Isolation and characterization of culturable thermophilic bacteria from hot springs in Benguet, Philippines

Socorro Martha Meg-ay V. Daupan¹ and Windell L. Rivera*¹,²

¹Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City, 1101, Philippines
²Natural Sciences Research Institute, University of the Philippines, Diliman, Quezon City, 1101, Philippines

Abstract—Despite the numerous geothermal environments in the Philippines, there is limited information on the composition of thermophilic bacteria within the country. This study is the first to carry out both culture and molecular-based methods to characterize thermophilic bacteria from hot springs in the province of Benguet in the Philippines. The xylan-degrading ability of each isolate was also investigated using the Congo red method. A total of 14 phenotypically-different isolates (7 from Badekbek mud spring and 7 from Dalupirip hot spring) were characterized. Phylogenetic analysis based on the nearly complete 16S rDNA sequences revealed that all the isolates obtained from Badekbek were affiliated with *Geobacillus*, whereas the isolates from Dalupirip clustered into 3 major linkages of bacterial phyla, Firmicutes (72%) consisting of the genera *Geobacillus* and *Anoxybacillus*; Deinococcus-Thermus (14%) consisting of the genus *Meiothermus*; and Bacteroidetes consisting of the genus *Thermonema* (14%). In addition, xylan-degrading ability was observed in all isolates from Badekbek and in 2 isolates from Dalupirip which showed high sequence similarity with *Geobacillus* spp. The results are also essential in understanding the roles of the physico-chemical properties of hot spring water as a driver of thermophilic bacterial compositions.

Keywords—16S rDNA sequencing, hot springs, Philippines, thermophilic bacteria

INTRODUCTION

The discovery of thermophilic bacteria capable of carrying out life processes in the boiling hot springs of Yellowstone National Park has become a foundation of developments in medicine and biotechnology. Since then, thermophiles have been isolated in geothermal features all over the world. Thermophilic bacteria are found in various geothermally-heated regions of the earth such as hot springs, deep sea hydrothermal vents and volcanic craters (Stetter et al. 1993). They can also live in fermenting materials that can produce heat such as compost piles and garbage landfills (Fujiio and Kume 1991). The ability of thermophiles to proliferate at elevated temperatures is attributed to the thermally-stable macromolecules they possess (Zeikus et al., 1998). As a consequence of growth at high temperatures and unique macromolecular properties, thermophiles exhibit high metabolic rates, thereby generating greater end-products despite lower growth rates compared to mesophiles. They also provide physically and chemically stable enzymes that are of significant use to industries (Haki and Rakshit 2003). Thermophiles have provided an interesting and challenging platform for researchers since the time of their discovery. However, due to difficulties in isolation and maintenance of the pure culture, their diversity in thermal habitats remains to be explored (Kikani and Singh 2011).

The Philippines is geographically situated in the Pacific Ring of Fire and is subject to numerous active volcanoes. The country boasts of bountiful natural resources including hot springs that provide good conditions for thermophilic bacterial growth. However, there is limited knowledge on the thermophile community in Philippine hot springs. Hot springs in Los Baños, Laguna, Philippines have been shown to harbor unique thermophiles. Fluorescence in situ hybridization (FISH) analysis showed that this site is dominated by microbial community belonging to domain Archaea, of which 63% were Crenarchaeota and 8% were Euryarchaeota and 17% were bacteria and the remaining 12% were unidentified (Lantican et al. 2011). Two novel hyperthermophilic crenarchaeotes have also been discovered from the same hot spring and the proposed names for these isolates were *Caldivirga maquilingensis* and *Caldisphaera lagunensis* (Itoh et al. 1999, Itoh et al. 2003). Hongmei and colleagues, on the other hand, described thermophilic microbial mats from Laguna hot springs (Hongmei et al. 2005).

This present study therefore aimed to isolate and characterize thermophilic bacteria from two hot springs in Benguet, Philippines and to assess their phylogenetic relationships.

MATERIALS AND METHODS

Sampling Sites

Benguet was selected because of its unique temperate climate compared to other provinces of the country (National Statistical Coordination Board 2005). Out of its 13 municipalities, hot springs are found in five of them. Two hot springs were
randomly selected for the isolation of thermophilic bacteria (Figure 1). These hot springs include Badekbek mud spring in Bokod and Dalupirip hot spring in Bokod. The temperature and pH of the hot springs were measured during sampling and the turbidity was visually assessed. The selected hot springs were found to vary greatly in terms of their physico-chemical properties. Badekbek mud spring had a temperature range of 78-80°C and Dalupirip hot spring had lower temperature range of 45-48°C. In terms of pH, the former had lower pH ranging from 3-4 and the latter had nearly neutral pH of 7-8.

**Figure 1.** Map showing the geographical location of the sampling sites in Benguet, Philippines (marked with red star) and municipalities with hot springs (marked with yellow circle).

### Sample Collection

Water samples were collected in triplicates at different points from the sampling sites using sterile thermal flasks (Ledbetter et al. 2007). All samples were immediately transported to the laboratory and directly inoculated onto solidified Thermus medium (ATCC medium 697) plates. This medium has the following composition (in micrograms per liter of deionized water): CaSO$_4$·7H$_2$O, 100; MgSO$_4$·7H$_2$O, 100; NaCl, 1,000; KNO$_3$, 103; NaNO$_3$, 689; Na$_2$HPO$_4$, 111; FeCl$_3$, 2.8; MnSO$_4$·H$_2$O, 22; ZnSO$_4$·7H$_2$O, 5; H$_2$BO$_3$, 5; CaSO$_4$, 0.2; Na$_2$MoO$_4$·2H$_2$O, 0.3; CoCl$_2$·2H$_2$O, 0.5; and EDTA, 6 (Brock and Freeze 1969). Yeast extract (0.08%) and peptone (0.05%) were added and the medium was solidified with Phytagel (Sigma) at 1% final concentration. MgSO$_4$ (0.5%) was also added to make the solidifying agent heat stable. Concentrated H$_2$SO$_4$ (0.5ml) was added to dissolve the salts and the pH of the medium was adjusted to 7.0. All plates were then incubated for a maximum of 3 days at 60°C.

### Purification and preservation of the isolates

Colonies were selected from each plate and were subjected to streaking on solidified Thermus medium at least three times. Single colony was picked and re-streaked on fresh solid media to obtain pure cultures. The colonies were observed under the microscope after several streaking to check for purity by assessing the homogeneity of cell morphology. Pure isolates were grown at 60°C for 24 hours and were suspended in Thermus broth containing 15% glycerol and stored at -70°C until use.

### Phenotypic characterization

The morphological and biochemical characteristics of the isolates were done according to the methods of Elmasser et al. (2007) and Narayan et al. (2008) using 18 to 24-hour old cultures. These include examination of cultural characteristics, Gram staining, motility test, Kovac’s oxidase and catalase test.

### Screening for xylan-degrading isolates

Pure isolates were tested for xylan-degrading ability because xylanases offer a wide range of industrial and environmental significance since these enzymes are being used to replace chlorinated compounds in bleaching of wood for paper production (Kaur et al. 2010). The screening was done by inoculating a loopful of each isolate to Thermus medium containing 0.5% xylan. After incubation of the plates for 48 hours, 0.1% Congo red solution was poured onto the plates. The plates were incubated for 30 minutes at 65°C and washed with 1M NaCl solution. Clear zones surrounding the colonies on the red background dyed with Congo red solution is indicative of positive activity for xylanase since Congo red binds only to carbohydrate polymers (Cordeiro et al. 2002; Teather and Wood 1982).

### DNA extraction

Cells from each of the 18 to 24-hour old cultures were harvested by centrifugation for 2 minutes at 10,000 rpm. The pellet was washed with 1X PBS; after which, it was resuspended in 10µl sterile distilled water. A 200µl aliquot of 5% Chelex was then added and the mixture was mixed vigorously. The mixture was incubated for 20 to 30 minutes in water bath at 56°C and spun at high speed using a vortex. The product was incubated for 8 minutes in a boiling water bath and spun for 2 to 3 minutes at 13,000 rpm. The supernatant was transferred to a new tube and stored at -20°C until use. The nucleic acid concentration of the crude DNA was estimated using NanoDrop 2000 (Thermo Scientific).

### PCR amplification

Amplification of the 16S rRNA gene was conducted using a pair of universal primers, 27F/1492R (Lau and Dal Jos 2009). All PCR reactions were carried out under the following conditions: 4 minutes at 94°C, 30 cycles of 1 minute at 92°C, 1 minute at 45°C, 1 minute at 72°C; followed by 10 minutes at 72°C. PCR products were purified using Expir™ PCR SV Purification Kit according to the manufacturer’s instructions. The PCR products were separated by gel electrophoresis at 100V for 25 minutes on 1X TAE buffer (Tris: Acetic acid: EDTA) and analyzed by staining with ethidium bromide under UV light. The purified PCR products were sent to Macrogen, Inc., South Korea for sequencing.

### Phylogenetic analyses

The sequences consisting of 1,400-1,500 nucleotides were determined and assembled using MEGA 5.05 (Tamura et al. 2011). The sequences were then compared with those available in GenBank using BLAST search (http://www.ncbi.nlm.gov/blast/). All sequences were aligned using Clustal W algorithm in BioEdit 7.0.5.3 (Hall, 1999). A total of 24 sequences were consolidated. The optimal model for DNA subsets was determined using Bayesian Information Criterion (BIC) as selection strategy in jModelTest (Posada 2008). The site saturation was tested using Swofford’s (2002) and PhyML 3.0 (Guindon 2010) programs, respectively.

### Nucleotide sequence accession numbers

All DNA sequences were deposited in GenBank, under accession numbers KC252975-KC252981 for Badekbek isolates and KC252983-KC252989 for Dalupirip isolates.

### RESULTS

Phenotypically different colonies that appeared after incubation on Thermus medium at 60°C from each hot spring site were selected for purification and characterization. A total of 14 thermophilic bacterial isolates (7 from each site) were obtained. It was noted that the isolates were either Gram-negative or Gram-positive. All the isolates had circular colony form and the cells were rod-shaped of variable. All the isolates had circular colony form and the cells were rod-shaped of different morphologies from slender, long rods (thread-like) to short, nearly rounded rods. Several fragmented colonies were also obtained. Motility varied among the isolates, of which 79% were found to be motile (Table 1).

### TABLE 1. Phenotypic characteristics of the 14 thermophilic bacterial isolates from Badekbek (Ba 1-7) and Dalupirip (Da 1-7) hot springs in Benguet, Philippines.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Culture characteristics</th>
<th>Gram stain</th>
<th>Cell shape</th>
<th>Catalase activity</th>
<th>Oxydase activity</th>
<th>Motility</th>
<th>Xylanase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba1</td>
<td>light yellow circular</td>
<td>+</td>
<td>small rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ba2</td>
<td>light yellow circular</td>
<td>-</td>
<td>small rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ba3</td>
<td>light yellow circular</td>
<td>+</td>
<td>small rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ba4</td>
<td>light yellow circular</td>
<td>-</td>
<td>small rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ba5</td>
<td>light yellow circular</td>
<td>+</td>
<td>small rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ba6</td>
<td>light yellow circular</td>
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<td>small rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ba7</td>
<td>light yellow circular</td>
<td>+</td>
<td>small rods</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Da1</td>
<td>light yellow circular</td>
<td>-</td>
<td>light rods</td>
<td>-</td>
<td>-</td>
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<td>-</td>
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<tr>
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<td>light rods</td>
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</tr>
<tr>
<td>Da4</td>
<td>light yellow circular</td>
<td>-</td>
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<tr>
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<td>-</td>
<td>light rods</td>
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<tr>
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<tr>
<td>Da7</td>
<td>light yellow circular</td>
<td>-</td>
<td>light rods</td>
<td>-</td>
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</tbody>
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The PCR amplification of the 16S rRNA gene was conducted using a pair of universal primers, 27F/1492R (Lau and Dal Jos 2009). All PCR reactions were carried out under the following conditions: 4 minutes at 94°C, 30 cycles of 1 minute at 92°C, 1 minute at 45°C, 1 minute at 72°C; followed by 10 minutes at 72°C. PCR products were purified using Expir™ PCR SV Purification Kit according to the manufacturer’s instructions. The PCR products were separated by gel electrophoresis at 100V for 25 minutes on 1X TAE buffer (Tris: Acetic acid: EDTA) and analyzed by staining with ethidium bromide under UV light. The purified PCR products were sent to Macrogen, Inc., South Korea for sequencing.
All the isolates from Badekbek mud spring and 2 more isolates from Dalupirip hot spring showed xylan-degrading ability as indicated by the cleared zones that formed on xylan plates following the Congo red method. Isolate Da3 exhibited the largest clearing zones. The other isolates had clearing zones with diameter ranging from 0.8mm to 1.5mm (Figure 2).

BLAST searches based on the nearly complete 16s rDNA sequences of the 14 isolates showed that there was a strong similarity (> 98%) between the test isolates and representative strains of Geobacillus, Thermus, Meiothermus and Anoxybacillus. Isolates from Badekbek had high sequence similarity with either G. thermoparaffinivorans or G. thermovorans. In contrast, isolates from Dalupirip hot spring showed high sequence homology to the aforementioned genera (Table 2).

Table 2. Identity of the 14 thermophilic bacterial isolates based on BLAST searches.

<table>
<thead>
<tr>
<th>Code (accession number)</th>
<th>Identity based on BLAST searches</th>
<th>GenBank Accession No.</th>
<th>E-value (Query Coverage %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ba3(KC252975)</td>
<td>Geobacillus</td>
<td>EU214615</td>
<td>0.0 (98)</td>
</tr>
<tr>
<td>BA3(KC252976)</td>
<td>thermoparaffinivorans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA3(KC252977)</td>
<td>Geobacillus</td>
<td>EU214615</td>
<td>0.0 (99)</td>
</tr>
<tr>
<td>BA3(KC252978)</td>
<td>thermoparaffinivorans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ba5(KC252979)</td>
<td>Geobacillus</td>
<td>EU214615</td>
<td>0.0 (99)</td>
</tr>
<tr>
<td>BA5(KC252980)</td>
<td>thermoparaffinivorans</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA5(KC252981)</td>
<td>Geobacillus</td>
<td>EU214615</td>
<td>0.0 (99)</td>
</tr>
<tr>
<td>Da1(KC252983)</td>
<td>Thermus roseus</td>
<td>Y09657.1</td>
<td>0.0 (96)</td>
</tr>
<tr>
<td>Da2(KC252984)</td>
<td>Geobacillus kustopphius</td>
<td>BA000043</td>
<td>0.0 (99)</td>
</tr>
<tr>
<td>DA2(KC252985)</td>
<td>Geobacillus stearchenthalphius</td>
<td>JF131205</td>
<td>0.0 (99)</td>
</tr>
<tr>
<td>Da4(KC252986)</td>
<td>Anoxybacillus</td>
<td>EU106698</td>
<td>0.0 (98)</td>
</tr>
<tr>
<td>Da5(KC252987)</td>
<td>Meiothermus sp. SK-2</td>
<td>GU129930</td>
<td>0.0 (94)</td>
</tr>
<tr>
<td>Da6(KC252988)</td>
<td>Geobacillus stearchenthalphius</td>
<td>AF491407</td>
<td>0.0 (99)</td>
</tr>
<tr>
<td>Da7(KC252989)</td>
<td>Geobacillus stearchenthalphius</td>
<td>AF491407</td>
<td>0.0 (99)</td>
</tr>
</tbody>
</table>

The aligned sequences for the nearly complete 16s rRNA had a length of 1,510 bp. Site saturation test (Xia test) revealed little saturation in the sequences. The optimal models of DNA substitution for the 16s rRNA as determined by ModelTest (Posada 2008) using Bayesian Information Criterion (BIC) was TRN+G. The phylogenetic relationships of the 14 thermophilic bacterial isolates and closely-related species were determined using the neighbor-joining (NJ) (Saitou and Nei 1987) and maximum-likelihood (ML) (Cavalli-Sforza and Edwards 1967; Felsenstein 1981) methods. The branches that corresponded to partitions reproduced below 50% bootstrap replicates were collapsed. The generated dendrogram revealed 3 clades supported by high bootstrap values (Figure 3). These clades are represented by 3 major lineages, namely: Firmicutes (72%) consisting of the genera Geobacillus and Anoxybacillus; Deinococcus-Thermus (14%) consisting of the genus Meiothermus; and Bacteroidetes consisting of the genus Thermus (14%). This third clade was observed to form distinct lineage from the first two clades. The xylan-degrading isolates clustered within the clade of Firmicutes and the brightly-pigmented isolates that did not exhibit xylan-degrading ability formed separate clades. Interestingly, 2 isolates (Da 6 and Da 7) that did not exhibit xylan-degrading ability formed a separate cluster within the first clade. It is also noted that isolates from Badekbek mud spring did not exhibit great diversity and all isolates fell under the first clade showing their close association with G. thermoparaffinivorans. The dendrogram also shows clustering of a member of the genus Bacillus to Geobacillus spp.

The xylan-degrading isolates clustered within the clade of Thermus ranges from 35 to 70°C and the major fatty acids are iso-branched saturated fatty acids (iso-15:0, iso-16:0 and iso 17:0) (Rahman et al. 2004). Members of the genus Geobacillus have rod-shaped cells, occurring singly or in short chains; colonies may show variable shape and may exhibit pigmentation (Nzina et al. 2001). All isolates from Badekbek mud spring had light yellow pigmentation and were found to be affiliated with Geobacillus. Although most species of Geobacillus are Gram-positive, their cell wall structure using Gram stain may vary between positive or negative (Nzina et al. 2001). Members of the genus Geobacillus have been previously shown to produce xylanases. G. stearchenthalphius T-6, for instance, produces two selective family 10 xylanases (complete the degradation of xylan (Solomon et al. 2007). Research has focused mainly on two of families of xylanase containing glycoside hydrolase and these are families 10 and 11. Family 10 xylanases have been isolated from various thermophilic microorganisms including species of Thermotoga, Caldocellulosa, Rhodothermus, and Bacillus. The other enzymes with xylanase activity that belong to other families are also studied, albeit to a lesser extent. Most of the xylanases that are of bacterial origin have optimum activity at approximately 60 to 65°C and a number of extremophilic xylanases have been described due to the industrial demand for such enzymes that can operate under process conditions (Collins et al. 2005). In the present study, only 2 isolates with high sequence similarity to Geobacillus did not exhibit xylan-degrading ability.

Isolate Da 4 was found to be affiliated with Anoxybacillus. Members of the genus Anoxybacillus are predominant in soil although aerobic growth has been observed in some species (Pikuta et al. 2003).

One isolate (Da 5) was found to be affiliated with Meiothermus ruber. In natural environments, representative strains of this genus are exclusively found in thermal limnetic systems, predominantly in terrestrial hot springs (Zhang et al. 2010). This isolate showed similar phenotypic properties with T-6, for instance, produces two selective family 10 xylanases (complete the degradation of xylan (Solomon et al. 2007).
pink pigmentation (Goh et al. 2011). Although this isolate was not able to degrade xylan, its pigmentation is noteworthy. Pigments from bacteria are being exploited for the production of natural dyes since bacteria produce better yields as opposed to dye extraction from eukaryotes. Natural dyes are preferred over synthetic ones since the former exhibit better biodegradability (Ahmad et al. 2012).

Another isolate (Da1) showed high sequence similarity with *Thermospora rossianum*, a bacterium that has been found to be polyextremophile being both thermophilic and halophilic. This isolate exhibited a yellow iridoid-based and filamentous cellular morphology typical of *T. rossianum* (Trenere et al. 1997). The yellow pigment is probably carotenoid.

The clustering of the genus *Geobacillus* with *Bacillus caldodenudens* as seen in Figure 3 may suggest the need for further reclassification of certain species of the genus *Bacillus*. However, only a small group of bacteria has been progressively been subdivided into the novel genera *Brevibacillus, Paenbacillus, Salibacillus*, and most recently, *Geobacillus* based on separate phylogenetic groupings derived from the 16S rRNA gene sequence information (Nacina et al. 2001). This also supports the conclusion of stating that 16S rDNA analysis alone may be insufficient to distinguish between some closely-related species possibly because of the existence of multiple 16S rRNA operons and the occurrence of recombination within the strain (Meintanis 2008; Vanden doo et al.). This is a more reliable approach to discriminate thermophilic bacteria even at strain levels would be the use of other genomic fingerprint method, like REP-PCR.

The constructed phylogenetic tree also shows that culturable thermophilic bacterial community in Dalupirip hot spring is more diverse in comparison to that of Baddekk, which is sulfur, acidic mud spring. This implies the importance of physico-chemical properties of hot spring water as a driver of thermophilic bacterial compositions.

**CONCLUSION**

This study has identified the thermophilic bacterial compositions of hot spring in the Philippines. This revealed the industrial significance of the isolates, particularly in xylan degradation. The sites were found to be dominated by species of *Geobacillus*. Members of *Meiothermus, Thermomonas, and Anoxybacillus* were also isolated in Dalupirip hot spring. Dalupirip hot spring showed greater diversity of bacteria because of the difference in physico-chemical characteristics of the location. It had a lower temperature range of 45-48°C that can support more bacteria. The higher temperature range in Baddekk hot spring supports the existence of the genus *Geobacillus*, which have previously been shown to produce xylanases. The clustering of *Bacillus caldodenudens* with *Geobacillus* ssp. in the generated dendrogram suggests the need for further analysis and possibly reclassification of certain species of *Bacillus* to *Geobacillus*. The results also imply the potential of isolated microbial communities in biobleaching of kraft pulp. Biores. Technol. 2010; 101: 9150-9155.

**ACKNOWLEDGEMENTS**

This work was supported by research grants from the Department of Science and Technology-Accelerated Science and Technology Human Resource Development Program (DOST-ASTHRDP) of the Philippines and the UNU- GIST project funded by the DOST and Science for Sustainability operated by the International Environmental Research Center, Gwangju Institute of Science and Technology (IERC-GIST) in South Korea. The authors are also grateful to the technical support given by Dr. In-Soo Chang of the Energy and Biotechnology Laboratory of the IERC-GIST.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interests regarding the publication of this article.

**CONTRIBUTIONS OF INDIVIDUAL AUTHORS**

SMMVD and WLR conceptualized the study. The experiments were conducted by SMMVD. SMMVD and WLR prepared the manuscript.

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