SHORT REPORT

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Biodiversity of thermotolerant *Bacillus* sp. producing biosurfactants, biocatalysts, and antimicrobial agents

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ABSTRACT: Thermotolerant bacteria isolated from soil and water samples taken from 76 hot springs in Thailand were investigated for their biosurfactant, biocatalytic, and antimicrobial properties. DNA samples purified from 148 pure isolates were PCR amplified using primers specific for the 16S rDNA hypervariable region of the genus *Bacillus*. The DNAPAR phylogenetic tree clearly demonstrated that these isolates were related to *Bacillus niacini*, *B. korensis*, *B. firmus*, *B. flexus*, *B. megaterium*, *B. pumilus*, *B. licheniformis*, *B. subtilis*, *B. fusiformis*, *Oceanobacillus picturae*, *Anoxybacillus gonensis*, *Aneurinibacillus thermoaerophilus*, and *Anoxybacillus flavithermus*. Determination of their biosurfactant properties using the oil spreading technique indicated that *B. megaterium* (SR8.5), *B. licheniformis* (CM8.2), and *B. pumilus* (SR4.4) possessed the highest biosurfactant activity. In addition, lipase, cellulase, and xylanase activities were detected from *B. megaterium* (CP1, SR8.5), *B. pumilus* (PR3.2), and *B. licheniformis* (CM1.1, LP3, MH11.3, LP3, NN2.3, PR2.3, PR2.2, PT1.2). While *B. subtilis* (CM3.3, CM14.1) had the highest cellulase activity, most isolates showed either undetectable or relatively low lipase and xylanase activities.

KEYWORDS: hot spring, thermophilic bacteria

INTRODUCTION

Microbial biodiversity in extreme environments is generally considered to be rare compared to most other environments[1]. A large number of aerobic or facultatively anaerobic, rod-shaped, endospore-forming bacteria, many of which belong to the genus *Bacillus*, are widely distributed in the environment. They are classified as acidophilic, alkalophilic, thermophilic or others according to their nature[3]. Thermophilic *Bacillus* species, with an optimal growth temperature lying between 45 °C and 70 °C, have been isolated from a wide range of environments[2], and the crucial features associated with heat-tolerance have been widely documented. Most of them are nonpathogenic, with high secretion capacity, and are competent in producing various biological substances such as secretory proteins, enzymes, biofilm, biosurfactants, and antibiotics.

Among the degradative enzymes that hydrolyse natural substrates, cellulases and xylanases are very important as cellulose and xylan are major components of hemicellulose and are the most abundant natural polymers. Cellulosic and hemicellulosic materials are of economic importance as they have many uses and are renewable. Xylans are heteropolysaccharides consisting of a β-1,4-xylopyranosyl backbone with branches of acetyl arabinosyl and glucuronyl residues[4]. Cellulose, a linear polymer, consists of glucose subunits linked by β-1,4-glucosidic linkage[5]. Xylanase and cellulase are valuable for use in various applications such as biomass production, feed additives treatments, biobleaching and biopulping processes, food processing, and enzymatic conversion for chemical production[6,7]. Lipase (triacylglycerol acyl hydrolase), which catalyses the hydrolysis of long chain acylglycerols to fatty acids, is another biotechnologically important enzyme widely used in the dairy and textile industries, production of detergents and surfactants, and oil processing[8,9]. Biosurfactants produced by several microorganisms also have potential applications for the food industry, cosmetics, pharmaceuticals, the chemical industry, enhanced recovery of oil, bioremediation, and also in medicine[10]. The present study examines the antimicrobial activity, biosurfactant production, and enzyme production of thermotolerant bacteria isolated from 76 hotsprings in Thailand.

MATERIALS AND METHODS

Sample collection

Samples were collected from 76 hot springs (34–92 °C, pH 5–9) in Thailand. Each sample containing an estimated ratio of 1:1 (water, solid) (v/w) hot spring water and surface sediment was collected using a sterile spoon, transferred to a 20 ml sterile bottle, and divided into aliquots (5 ml of each) for parallel analyses. All specimens were kept on ice until reaching the laboratory.

Bacterial culture and DNA extraction

Thermotolerant bacteria were isolated as single individual colonies and were cultured in Luria-Bertani (Difco) at 45 °C for 14 h with agitation at 250 rpm. The cells were collected by centrifugation at 10000 g for 10 min prior to lysis of the cells at room temperature for 1 h in lysis buffer (10 mM Tris-HCl, 20 mM glucose, 100 mg/ml lysozyme, 2% SDS). The soluble fraction obtained after centrifugation was used for determination of antimicrobial, biosurfactant, and enzyme activities. DNA of thermotolerant bacteria was prepared by the conventional phenol/chloroform DNA extraction method [11].

PCR amplification and DNA sequencing of the 16S rDNA hypervariable region

PCR amplification of the 16S rDNA hypervariable region was performed according to the method of Keiichi et al [3]. PCR fragments were purified using the QIAquick gel extraction kit. DNA sequences of the PCR product were determined using the dideoxy DNA sequencing method based on the big dye terminator cycle sequencing procedure and were analysed using a genetic analyser (ABI Prism 3100, Perkin Elmer).

Phylogenetic analysis

Using CLUSTALX version 1.83 [12], the sequences of the 16S rDNA hypervariable region of the bacterial isolates were aligned and clustered against those available from GenBank. *Thermus thermophilus* was used as the outgroup to root the 16S rDNA hypervariable regions. Blocks of sequence data contain 255 characters with a data matrix of 173 taxa. Phylogenetic trees were constructed by using PAUP version 4.0b10 [13], and cladograms were created by using maximum parsimony methods. Bootstrap values were replicated 1000 times and computed using PAUP.

Screening for antimicrobial activities

The antimicrobial activity of the soluble fraction of bacterial lysate against gram positive and gram negative bacteria was determined using the disc diffusion method [14]. The diameter of clear zone around the disc was measured and scored in a range from 1+ to 3+ corresponding to weak to strong inhibitory effects of the isolates on the test organisms.

Screening for biosurfactant production

The oil spreading technique was used to screen the isolates for biosurfactant activity [15]. The diameter of the clear zone on the oil surface was measured and scored from '1+' to '4+' corresponding to the diameter of the clear zone produced by each isolate.

Screening for enzyme activity

All isolates were screened for lipase activity on agar plates containing tributyrin agar (Difco) as substrate. After point inoculation, the plates were incubated at 45 °C for 48 h and the presence of lipase producing bacteria was indicated by a clear halo around the colonies [16].

To test for cellulase activity, the bacterial isolate was inoculated onto a CMC agar plate containing carboxymethyl cellulose as the sole carbon source and incubated at 45 °C for 3 days. Cellulase producing bacteria were identified by the presence of clear halo around the colony after flooding the plate with Congo red [17].

Determination of xylanase activity was accomplished by using xylan agar. Xylanase-producing bacteria were identified by a clear halo around the colony [17].

RESULTS

Phylogenetic analysis

In the biodiversity study of the culturable bacteria isolates from 76 hot springs in southern Thailand, 148 bacterial isolates were obtained (Table 1). The phylogenetic analysis of the 16S rDNA hypervariable region from the isolates suggested classification of the bacteria into 13 groups of *Bacillus* spp.: *B. niacini*, *B. korensis*, *B. firmus*, *Oceanobacillus picturae*, *B. flexus*, *B. subtilis*, *B. licheniformis*, *B. megaterium*, *B. pumilus*, *B. fusiformis*, *Anoxybacillus flavithermus*, *Anoxybacillus gonensis*, and *Anneurinibacillus thermoaerophilus* (Fig. 1).

Table 1 Antimicrobial activity (B = *B. subtilis*, S = *S. aureus*, E = *E. coli*, P = *P. aeruginosa*, A = *Aspergillus* sp., F = *Fusarium* sp., R =*Rhizopus* sp., C = *Candida* sp.), biosurfactant production (BP) at 144 h, and enzyme production (EP, lip = lipase, cel = cellulase, xyl = xylase) of the isolates.

Isolate	Accession	Strain	Antimicrobial activity at 7 days[a]								BP[b]	EP[c]		
	number		B	S	E	P	A	F	R	C		lip	cel	xyl
CM1.1	EF547255	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	2+	1+	1+
CM1.2	EF547256	*A. flavithermus*	-	-	1+	-	-	-	-	-	1+	1+	3+	-
CM1.3	EF547254	*B. licheniformis*	-	1+	-	-	-	-	-	-	1+	1+	1+	-
CM2	EF547257	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	3+	-
CM3.1	EF547259	*B. subtilis*	-	-	-	-	-	-	-	-	1+	-	1+	-
CM3.2	EF547260	*B. subtilis*	-	-	-	-	-	-	-	-	2+	-	1+	2+
CM3.3	EF547258	*B. subtilis*	-	-	-	-	-	3+	-	-	1+	-	4+	2+
CM4.1	EF547261	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	1+	1+
CM4.2	EF547262	*A. thermoaerophilus*	-	-	-	-	-	-	-	-	-	-	-	-
CM5.1	EF547263	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
CM5.2	EF547264	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
CM5.3	EF547265	*B. licheniformis*	-	-	-	-	-	-	-	-	-		-	-
CM7	EF547266	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	1+	1+
CM8.1	EF547267	*B. licheniformis*	-	1+	-	-	-	-	-	-	1+	-	1+	-
CM8.2	EF547268	*B. licheniformis*	-	-	-	-	-	-	-	-	4+	-	1+	-
CM9	EF547269	*B. megaterium*	-	-	-	-	-	-	-	-	1+	-	-	-
CM11	EF547270	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
CM12	EF547271	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
CM13.1	EF547272	*B. subtilis*	-	-	-	-	-	-	-	-	-	-	-	-
CM13.2	EF547273	*B. megaterium*	-	-	-	-	-	-	-	-	3+	1+	-	-
CM14.1	EF547274	*B. subtilis*	-	-	-	-	-	-	-	-	1+	-	4+	-
CM14.2	EF547275	*B. subtilis*	-	-	-	-	-	-	-	-	-	-	-	-
CM14.3	EF547276	*B. subtilis*	-	-	-	-	-	2+	-	-	2+	-	2+	-
CM15.1	EF547277	*B. subtilis*	-	-	-	-	-	-	-	-	-	-	-	-
CM15.2	EF547278	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	1+	1+	1+
CM15.3	EF547279	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
CM15.4	EF547280	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
CM15.5	EF547281	*B. licheniformis*			-	-	-	-	-	-			-	-
CM16	EF547282	*B. subtilis*	-	-	-	-	-	2+	-	-	1+	-	1+	2+
MH1.1	EF547283	*B. licheniformis*	-	1+	-	-	-	-	-	-	2+	-	-	-
MH1.2	EF547284	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	1+	-
MH1.3	EF547285	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
MH8.1	EF547287	*B. megaterium*	-	-	-	-	-	-	-	-	1+	-	-	-
MH8.2	EF547288	*B. licheniformis*	-	-	-	-	-	-	-	-	2+	-	1+	1+
MH8.3	EF547289	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
MH9	EF547290	*B. subtilis*	-	-	-	-	-	2+	-	-	2+	-	3+	-
MH10	EF547291	*B. licheniformis*	-	1+	-	-	-	-	-	-	1+	-	1+	-
MH11.1	EF547292	*B. licheniformis*	-	1+	-	-	-	-	-	-	1+	-	1+	-
MH11.2	EF547294	*B. licheniformis*	-	-	-	-	-	1+	-	-	3+	-	2+	1+
MH11.3	EF547293	*B. licheniformis*	-	1+	-	-	-	-	-	-	2+	1+	1+	1+
CR1.1	EF547295	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	1+	-
CR1.2	EF547296	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	-	-
CR2	EF547297	*B. subtilis*	-	-	-	-	-	2+	-	-	2+	-	3+	1+
CR3	EF547298	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	-	-
CR4.1	EF547299	*B. licheniformis*	-	-	1+	-	-	-	-	-	1+	-	1+	-
CR4.2	EF547300	*B. subtilis*	-	1+	1+	-	-	-	-	-	1+		1+	1+
CR5	EF547301	*B. subtilis*	-	-	-	-	-	2+	-	-	2+	-	3+	-
CR7	EF547303	*B. koreensis*	-	-	-	-	-	-	-	-	-	-	-	-

[a] antimicrobial activity clear zone diameter: 1+ = < 10 mm, 2+ = 10–20 mm, 3+ = > 20 mm

[b] biosurfactant property clear zone diameter: 1+ = < 10 mm, 2+ = 10–20 mm, 3+ = 21–30 mm, 4+ = 31–40 mm

[c] enzyme activity clear zone diameter: 1+ = < 10 mm, 2+ = 10–20 mm, 3+ = > 20 mm - = not detected

Isolate	Accession number	Strain	Antimicrobial activity at 7 days[a]								BP[b]	EP[c]		
			B	S	E	P	A	F	R	C		lip	cel	xyl
CR8	EF547304	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
CR9	EF547305	*B. licheniformis*	-	1+	-	-	-	-	-	-	1+	1+	1+	-
CR10.1	EF547306	*B. subtilis*	-	-	-	-	-	-	-	-	-	-	-	-
CR10.2	EF547307	*B. megaterium*			-								-	-
LP1.1	EF547308	*B. licheniformis*	-	-	-	1+	-	-	-	-	1+	1+	-	-
LP1.2	EF547309	*B. licheniformis*	-	1+	-	-	-	-	-	-	2+	1+	1+	-
LP3	EF547310	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	1+	1+	1+
LP4	EF547311	*B. licheniformis*	-	-	-	-	-	-	-	-	2+	1+	1+	-
LP5	EF547312	*B. subtilis*	-	-	-	-	-	2+	-	-	-	-	-	-
LP7.1	EF547313	*B. megaterium*	-	-	-	-	-	-	-	-	1+	1+	1+	-
LN2.1	EF547315	*B. pumilus*	-	-	-	-	-	-	-	-	-	-	-	-
LN2.2	EF547316	*B. megaterium*		-									-	-
NN1.1	EF547317	*B. pumilus*	1+	-	-	-	-	-	-	-	2+	1+	1+	-
NN1.2	EF547318	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
NN1.3	EF547319	*B. pumilus*	1+								1+	1+	3+	-
NN2.1	EF547320	*B. licheniformis*	1+	-	-	-	-	-	-	-	1+	-	1+	-
NN2.2	EF547321	*B. fusiformis*	-	1+	-	-	-	1+	-	-	1+	-	-	-
NN2.3	EF547322	*B. licheniformis*	1+	-	-	1+	-	-	-	-	2+	1+	1+	1+
PR1.1	EF547323	*B. pumilus*	-	1+	-	-	-	-	-	-	1+	1+	1+	-
PR1.2	EF547324	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	1+	-
PR2.1	EF547325	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
PR2.2	EF547326	*B. pumilus*	-	1+	-	-	-	-	-	-	1+	1+	2+	-
PR2.3	EF547327	*B. licheniformis*	-	2+	-	-	-	-	-	-	1+	1+	1+	1+
PR2.4	EF547328	*B. pumilus*		-	-	-	-	-	-	-	1+	1+	1+	-
PR3.1	EF547329	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
PR3.2	EF547330	*B. pumilus*	-	-	-	-	-	-	-	-	1+	1+	2+	1+
PR4.1	EF547331	*B. licheniformis*	-	-	-	-	-	2+	-	-	1+	-	-	-
PR4.2	EF547332	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	1+	-
PR4.3	EF547333	*B. pumilus*	1+	-	1+	-	-	-	-	-	3+	1+	-	-
CP1	EF538838	*B. megaterium*	-	-	-	-	-	-	-	-	1+	1+	2+	2+
RN1	EF538823	*B. pumilus*	-	-	-	-	-	-	-	-	-	-	-	-
RN7.1	EF538826	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
RN8	EF538827	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	-	-
PG2.1	EF538832	*B. megaterium*	1+	1+	-	-	-	-	-	-	1+	-	-	-
PG2.2	EF538829	*B. niacini*	-	-	-	-	-	-	-	-	-	-	-	-
PG2.3	EF538831	*B. megaterium*		-	-	-	-	-	-	-	-	-	-	-
KB1	EF538834	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	1+	-
KB3.2	EF538836	*B. megaterium*	-	-	1+	1+	-	-	-	-	1+	-	1+	-
KB5	EF538839	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	1+	1+	-
TR1.2	EF538847	*B. pumilus*	-	1+	-	-	-	-	-	-	1+	1+	-	-
TR2.1	EF538846	*B. fusiformis*	-	-	-	1+	-	1+	-	-	1+	-	1+	-
TR2.2	EF538848	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
PL2.1	EF538853	*B. pumilus*	-	-	-	-	-	-	-	-	-	-	-	-
ST1.1	EF538849	*B. subtilis*	-	-	-	-	-	-	-	-	2+	-	1+	-
ST1.2	EF538850	*B. megaterium*	-	-	-	-	-	-	-	-	1+	-	-	-
YL1	EF538844	*B. pumilus*	-	1+	-	-	-	-	-	-	1+	2+	1+	-
NS1	EF538840	*B. korensis*	-	-	-	-	-	-	-	-	-	-	-	-
NS2.1	EF538841	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
NS2.2	EF538842	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
NS2.3	EF538843	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
SR1.1	EF538807	*B. pumilus*	-	-	-	1+	-	-	-	-	1+	-	1+	-
SR1.2	EF538808	*O. picturae*	-	-	-	-	-	2+	-	-	3+	-	-	2+

[a] antimicrobial activity clear zone diameter: 1+ = < 10 mm, 2+ = 10–20 mm, 3+ = > 20 mm

[b] biosurfactant property clear zone diameter: 1+ = < 10 mm, 2+ = 10–20 mm, 3+ = 21–30 mm, 4+ = 31–40 mm

[c] enzyme activity clear zone diameter: 1+ = < 10 mm, 2+ = 10–20 mm, 3+ = > 20 mm - = not detected

Isolate	Accession number	Strain	Antimicrobial activity at 7 days[a]								BP[b]	EP[c]		
			B	S	E	P	A	F	R	C		lip	cel	xyl
SR4.1	EF538812	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
SR4.2	EF538813	*B. megaterium*	1+	-	2+	1+	-	-	-	-	1+	-	-	-
SR4.3	EF538814	*B. flexus*	-	-	-	-	-	-	-	-	-	-	-	-
SR4.4	EF538815	*B. pumilus*	-	1+	1+	-	-	-	-	-	3+	-	1+	-
SR5	EF538816	*B. megaterium*	-	-	-	-	-	-	-	-	3+	-	1+	-
SR7	EF538817	*B. firmus*	1+	1+	1+	-	-	-	-	-	1+	-	-	-
SR8.1	EF538818	*B. megateriums*	-	-	-	-	-	-	-	-	-	-	-	-
SR8.2	EF538820	*B. megaterium*	-	1+	1+	1+	-	-	-	-	2+	1+	1+	-
SR8.3	EF547360	*B. megaterium*	-	1+	1+	-	-	-	-	-	1+	-	1+	1+
SR8.4	EF538821	*B. flexus*	-	-	-	-	-	-	-	-	-	-	-	-
SR8.5	EF538819	*B. megaterium*	-	1+	1+	-	-	-	-	-	4+	1+	1+	1+
UT1.1	EF538855	*B. pumilus*	-	1+	-	-	-	-	-	-	1+	-	1+	-
UT1.2	EF538856	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
KP3.1	EF538859	*B. pumilus*	-	-	1+	-	-	-	-	-	4+	-	1+	-
KP3.2	EF538860	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
KP3.3	EF538861	*B. megaterium*	-	1+	-	-	-	-	-	-	1+	-	-	-
KP4.1	EF538862	*B. licheniformis*	-	1+	-	-	-	-	-	-	2+	1+	-	1+
SK1.1	EF538864	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
SK2.1	EF538865	*B. pumilus*	-	-	-	-	-	-	-	-	-	-	-	-
SK2.2	EF538866	*B. pumilus*	-	-	-	-	-	-	-	-	1+	-	1+	-
SK2.3	EF538867	*B. subtilis*	-	-	-	1+	-	2+	-	-	1+	-	1+	-
PB1.1	EF538873	*B. pumilus*	-	-	-	-	-	-	-	-	1+	-	1+	-
PB1.2	EF538874	*B. subtilis*	-	-	-	1+	-	-	-	-	1+	-	1+	-
PB2.1	EF538869	*B. pumilus*	-	-	-	-	-	-	-	-	1+	-	2+	2+
PB2.2	EF538870	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	1+	3+	1+
TK1	EF547335	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
TK2.1	EF547336	*B. pumilus*	-	-	-	-	-	-	-	-	1+	-	1+	2+
TK2.2	EF547337	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
TK2.4	EF547339	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
TK2.5	EF547340	*B. subtilis*	-	-	-	-	-	-	-	-	1+	-	1+	1+
TK2.6	EF547341	*B. pumilus*	-	-	-	-	-	-	-	-	1+		1+	-
TK3.1	EF547342	*B. licheniformis*	1+	-	-	1+	-	-	-	-	1+	1+	1+	-
TK4.1	EF547344	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
TK4.2	EF547345	*B. subtilis*	-	-	-	-	-	-	-	-	-	-	-	-
TK4.3	EF547346	*B. pumilus*	-	-	-	-	-	-	-	-	-		-	-
KC2	EF547347	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	-	2+	-
KC4.1	EF547348	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
KC5.1	EF547349	*B. subtilis*	-	-	-	-	-	2+	-	-	2+	-	1+	-
KC5.2	EF547350	*B. subtilis*	-	-	-	-	-	-	-	-	-	-	-	-
KC5.3	EF547351	*B. pumilus*	-	-	-	-	-	-	-	-	2+	-	1+	-
KC5.4	EF547352	*B. pumilus*	-	-	-	-	-	-	-	-	1+	-	2+	-
KC5.5	EF547353	*A. gonensis*	-	-	-	-	-	-	-	-	-	-	-	-
KC5.6	EF547354	*B. megaterium*	-	-	-	-	-	-	-	-	-	-	-	-
PT1.1	EF547355	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	1+	1+	-
PT1.2	EF547356	*B. licheniformis*	-	-	-	1+	-	-	-	-	1+	1+	1+	1+
PT1.3	EF547357	*B. licheniformis*	-	-	-	-	-	-	-	-	-	-	-	-
PT1.4	EF547358	*B. licheniformis*	-	-	-	-	-	-	-	-	1+	1+	-	-
RB1	EF547359	*B. subtilis*	-	-	-	1+	-	-	-	-	2+	-	2+	-

[a] antimicrobial activity clear zone diameter: 1+ = $<$ 10 mm, 2+ = 10–20 mm, 3+ = $>$ 20 mm

[b] biosurfactant property clear zone diameter: 1+ = $<$ 10 mm, 2+ = 10–20 mm, 3+ = 21–30 mm, 4+ = 31–40 mm

[c] enzyme activity clear zone diameter: 1+ = $<$ 10 mm, 2+ = 10–20 mm, 3+ = $>$ 20 mm - = not detected

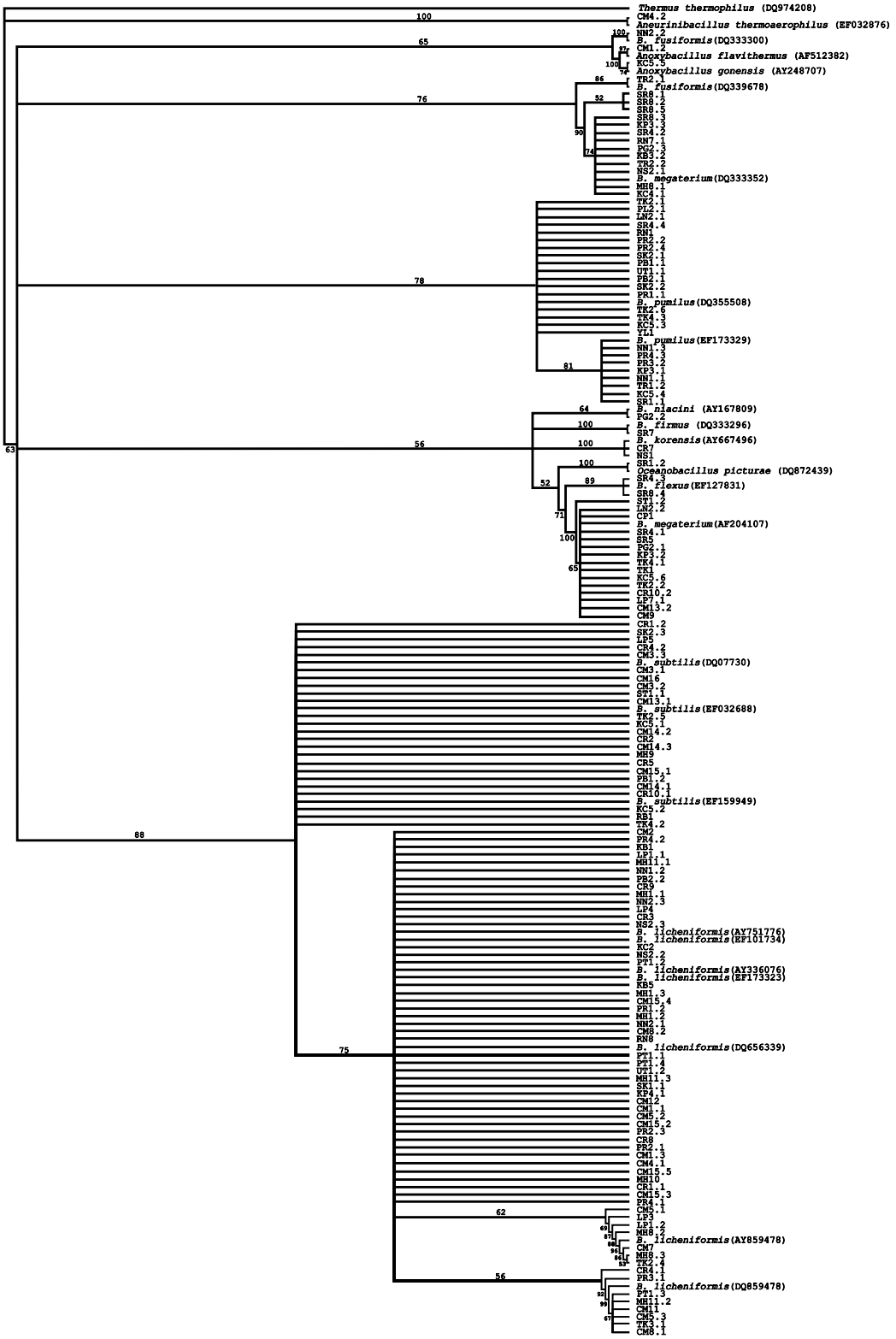

Fig. 1 Phylogenetic relationship among moderately thermotolerant bacteria based upon maximum parsimony analysis of sequences of the 16S rDNA hypervariable region. Sequence codes refer to those given in Table 1. The values indicate the percentage of occurrence in 1000 bootstrapped trees.

Antimicrobial activities

The bacterial lysate of many *Bacillus* isolates from hot spring water in Thailand showed variable inhibitory effects against the test organisms (Table 1). The lysate from *B. megaterium* (SR4.2) could inhibit the gram positive bacteria (*B. subtilis* and *S. aureus*), while that from *B. megaterium* (SR8.2, KB3.2) and *B. subtilis* (CR4.2) inhibited the gram negative bacteria (*E. coli* and *P. aeruginosa*). The lysate from *B. firmus* (SR7), however, was able to inhibit both gram positive (*B. subtilis* and *S. aureus*) and the gram negative bacterium (*E. coli*). Likewise, the lysate from *B. licheniformis* (TK3.1 and NN3.3) and *B. pumilus* (PR4.3) inhibited both gram positive and gram negative bacteria. Some *Bacillus* isolates exhibited inhibitory activity against only one gram positive and one gram negative bacteria. For example, the lysate from *B. megaterium* isolates SR4.4, SR8.3, SR8.5 inhibited the growth of *S. aureus* and *E. coli*, while the lysate from *B. megaterium* isolate PG2.1 inhibited the growth of *B. subtilis* and *S. aureus*. Interestingly, some species of *Bacillus* isolates could inhibit the test organisms belonging to the same genus, i.e. lysate from *B. subtilis* inhibited the growth of *B. pumilus* (NN1.1, NN1.3, PR4.3, TK3.1), *B. licheniformis* (NN1.2, NN2.1, NN2.3), *B. megaterium* (PG2.1, SR4.2) and *B. fermus* (SR7), while some strains such as *B. pumilus* (PR3.2) and *B. megaterium* (CP1, RN7.1, RN8, PG2.3) showed no effect on the growth of *B. subtilis*. *Fusarium* sp., the only test fungus in the present study, was inhibited by lysate of the *Bacillus* isolates, *B. subtilis* (CM3.3), *B. subtilis* (CM16, MH9, CR2, CR5, LP5, KC5.1), *B. fusiformis* (NN2.2, TR2.1), and *B. licheniformis* (PR4.1), and *B. subtilis* (SK2.3) (Table 1). Our results also revealed that *O. picturae*, the only non-*Bacillus* in this study, could inhibit *Fusarium* sp.

Biosurfactant capability

Almost all isolates produced biosurfactant agents. *B. licheniformis* (CM8.2), *B. pumilus* (KP3.1) and *B. megaterium* (SR8.5) produced biosurfactant at the highest level. The others that produced a rather high level were *B. licheniformis* (MH11.3, KP4.1), *B. subtilis* (MH11.2), *B. megaterium* (CM13.2 , SR5), and *B. pumilus* (SR4.4, KP4.4, PR4.3). The only non-*Bacillus* that also produced biosurfactant at a rather high level was *O. picturae*.

Enzyme activities

Most *Bacillus* isolates showed cellulase activity while only some species showed lipase and xylanase activities. The *Bacilli* that possessed activities for all three enzymes were *B. megaterium* (CP1, SR8.5), *B. pumilus* (PR3.2) and *B. licheniformis* (CM1.1, LP3, MH11.3, LP3, NN2.3, PR2.3, PR2.2, and PT1.2). *B. subtilis* (CM3.3 and CM14.1) were among those that had the highest cellulose hydrolysing activity. However, most isolates were found to have either low or no lipase or xylanase activities. Those that had lipase activity were *B. licheniformis* (CM1.1, CM1.3, MH11.3, CR9, LP1.1, LP1.2, LP1.3, LP4, NN2.3, PR2.3, KP1.1, PR2.2, TK3.1), *B. pumilus* (NN1.1, NN1.3, PR1.1, PR2.2, PR2.4, PR3.2, PR4.3, TR1.2, YL1), *B. megaterium* (LP7.1, CP1, R8.2, SR8.5, LP4.1) and *B. subtilis* (CM13.1), and those that possessed xylanase activity were *B. licheniformis* (CM4.1, CM7, MH8.2, MH11.2, LP1.2, NN2.3, PR2.3, KB5, PR2.2, PT1.2), *B. subtilis* (CM3.2, CM3.3, CM16, CR4.2, TK2.5) *B. pumilus* (PR3.2, PB2.1), and *B. megaterium* (CP1, SR8.3, SR8.5, LP4.1).

DISCUSSION

The results from all testing and assays revealed that all *Bacillus* isolates could produce biosurfactants. It has been previously reported that the common biosurfactants produced by *Bacillus* spp. are lipopeptides[18,19]. Production of surfactants is important for the survival of the organisms as the compounds facilitate the adhesion and attachment of bacterial cells to natural substrates. In addition, considerable evidence suggests that biosurfactants may be involved in a protective mechanism against unfavourable environmental conditions[20,21].

The data revealed that the soluble fraction from *B. megaterium* (SR4.2, SR8.2, SR4.4, SR8.3, SR8.5), *B. firmus* (SR7), *B. licheniformis* (TK3.1 and NN3.3), and *B. pumilus* (PR4.3) exhibited antimicrobial activity against both gram positive and gram negative bacteria[22,23]. The antibacterial activity of many isolates are very interesting because they showed an inhibitory effect against a broad spectrum of bacteria. In addition to antimicrobial activity, the soluble fraction from *B. megaterium* (TR2.1) also exhibited antifungal activity which was similar to that of *B. subtilis*[24]. The antifungal and antibacterial activities of the *Bacillus* isolates could presumably enable the bacteria to compete for survival against other species.

The bacterial isolates also showed some hydrolytic activities. Almost all of them exhibited cellulase activity, indicating that the organisms could use cellulose which is abundant in the environment and is a substrate that can be used for producing glucose for the generation of energy. Among the cellulase producing *Bacillus* spp., *B. licheniformis*, *B. subtilis*, *B. pumilus* and *B. megaterium* could produce this

enzyme[25]. However, most of the *Bacillus* isolates did not exhibit xylanase activities, suggesting that xylan was not their actual substrate. The complete degradation of xylan requires several hydrolytic activites of many enzymes such as xylanases, β-xylosidases, α-glucuronidases, and esterases. Therefore our data suggests that the isolates did not have specific enzymes for xylan degradation. Similarly, most of the bacterial isolates exhibited either low or no lipase activity which is presumably due to the low level of lipid substrates in hot spring areas.

In conclusion, the 148 pure isolates from high-temperature environments proved to be promising candidates for further studies on the characterization and identification of biosurfactants, cellulases and antimicrobial agents. The isolates of some *Bacillus* spp. could be used as sources of thermostable biologically active compounds.

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