

Characterization of Moderately Thermophilic Bacteria Isolated from Saline Hot Spring in Japan

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Twelve strains of moderately thermophilic bacteria were isolated from a saline hot spring (55 °C, pH 8.3) located in Odaito, Hokkaido, Japan. All strains showed over 99% sequence similarities in the 16S rRNA gene. However, only eight strains grew well at 55 °C and demonstrated detectable β -galactosidase activities. The representative strains, BEK6 and BEK11, were chosen from individual phenotypic groups and were further characterized. The nucleotide sequence of the almost full-length 16S rRNA genes of the representative strains showed 97.1% and 96.6% similarity with *Bacillus aeolius* and *Bacillus alveayuensis*, respectively, indicating that the strains belonged to a novel species of the genus *Bacillus*. The cells of both strains were Gram-positive and showed catalase and oxidase activities. The optimal growth temperature and pH of strains BEK6 and BEK11 was approximately 50 °C and 7-8, respectively. These strains grew in a medium containing 10% NaCl, in contrast to *B. aeolius* and *B. alveayuensis* that grew in a medium containing maximum of 4-5% NaCl. The strain BEK11 showed relatively strong protease and amylase activities in the presence of 3% NaCl, suggesting the potential industrial uses of these enzymes under saline conditions.

Key words: thermophile, hot spring, novel species, *Bacillus*

Dua belas galur bakteri termofilik telah diisolasi dari sumber air panas berkadar garam tinggi (55 °C, pH 8.3) yang terletak di Odaito, Hokkaido, Jepang. Semua galur bakteri menunjukkan lebih dari 99% persamaan sekuens dalam gen rRNA 16S. Hanya delapan galur bakteri yang dapat tumbuh dengan baik pada suhu 55 °C dan mempunyai aktivitas β -galaktosidase. Dari kelompok fenotipe individu BEK6 dan BEK11 dipilih sebagai perwakilan. Urutan nukleotida 16S rRNA secara parsial dari BEK6 dan BEK11 menunjukkan masing-masing 97.1% dan 96.6% kesamaan dengan *Bacillus aeolius* dan *Bacillus alveayuensis*, menunjukkan bahwa galur tersebut adalah spesies baru dari genus *Bacillus*. Kedua bakteri adalah Gram-positif dan mempunyai aktivitas katalase dan oksidase. Suhu pertumbuhan optimal galur BEK6 dan BEK11 ialah sekitar 50 °C dan pH 7-8. Berbeda dengan *B. aeolius* dan *B. alveayuensis* yang hanya dapat tumbuh dalam medium yang mengandung NaCl maksimum 4-5%, kedua galur bakteri tersebut dapat tumbuh dalam medium yang mengandung NaCl 10%. Galur BEK11 menunjukkan aktivitas protease dan amilase yang relatif tinggi dalam kondisi NaCl 3%, sehingga menjanjikan potensinya sebagai produsen enzim industri.

Kata kunci: termofil, sumber air panas, spesies baru, *Bacillus*

Thermophiles are microbes that live in geothermal hot springs, hydrothermal vents, and artificially hot environments such as fermented compost and show optimal growth above 45-50 °C (Lebedinsky *et al.* 2007). These microbes are roughly classified as moderately thermophilic and extremely thermophilic. The former have an optimal growth temperature ranging from 45-50 °C to approximately 65 °C; microbes belonging to both classes may have the potential for industrial application. Moreover, thermophiles and their enzymes are interesting subjects for research in fundamental microbiology, evolutionary biology, biochemistry, and protein engineering. The biodiversity of thermophiles has been well studied until date using cultivation-dependent and independent methods; however, the existence of numerous uncultured thermophilic species in hot environments is indicated. During a recent attempt to

identify novel thermophiles, we isolated twelve strains of moderately thermophilic bacteria from a saline hot spring in Hokkaido, Japan. Preliminary experiments for the taxonomic classification of the isolated strains based on the 16S rRNA gene (16S rDNA) sequences indicated that the strains were closely related to each other and could be classified as a novel species of the genus *Bacillus*, which consists of many species including the biotechnologically valuable strains (Helianti *et al.* 2010). In this paper, we describe the physiological properties, chemotaxonomic characteristics, and phylogeny of the newly isolated *Bacillus* strains and analyze their enzymatic activities.

MATERIALS AND METHODS

Sampling, Isolation, and Cultivation of Microbes. A pumped, hot, spring water sample was obtained from the Odaito hot spring in Hokkaido, Japan, with the permission of the pumping facility

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owner. The temperature and pH of the water were approximately 55 °C and 8.3, respectively. The sample was plated onto modified Brock's basal salts (Kurosawa *et al.* 1998) supplemented with 0.1% (w/v) yeast extract and artificial sea salts (1% w/v) at pH 8.3, solidified by 0.7% (w/v) Gelzan (Sigma), and incubated at 50 °C for two days. Colonies were picked up and purified by repeated single-colony isolation. For further characterization of the isolates, a "routine medium," in which artificial sea salts and Gelzan were replaced with NaCl and 1.5% agar, respectively, was used. The cultures were maintained at 50 °C and pH 8.0 for routine cultivation.

Morphology and Phenotypic Characteristics.

The representative strains (BEK6 and BEK11) were subjected to standard tests including Gram staining and tests for motility, catalase, and oxidase activities according to standard method (Aygani and Arian 2007). Anaerobic growth was examined by an AnaeroPack jar (Mitsubishi Gas Chemical), and cell morphology was examined by a phase contrast microscope (Axioskop 40; Carl Zeiss). To determine the temperature and pH optima, the strains were cultivated in the "routine medium" and various pH values were used to determine the pH dependence of growth. In addition, the NaCl tolerance of growth was examined. All cells were grown aerobically in standing cultures of loosely-capped glass tubes. Growth was monitored by measuring the optical density (OD₆₀₀) of the culture; utilization of lactose, maltose, glucose, D-sorbitol, D-galactose, melibiose, D-mannose, D-fructose, D-cellobiose, sucrose, D-xylose, trehalose, and citrate (1.0 g L⁻¹ of each carbohydrate) was tested. Fatty acids of the cells in the exponential growth phase were hydrolyzed and methylated by tetramethylammonium hydroxide at 315 °C. The resultant fatty acid methyl esters were then analyzed using GC/MS (6850 Network GC system and 5975C-VL-MSD with a Triple-Axis Detector; Agilent). Various enzymatic activities were analyzed by an API ZYM kit (BioMerieux) according to the manufacturer's instructions except for the incubation temperature, which was adjusted to 45 °C. The protease, amylase, chitinase, cellulase, and xylanase activities of the strains were examined using solidified media (plates) containing individual substrates and detected by a clear zone (halo) appearing around the colonies.

Phylogenetic Analysis. The 16S rDNA of the strains was amplified by PCR using the bacterial universal primers B27F (forward; 5'-AGAGTTTGATCMTGGCTCAG, positions 8-27 based on *Escherichia coli* numbering) and U1492R (reverse; 5'-GGYTACCTTGTTACGACTT, positions

1510-1492) (Kurosawa *et al.* 2010). The following thermal cycle was used 30 times: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1.5 min. DNA sequencing was performed by the dideoxynucleotide chain termination method. The 16S rDNA sequences of strains BEK6 and BEK11 were compared with the available 16S rDNA sequences in the Genbank DNA database using BLASTN (<http://www.ncbi.nlm.nih.gov/blast/>; Altschul *et al.* 1990). Seventeen 16S rDNA sequences of related species belonging to the family *Bacillaceae* were aligned using CLUSTALW (Thompson *et al.* 1994); all sites with gaps in any of the sequences and PCR primers were eliminated from the alignment. Phylogenetic trees with bootstrap sampling were reconstructed by the neighbor-joining method (Saitou and Nei 1987) using the Genetyx-Win software (Genetyx). The almost full-length 16S rDNA sequences of strains BEK6 and BEK11 were deposited in the DNA database with the accession numbers AB607229 and AB607228, respectively.

RESULTS

We isolated twelve moderately thermophilic bacterial strains designated BEK3, BEK5, BEK6, BEK7, BEK9, BEK10, BEK11, BEK12, BEK13, BEK14, BEK15, and BEK16 from a saline hot spring in Hokkaido, Japan. Preliminary analysis of the partial 16S rDNA sequences (approximately 500 bp) of these isolates indicated that they were closely related to each other and belonged to the genus *Bacillus*. However, the analysis also indicated that strains BEK6, 7, and 10 showed no or very low β -galactosidase activity compared with other strains such as BEK3, 5, 9, 11, 12, 13, 14, 15, 16. Moreover, these β -galactosidase-negative strains, particularly BEK6, showed poor growth at 55 °C compared with the other strains. Strain BEK11 showed the highest cell density at 55 °C. Therefore, we chose strains BEK6 and BEK11 as representatives of the individual phenotypes for further characterization (Table 1).

Strains BEK6 and BEK11 were obligate aerobes and Gram-positive motile rods. The colonies of both strains were matt white, circular, and convex (average, 0.5-1.0 mm) with slightly undulate margins. The lengths of BEK6 and BEK11 cells were 0.6 × 2-3 μ m and 0.6 × 3-7 μ m, respectively (Fig 1). In the stationary growth phase, both strains formed endospores. In addition, they showed catalase and oxidase activities. Nitrate reduction and indole production by these strains were not detected. Optimal growth temperatures of strains BEK6 and BEK11 were approximately 45 °C and 50 °C, respectively. Both strains appeared to be slightly alkaliphilic and grew at pH 7-10. The strains

were relatively halotolerant and grew in a medium containing 10% (w/v) NaCl. The major fatty acid identified was C₁₆, which constituted approximately 50% of the total fatty acid content; no unsaturated fatty acid was found in either strain (Table 2). Phylogenetic analysis indicated that strains BEK6 and BEK11 were most closely related to *B. aeolius* (Gugliandolo *et al.* 2003) and *B. alveayuensis* (Bae *et al.* 2005; Fig 2). The 16S rDNA sequences of the isolated strains showed 97.1% and 96.6% similarity with those of *B. aeolius* and *B. alveayuensis*, respectively.

We used the API-ZYME kit to analyze the enzymatic activities of strains BEK6 and BEK11; both strains showed alkaline phosphatase and esterase activities. As mentioned earlier, the β -galactosidase

activity was detected only in strain BEK11. Protease and amylase activities were detected by the plate tests performed at 45 °C and 1% NaCl, and the strain BEK11 produced larger halos on both substrates. Activities of chitinase, cellulose, and xylanase were not detected. Subsequently, the protease and amylase activities of strain BEK11 were examined at higher temperatures and NaCl concentrations; these activities were observed at 50 °C in the presence of 3% NaCl. The utilization of carbohydrates also differed between strains BEK6 and BEK11. Strain BEK6 utilized maltose, glucose, fructose, cellobiose, sucrose, and trehalose, while strain BEK11 was able to use galactose and melibiose, in addition to those used by strain BEK11.

Table 1 Phenotypic characteristics of the strain BEK6, BEK11 and relative species.

Properties	BEK6	BEK11	<i>Bacillus aeolius</i>	<i>B. alveayuensis</i>
Colony color	white	White	ND	cream
Cell form	rods	Rods	rods	Rods
Cell width×length (μm)	0.6×2-3	0.6×3-7	0.5×2.0	0.5-1.5×2.5-5
Motility	+	+	+	+
Spore forming	+	+	+	+
Aerobic growth	+	+	+	+
Anaerobic growth	-	-	-	-
Growth temperature range (°C)	25-55	25-55	37-65	40-65
Optimal growth temperature (°C)	45	50	55	55
Growth pH range	7.0-10.0	6.0-10.0	7.0-9.0	6.5-9.0
Optimal growth pH	7.0-8.0	7.0-8.0	8.0	7.0-7.5
NaCl range for growth (%)	0-13	0-11	0.5-5.0	0-4.0
Optimal NaCl concentration (%)	2-3	2-3	2	3
Catalase	+	+	-	+
Oxidase	+	+	+	-
Nitrate reduction	-	-	-	-
Indole production	-	-	-	-
Gram reaction	+	+	+	+

B. aeolius, *B. aeolius* 4-1^T(Gugliandolo *et al.* 2003); *B. alveayuensis*, *B. alveayuensis* TM1^T(Bae *et al.* 2005); ND, not determined.

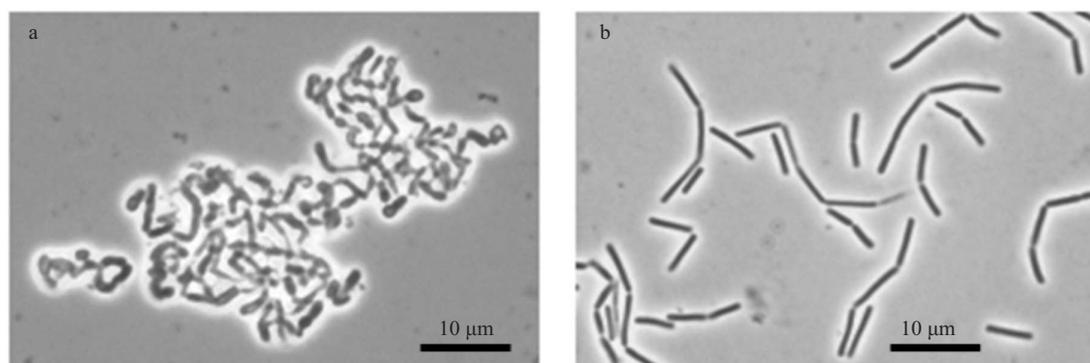


Fig 1 Phase contrast micrographs of the strains. a, BEK6 and b, BEK11

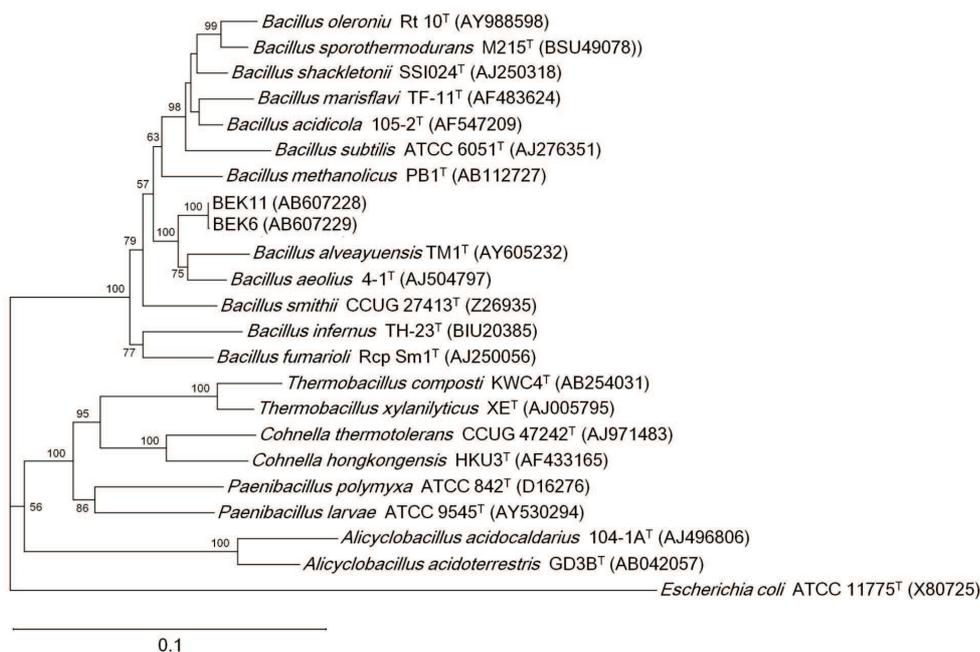


Fig 2 NJ-tree for the strain BEK6, BEK11 and relative species. The bootstrap values (1000 sampling) above 50% are shown at nodes.

Table 2 Fatty acid composition of the strain BEK6, BEK11 and relative species.

Fatty acid composition	BEK6	BEK11	" <i>B. aeo</i> " ¹	<i>B. alv</i> ²
10:0		1.7		
12:0				4.4
13:0				2.2
14:0			2.3	2.2
iso-14:0				0.6
15:0			2.0	3.5
iso-15:0			8.5	15.5
anteiso-15:0			2.5	3.5
16:0	51.5	49.6	33.2	11.1
iso-16:0			12.5	10.1
anteiso-16:0	7.3	8.8		
16:1 ω 9				0.9
16:1 ω 13 <i>t</i>				1.4
17:0	9.6	8.7	4.9	0.7
iso-17:0	8.2	10.5	11.1	15.4
anteiso-17:0			10.1	11.1
Cyclohexan-17:0				1.5
18:0	14.8	11.1	12.8	4.8
anteiso-18:0	8.6	8.0		
18:1 ω 9				3.0
19:0		1.6		

¹*B. aeolius* 4-1^T(Gugliandolo *et al.* 2003), ²*B. alveayuensis* TM1^T(Bae *et al.* 2003).

DISCUSSION

Phylogenetic analysis using 16S rDNA sequences indicated that strains BEK6 and BEK11 were affiliated with the genus *Bacillus*. As mentioned in the results section, the phylogenetically closest species was *B. aeolius*. However, strains DSM 15084 and CIP 107628, which have been deposited as the type strains of *B. aeolius*, are not representative of the original type

strain, strain 4-1. Attempts to obtain a subculture of the original strain from the depositor were in vain because the strain is no longer available in the depositor's laboratory (Pukall *et al.* 2008). According to Rule 18c of the International Code of Nomenclature of Bacteria (Lapage *et al.* 1992), Pukall *et al.* 2008 proposed that the Judicial Commission of the International Committee on Systematics of Prokaryotes places the name *B. aeolius* (Gugliandolo *et al.* 2003) on the list of rejected specific and subspecific epithets in names of species and subspecies of bacteria, if a suitable replacement for the type strain or a neotype is found within two years of publication of their request. Although *B. aeolius* has not been placed on the rejected list until date, the closest available strain (to strains BEK6 and BEK11) is possibly *B. alveayuensis* strain TM1^T (Bae *et al.* 2005). The similarity of 16S rDNA sequences between the representative strains and *B. alveayuensis* was 96.6%, suggesting that strains BEK6 and BEK11 differ from *B. alveayuensis*. As shown in Tables 1 and 2, many phenotypic differences between the isolates and *B. aeolius* or *B. alveayuensis* were also identified. In particular, strains BEK6 and BEK11 were more tolerant to alkaline and saline conditions than strains of the most closely related species, *B. alveayuensis*. Phenotypic and phylogenetic evidence suggested that the newly isolated strains were novel species of the genus *Bacillus*. Moreover, we examined the secreted hydrolyzing enzymatic activities of strains BEK6 and BEK11 and detected relatively strong protease and amylase activities from strain BEK11 at 50 °C in the presence of 3% NaCl, indicating that these

enzymes can be used at relatively high temperatures and saline environments. Further enzymatic characterization and chemotaxonomic analysis for the validation of the isolates as a novel species are now in progress.

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