroph identified as new planctomycete. *Nature* **400**, 446–449.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **28**, 2731–2739.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680.

Tobler, D.J. and Benning, L.G. 2011. Bacterial diversity in five Icelandic geothermal waters: temperature and sinter growth rate effects. *Extremophiles* **15**, 473–485.

Torre, J.R., Walker, C.B., Ingalls, A.E., Könneke, M., and Stahl, D.A. 2008. Cultivation of a thermophilic ammonia oxidizing archaeon synthesizing crenarchaeol. *Environ. Microbiol.* **10**, 810–818.

Tsuyuki, T. 1976. Trutium content of the thermal-waters of Hitoyoshi, Kirishima and Ibusuki hot springs, south Kyusyu, Japan. *Kagoshima University Repository*. **9**, 75–86 (in Japanese).

Vandecandelaere, I., Nercessian, O., Segaert, E., Achouak, W., Mollica, A., Faimali, M., and Vandamme, P. 2009a. *Nautella italica* gen. nov., sp. nov., isolated from a marine electroactive biofilm. *Int. J. Syst. Evol. Microbiol.* **59**, 811–817.

Vandecandelaere, I., Segaert, E., Mollica, A., Faimali, M., and Vandamme, P. 2009b. *Phaeobacter caeruleus* sp. nov., a blue-coloured, colony-forming bacterium isolated from a marine electroactive biofilm. *Int. J. Syst. Evol. Microbiol.* **59**, 1209–1214.

Wagner, M. and Horn, M. 2006. The *Planctomycetes*, *Verrucomicrobia*, *Chlamydiae* and sister phyla comprise a superphylum with biotechnological and medical relevance. *Curr. Opin. Biotech.* **17**, 241–249.

Wang, S., Xiao, X., Jiang, L., Peng, X., Zhou, H., Meng, J., and Wang, F. 2009. Diversity and abundance of ammonia-oxidizing archaea in hydrothermal vent chimneys of the Juan de Fuca Ridge. *Appl. Envion. Microbiol.* **75**, 4216–4220.

Ward, D.M., Ferris, M.J., Nold, S.C., and Bateson, M.M. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. *Microbiol. Mol. Biol. Rev.* **62**, 1353–1370.

Ward, D.M., Tayne, T.A., Anderson, K.L., and Bateson, M.M. 1987. Community structure and interactions among community members in hot spring cyanobacterial mats. *Symp. Soc. Gen. Microbiol.* **41**, 179–210.

Yang, C.H., Huang, Y.C., and Chen, C.Y. 2009. Degradation of rutin by *Thermoactinomyces vulgaris* and other thermophilic compost isolates. *J. Agric. Food Chem.* **57**, 5095–5099.

Yoon, J.H., Kang, S.J., Lee, S.Y., Oh, K.H., and Oh, T.K. 2009. *Seohaeicola saemankumensis* gen. nov., sp. nov., isolated from a tidal flat. *Int. J. Syst. Evol. Microbiol.* **59**, 2675–2679.

Yoon, J.H. and Oh, T.K. 2005. *Sphingopyxis flavimaris* sp. nov., isolated from sea water of the Yellow Sea in Korea. *Int. J. Syst. Evol. Microbiol.* **55**, 369–373.

Zhang, C.L., Ye, Q., Huang, Z., Li, W.J., Chen, J., Song, Z., Zhao, W., Bagwell, C., Inskeep, W.P., Ross, C., and *et al.* 2008. Global occurrence of archaeal *amo*A genes in terrestrial hot springs. *Appl. Envion. Microbiol.* **74**, 6417–6426.