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Original Article

Molecular Characterization and Phenotype Analysis of a Novel Psychrotrophic *Exiguobacterium* Species from the Ilam Mountains

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Abstract

Introduction: The genus *Exiguobacterium* includes psychrotrophic, mesophilic, and moderate thermophilic strains and species that have been isolated from extreme environments, including very cold or hot environments. The genus *Exiguobacterium* has received much attention in biotechnology, and related industries due to secretory enzymes with high enzymatic activity and consequently stable enzymes capable of tolerating extreme conditions. The aim of the present study was to introduce and describe the phenotypic and the genotypic characterization of a novel species of genus *Exiguobacterium* isolated from the Ilam mountains, Iran.

Materials and Methods: Genotypic features were analyzed using universal genes (*gyrB, hsp70, rpoB, and citC*) belonging to the *Exiguobacterium* genus. Also, *cspC1, nusA* and *dnaJ* genes were used to confirm the profile of new psychrotrophic strains. The distinctive phenotypic characteristics of the new strain with the most famous strains of the genus *Exiguobacterium* were investigated. All statistical analyses were conducted using R Packages for data visualization and exploratory data.

Results: A motile, Gram-stain-positive, rod-shaped, non-sporing, tolerant up to 5% NaCl, grew at 0-25 °C and pH 6 and 9, designated *Exiguobacterium* sp. HA2 was isolated from the soil. The major isoprenoid quinone is MK-7 and in the smaller amount are MK-6 and MK-8. The major cellular fatty acids (>10 %) were isoC13:0, isoC15:0 and C16:0. To adapt to low temperatures, *Exiguobacterium* sp. HA2 upregulated expresses cold shock response including cold shock protein (CSP) and transcription elongation protein NusA. Also, downregulated expression of heat shock protein DnaJ domain protein.

Conclusions: In the current study we investigated the difference in the genotype, phenotypic, and functional characteristics of *Exiguobacterium* sp. HA2. It can be regarded as representing a novel psychrotrophic species within the genus *Exiguobacterium*.

Keywords: Psychrotrophic, Genus *Exiguobacterium*, Phenotype Analysis, R packages, Data Analysis

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Introduction

The genus *Exiguobacterium* belonging to the family *Bacillaceae* of the phylum Firmicutes was first described in 1983 with isolation and characterization of *Exiguobacterium aurantiacum* from potato-processing effluent, and now, this genus accommodates 24 recognized species.[1] The members of the genus *Exiguobacterium* are low G + C, Gram-positive facultative anaerobic bacteria, with morphological variety (ovoid, rods, double rods, and chains) depending on species and environmental conditions.[2,3] This versatile genus includes psychrotrophic, mesophilic, and moderate thermophilic strains and species, that have been isolated from a wide range of habitats, including ancient Siberian permafrost, Greenland glacial ice, hot springs at Yellowstone National Park and the food processing environments.[4,5] Hence, the isolation sources of this bacterium are totally different and often extreme environments.[3]

The comparative analysis of *Exiguobacterium* genomes has revealed a high degree of divergence in the genomes of various strains. In general, this genus has been falling into two distinct groups based on the *16S rRNA* gene sequence analysis: group I forms the psychrophilic strains, and group II includes thermophilic strains, except *Exiguobacterium* sp. GIC3.[3,6] Each of these strains appear to have specifically and exclusively adapted to the environments they were isolated from, and the diversity in their genome has been responsible for their adaptation to different conditions.[7] For instance, *Exiguobacterium sibiricum* is one of the main members of this family, which is reputed to survive at -20 °C for several

weeks in soy culture medium with no bacterial protection against the cold.[8,9]

Some of the *Exiguobacterium* strains from different niches have unique traits that make them suitable for applications in biotechnology, detergent industry, sewage treatment, and agriculture, which include: neutralizing highly alkaline wastewater and treatment of azo dye wastewater, reducing arsenate to arsenite and, chrome and mercury, pesticide removal and plant growth-promoting.[10] Furthermore, different *Exiguobacterium* strains can produce several enzymes that exhibit considerable thermal stability, such as alkaline protease, EKTA catalase, guanosine kinase, ATPases, dehydrogenase, esterase. This bacterium also possesses genes encoding proteins that make them resistant to oxidative and UV stress and Heavy metals such as mercury, arsenic, copper, cadmium.[10,11] The remarkable properties and wide applications range of this bacterium can be of particular importance to the industry, the environment, and even space research projects.

Psychrotrophic microorganisms have been described that can be growing at 4 °C and are restricted to <25 °C.[12] Typically, psychrophilic and psychrotrophs bacterium contains cold-adapted enzymes that show higher catalytic activity at very low temperatures. From the biotechnological point, cold-adapted enzymes can the provide potential for the biotechnology industry. Cold-adapted enzymes can help to save energy in washing processes, food processing or bioremediation. Using this enzyme can prevent the growth of mesophilic contaminants at low temperatures in food processing.[7,8]

In this study, an orange-pigmented bacterial strain, designated sp. HA2, was isolated from the Ilam Mountains and subjected to detailed characterization phenotypic, genotypic and chemotaxonomic investigation. We analyzed the association of genes previously reported involved in adaptation to cold stress condition through real-time quantitative PCR.

Materials and Methods

Sample Collection and Isolation

Strain sp. HA2 was isolated while investigating the bacterial diversity of a soil sample which was collected from the kabir mountain (33° 13′ 16″ N 47° 06′ 18″ E), Ilam Province, Iran. The soil sample was serially diluted and dispensed on Tryptic Soy Agar (TSA) medium (Difco Laboratories, Detroit, MI, USA) at the 4 °C for 10 days to determine the bacterial density. The strain sp. HA2 was purified by repeatedly transferring a single colony on the same medium and stored at -80 °C in tryptic soy broth (TSB, Difco, Sparks, MD, USA) supplemented with 20% (w/v) glycerol. The reference strains *E. antarcticum* DSM 14480T, *E. sibiricum* DSM 17290T, *E. undae* DSM 14481T, *E. Indicum* IAM 15368T, *E. oxidotolerans* JCM 12280T, *E. acetylicum* DSM 20416T were used for phenotypic analysis.

Microscopic Analyses

For scanning electron microscopy (TESCAN, VEGA3), cells grown were suspended in 50 mM of PBS and fixed with 3% glutaraldehyde overnight at 4 °C. Cells were washed again and resuspension in PBS solution. The final pellet was resuspended in 4% R2A agar. Secondary fixation was achieved by incubation in 1% (w/v) aqueous osmium tetroxide solution (OsO_4) for 1 h and dehydrated through a graded ethanol series solution (15% for 15 min, 50%, 75% and 90% for 30 min, and 100% for 1 h), and embedded in Spurr's resin (EM Sciences, Fort Washington, PA), then polymerized for 24 h at 50 °C.

Physiology and Biochemical Characterization

Gram-staining was performed as described previously.[13] Minimum and maximum temperatures for growth was investigated on R2A agar after incubation for 5 days at 0 to 25 °C (0, 4, 10, 15, 20, 25 °C). Tolerance to NaCl was determined by incubating R2A broth supplemented with NaCl (0-10%, w/v, at 0.5% intervals) concentrations in 1.5 ml. The pH range was evaluated using R2A broth containing various pH 6-9 (at intervals of 0.5 pH unit) prior to autoclaving using appropriate buffers. Presence of spores was evaluated by staining with malachite green. API 20NE, API ID 32GN and API ZYM (bioMerieux) were used for analyzing the production of different enzymes. Fatty acid extracted and analysis was determined by gas chromatography (GC) as described previously.[14] Antibiotic sensitivity testing was performed using standard methods.[15-17] The culture's ability to assimilate different carbon compounds was assessed using minimal medium [K_2HPO_4 2% (w/v); KH_2PO_4 0.5% (w/v); agar 1% (w/v)]. The analysis of chemotaxonomic markers was performed through cell wall amino acids,[18] polar lipids,[19] peptidoglycan structure[20] and isoprenoid quinones.[21] *E. sibiricum* DSM 17290, *E. antarcticum* DSM 14480T, *E. oxidotolerans* JCM 12280T, *E. acetylicum* DSM 20416T, and *E. undae* DSM 14481T were used as reference strains in morphological and biochemical assessments and fatty acids identification.

Genomic Characterization

Genomic DNA was extraction and purification using the THP (Triton/Heat/Phenol) slight modification of protocol.[22] DNA quantity and quality were determined using the NanoDrop (NanoDrop 3300, Thermo Fisher Scientific Inc. USA). Also, Phylogenetic comparisons were performed between the mentioned new strain and sequences of four other genes including *gyrB* (gyrase subunit B), *hsp*70 (Class I-heat shock protein-chaperonin), *rpoB* (DNA-directed RNA polymerase beta subunit), and citC (isocitrate dehydrogenase). To confirm bacterial identification, we amplified the genes (*16S rRNA*, *gyrB*, *hsp*70, *rpoB*, *citC*) directly by using the universal primers for *Exiguobacterium*

(Table1). To analysis genes related cold shock proteins response, we used the primer genes (*csp1*, *nusA*, *dnaJ)* in Table 2. The annealing temperatures were 53 °C for *rpoB*, *csp1*, and 49 °C for *citC*, *nusA*, *dnaJ* and *gyrB*, and 47 °C for *hsp*70. The PCR amplified products were purified using QIAquick Kit (PCR Purification Kit, Qiagen, Valencia, CA, USA) and then sequenced.[8] The TOPO TA Cloning Kit (Life Technologies) was used for the cloning and sequencing of the amplicons of other genes. The Qiagen Plasmid Kit (Qiagen, Valencia, CA, USA) was then used to extract the obtained clones. An ABI 373a DNA sequencer was used to analyze the products of cycle sequencing performed by a Perkin-Elmer 9600 thermal cycler and an ABI Dye Terminator Chemistry (PE Applied Biosystems).

Table 1. Primers Used for PCR Amplification for *Exiguobacterium* Genus Analysis

Gene Amplified	Sequence (5´– 3´)	Fragment Size (bp)
16S rRNA		1506
16S-F	AGG GTT GCG CTC GTT G	
16S-R	AAG GAG GTG WTC CAR CC	
DNA Gyrase-beta Subunit		930
gyrB-F	AAA CGT CCG GGT ATG TAT ATC GGA TCG AC	
gyrB-R	CGG CGG CTG SGC AAT RTA SAC GTA	
ClassI-heat Shock Protein-chaperonin	CCC GAA TTC GGT AHA GTA AAA TGG TTY AAC KC	1453
hsp70-F	CCC GGA TCC GGT TAC GTT ASC WGC TKS HGG	
hsp70-R	DCC	
DNA-directed RNA Polymerase Beta Subunit		1072
rpoB-F	CGA ACA TGC AAC GTC AGG C	
rpoB-R	ACA TCY TCY TCA CGN GCA CC	
Isocitrate Dehydrogenase		1163
citC-F	GGD GAY GGM ACW GGW CCW GAY ATT TGG	
citC-R	AAT TCW GAA CAT TTM ACT TCT GT	

N = A:T:C:G; H = A:T:C; D = T:G:A; K = T:G; Y = C:T; M = C:A; W = A:T; R = A:G; S = C:G; all 1:1

Table 2. Primers Used for qPCR for Influence of Cold Stress on *E.* sp. HA2

Gene Amplified	Sequence (5´– 3´)	Fragment Size (bp)
Cold Shock Response		91
cspC1-F	AAC AAG GTA AAG TGA AAT GG	
cspC1-R	GCT GAG AAG TGA ACG AA	
Cold Shock Response		206
nusA-F	CGT TGC TTT CGC TGG TT	
nusA-R	AGA TTG CCG TTG CTT CC	
Protein Folding		157
dnaJ-F	TTG GTC GTG TCG TGA	
dnaj-R	ATC TGT TGT CCG TTA TCG	
House Keeping Gene		103
16S rRNA-F	TAC AGA AGA GAA GAG TGG AA	
16S rRNA-R	TCA GCG TCA GTT ACA GA	

RNA Extraction and Real-Time PCR of Genes Influencing Cold Stress

Total RNA was extracted from *Exiguobacterium* sp. HA2 using the RNA extraction reagent TRIzol (DNA Biotech) per manufacturer's instructions after growing in LB agar at 4 °C and 25 °C for 48-96 h. To investigate the effect of temperature on genes involved in the cold-adapted process different temperatures were used. Total RNA quantity and quality were determined using the NanoDrop (NanoDrop 3300, Thermo Fisher Scientific Inc. USA). For cDNA synthesis, BioFact™ RT kit was used and reaction mixture were followed as mentioned in the protocol given with the kit. Primers were designed using AlleleID software version 7.5 (Premier Biosoft, USA). The relative expression of genes was calculated using the ($2^{-\Delta\Delta Ct}$) method and normalized using the *16S rRNA* as the internal control. All sample reactions were performed in triplicates and the mean of the three reactions was considered as a representative value for each sample.

Data Analysis

Four genes belonging to the *Exiguobacterium* genus were used for analysis (*gyr*B, *cit*C, *rpo*B, and *hsp*70). All statistical analysis and graphics were conducted using R version 4.1.1 (Kick Things) including the "Adegenet", "ape", "ggtree", "ggplot2", "stats", "ips", and "msa" R-package. We constructed phylogenetic trees based on the neighbor-joining and distance methods. The phylogenetic tree was reconstructed with generated by the maximum-likelihood method and the reliability of each node was assessed with 1000 bootstrap test replications. The scale bar represents 0.1 substitution per nucleotide position. The roots of the trees were determined using those genes from Bacillus.

Results

Phenotypic Characteristics

The major isoprenoid quinone is MK-7 and in the smaller amount are MK-6 and MK-8. Polar lipids compositions including diphosphatidylglycerol, phosphatidylglycerol, phosphatidylserine,

phosphatidylinositol, phosphatidylethanolamine. Major fatty acids (>10%) are iC13:0, iC15:0 and C16:0; minor components are listed in Table 3. The peptidoglycan type is lysine-glycine.

Cells of strain sp. HA2 were Gram-positive, non-sporing, motile, facultatively anaerobic, rod-shaped, approximately 0.8-1 µm in width and 1.5-2 µm in length (Figure 1). Growth occurred at between 0 and 25 °C (optimally, 15 °C), pH grows at 6-10 (optimally, pH 7.0) and in the presence of 0-10 (w/v) NaCl with an optimum of approximately 5 (w/v) NaCl. Positive in tests for oxidase, gelatinase, b-galactosidase, DNase, catalase, caseinase, phosphatase, lysine decarboxylase, but are negative for, urease and H_2S production and for the indole test. *Exiguobacterium* sp. HA2 utilize D-fructose, D-galactose, D-mannose, L-rhamnose, cellobiose, D-lactose, maltose, starch, amygdalin, arbutin, glycogen, citrate utilization. Strain sp. HA2 was susceptible to amikacin (30 µg), amoxicillin (30 µg), clindamycin (25 µg), colistin (10 µg), doxycycline (25 µg), co-trimoxazole (25 µg), nalidixic acid (30 µg), norfloxacin (10 µg), nitrofurantoin (300 µg), sulfamethoxazole (50 µg), tobramycin (15 µg), lomefloxacin (30 µg), roxithromycin (30 µg), ciprofloxacin (30 µg), lincomycin (15 µg), cefotaxime (30 µg), cefazolin (30 µg), kanamycin (30 µg), novobiocin (30 µg), chloramphenicol (30 µg), ampicillin (25 µg), tetracycline (30 µg), streptomycin (25 µg), erythromycin (15 µg), bacitracin (10 µg), polymyxin B (50 µg), oleandomycin (15 µg), spectinomycin (100 µg), rifampicin (25 µg) and carbenicillin (100 µg). But resistant to ceftriaxone (30 µg) and gentamycin (10 µg). The phenotypic features and biochemical profile of sp. HA2 compare with other reference strains are description and illustrated in Table 4.

Table 3. Fatty Acid Composition of the Siberian Permafrost Isolates and the Type Strains of *Exiguobacterium*

	HA2	7-3[b]	255-15[b]	190-11[b]	*E. undae*[a]	*E. antarcticum*[a]	*E. aurantiacum*[a]
iC11:0							2
iC12:0	3	2	2	2	2	3	3
C12:0	1	1		1		1	2
iC13:0	11	9	13	8	9	12	18
AiC13:0	8	11	15	10	9	11	12
iC14:0	9	1	1	1	2	1	
C14:0	2	3	1	2	2	2	3
iC15:0	11	13	12	13	10	11	4
aiC15:0	4	3	4	3	3	2	
C16:1-11c	9	8	3	7	8	18	10
iC16:0	3	2	2	2	2		
C16:1-5c		1		1			
C16:1-7c		1		1	7	3	
C16:0	16	17	20	12	17	13	27
C17:1-10c	4	2	2	3	2	3	
iC17:0	5	9	12	8	7	5	6
aiC17:0	3	3	3	3	2		
C18:1-9c		2	1	2	3	6	2
C18:1-7c	2	2	1	3	3		
C18:0	5	4	5	3	6	5	5

Strains: 1, *Exiguobacterium* sp. HA2; 2, *Exiguobacterium* strains 7-33; 3, *Exiguobacterium* strains 255-15; 4, *Exiguobacterium* sp. 190-11; 5, *E. undae* DSM 14481T; 6, *E. antarcticum* DSM 14480T; 7, *E. aurantiacum* DSM 6208T.
Only values >1% are indicated; values ≥5% are given in bold.
[a]Data obtained from Rodrigues et al. (2006)[9]
[b]Data obtained from Fruhling et al. (2002)[18]

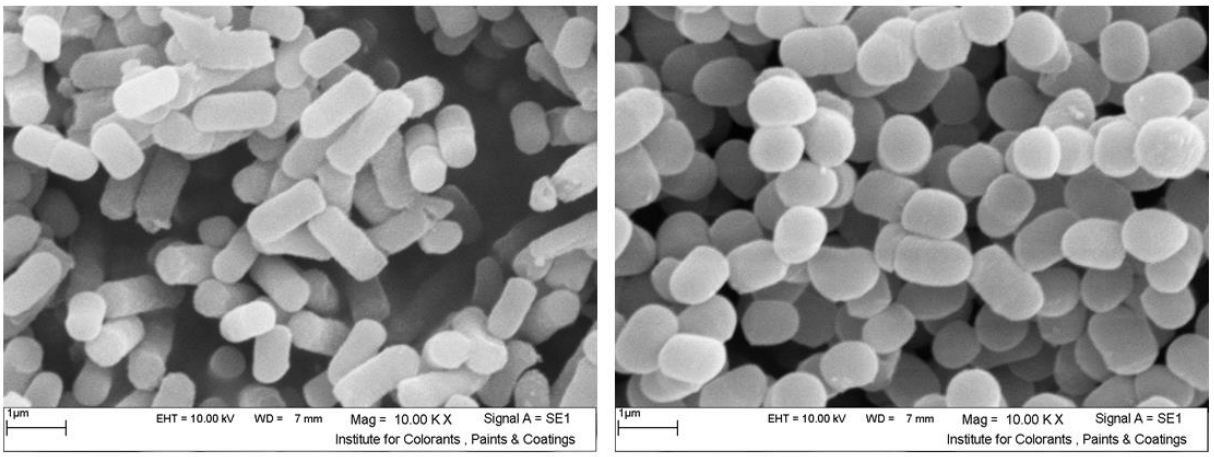


Figure 1. Scanning Electron Microscopy Images of *Exiguobacterium* sp. HA2 with Different Resolutions.

Table 4. Phenotypic Features Distinguishing Strain *Exiguobacterium* sp. HA2 from the Six Most Closely Related Species of the Genus *Exiguobacterium*

Characteristics	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Source	Soil	Microbial Mat	Siberian	Garden Pond	Glacial Water	Drainage From	Creamery Waste
Colony Size (cm)	2-3	2-3	2-3	2-4	2-4	1-5	2-5
Colony Shape	Round	Round	Round	Round	Round	Round	Irregular
Colony Color	Yellowish Orange	Orange	Orange	Orange	Yellowish Orange	Orange	Yellowish Orange
Growth Temperature (°C)							
2.5 °C	+	-	+	-	W	W	-
5 °C	+	-	+	-	+	+	-
37 °C	–	+	+	+	-	+	+
Maximum Growth Temperature (°C)	25	41	40	41	30	40	37
Gelatinase	+	+	+	+	-	+	+
DNase	+	+	-	+	-	+	+
Caseinase	+	+	+	+	-	+	+
Phosphatase	+	+	+	-	+	+	+
Lysine decarboxylase	+	-	+	+	+	-	-
Arginine decarboxylase	+	+	+	+	+	-	-
Tryptophan Eamination	+	+	+	-	-	+	+
Aesculin Hydrolysis	+	+	+	+	-	+	+
Starch hydrolysis	+	+	+	+	-	+	+
Citrate Utilization	-	-	+	-	+	+	+
Malonate Utilization	+	-	-	-	+	-	-
Reduction of Nitrate to Nitrite	-	-	-	-	+	-	-
Methyl Red Test	+	+	-	+	–	+	+
Acid Production From							
Melibiose	-	-	+	-	-	-	-
D-adonitol	-	-	-	-	-	-	+
Cellobiose	+	+	-	-	-	-	+
D-fructose	+	+	+	+	-	+	+
D-glucose	+	+	+	+	-	+	+
D-mannose	+	-	+	+	-	+	+
Maltose	+	+	+	+	-	+	+
D-ribose	+	+	+	+	-	+	+
L-rhamnose	+	+	-	+	-	+	+
Sucrose	+	+	+	+	-	+	+
Trehalose	+	+	+	+	-	+	+
L-xylose	+	-	+	-	-	-	-
Inulin	-	-	+	-	-	-	-
Carbon Source Utilization							
L-arabinose	-	-	+	-	-	-	-
D-arabinose	-	-	+	-	-	-	-
D-ribose	+	+	+	+	-	+	+
D-xylose	-	-	-	-	+	-	-
L-xylose	-	-	-	-	+	-	-
D-fructose	+	+	+	+	-	+	+
D-galactose	+	-	-	+	+	-	-
D-mannose	+	+	-	+	-	+	+
L-rhamnose	+	-	-	-	+	-	-
Cellobiose	+	+	-	+	+	-	+
D-lactose	+	-	+	-	-	-	-
Maltose	+	+	-	+	-	+	+
L-fucose	-	-	-	-	-	-	+
Melibiose	-	-	+	-	+	-	-
Trehalose	+	+	+	+	-	+	+
Melezitose	-	-	-	-	-	-	+
Raffinose	+	+	-	+	+	-	-
Cellulose	+	-	-	+	-	-	-
Inulin	-	-	-	-	+	-	-
Starch	+	+	+	+	-	+	+
Amygdalin	+	+	+	+	-	+	-
Arbutin	+	+	-	+	+	+	-
Salicin	-	-	ND	+	-	-	+
Glycogen	+	+	-	+	+	-	-
Myo-inositol	-	-	-	-	+	-	-
Dulcitol	-	-	-	-	+	-	-
Erythritol	+	-	ND	+	+	+	-
D-mannitol	-	-	-	+	-	+	+
D-sorbitol	+	-	+	-	+	-	-
Xylitol	-	-	-	-	-	-	-
Methyl a-D-mannoside	+	+	-	-	-	-	+
Methyl a-D-glucoside	+	-	-	+	-	+	+
Methyl a-D-galactoside	-	-	-	-	+	-	-

Continue							
Methyl b-D-Galactoside	-	-	-	-	+	-	-
a-ketoglutaric Acid	-	-	-	-	+	-	-
Citric Acid	+	+	-	+	+	-	-
Fumaric Acid	-	-	-	-	+	-	-
C-glucuronic Acid	+	-	-	+	+	-	-
Lactic Acid	+	-	-	-	+	-	-
L-malic Acid	-	-	-	-	+	-	-
Valeric Acid	-	+	ND	-	+	-	-
Sodium Acetate	+	+	-	+	+	-	-
5-ketogluconate	-	-	-	-	+	-	-
Sodium Gluconate	-	-	ND	-	+	-	-
Sodium Fumarate	+	+	ND	-	+	-	-
Sodium Formate	+	+	ND	-	+	-	-
Sodium Succinate	-	-	ND	+	+	-	-
Potassium Acetate	-	-	-	+	+	-	-
Sodium Pyruvate	+	+	-	+	-	+	+
Amino Acid Utilization							
L-alanine	+	+	+	+	+	-	-
L-arginine	+	+	+	+	+	-	-
L-aspartic acid	-	-	-	+	+	+	-
L-asparagine	+	+	-	+	+	-	-
L-cysteine	+	+	-	+	-	-	-
L-creatinine	+	-	-	-	+	-	-
L-glycine	+	-	-	+	+	-	-
L-glutamic acid	+	+	+	-	+	-	-
L-histidine	+	+	+	+	+	-	-
L-isoleucine	+	+	-	+	+	+	-
L-leucine	+	+	-	+	+	-	+
L-lysine	+	+	+	+	+	+	-
L-methionine	+	+	+	+	+	+	-
L-ornithine	+	+	-	+	+	-	-
L-serine	+	-	+	+	+	+	-
L-threonine	+	+	+	+	+	-	-
L-proline	+	+	+	+	+	-	-
L-tryptophan	+	+	ND	-	+	-	-
L-tyrosine	+	+	ND	+	+	-	+
L-valine	+	+	ND	+	+	-	-
Antibiotic Tolerance (µg)							
Amikacin (30)	S	S	S	S	R	S	S
Amoxicillin (30)	S	S	S	S	R	S	S
Clindamycin (25)	S	S	S	S	R	S	R
Colistin (10)	S	R	R	S	R	R	S
Doxycycline (25)	S	S	R	S	R	S	S
Co-trimoxazole (25)	S	S	S	S	R	S	S
Nalidixic acid (30)	S	S	S	S	R	S	S
Norfloxacin (10)	S	S	R	S	R	S	S
Nitrofurantoin (300)	S	S	S	S	R	S	S
Sulfamethoxazole (50)	S	S	R	R	R	S	S
PeptidoglycanD	Lys–Gly	ND	Lys–Gly	Lys–Asp	Lys–Gly	Lys–Gly	Lys–Gly
Quinone (s)	MK-6, MK-7, MK-8	MK-6, MK-7, MK-8	MK-6, MK-7, MK-8	MK-6, MK-7, MK-8	MK-7, MK-8	MK-6, MK-7, MK-8	MK-7
Polar Lipids	PG, DPG, PE, PS, PI,	PG, DPG, PE, PS, PI,	PG, DPG, PE	PG, DPG, PE, PS, PI,	PG, DPG, PE (tr)	PG, DPG, PE, PS, PI,	DPG, PS

Strains: 1, *Exiguobacterium* sp. HA2; 2, *E. antarcticum* DSM 14480T; 3, *E. sibiricum* DSM 17290T; 4, *E. undae* DSM 14481T; 5, *E. indicum* IAM 15368T; 6, *E. oxidotolerans* JCM 12280T; 7, *E. acetylicum* DSM 20416T. +, Positive; 2, Negative; ND, not determined; W, Weakly positive; R, Resistant; S, Sensitive; DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.
Data belonging to strains 2 to 6 obtained from Rodrigues et al. (2006)[9]

Analysis of Genotype

Genotypic analyses were conducted to clarify the phylogenetic relationship between the new strain and the most relevant set of five reference strains. The sequence analysis of the *16S rRNA* gene indicated that new strain was categorized in the genus *Exiguobacterium* (Figure 2). The sequence was deposited in GenBank with the accession number KT967971. The sequences of *gyrB*, *citC*, *rpoB*, *hsp*70, and *csp*, also were deposited in GenBank with the accession numbers of KX574228, MH370480, MH378445, MH378775, and MH379139, respectively. Phylogeny analysis of other genes was performed to confirm identification the phylogenetic relationships of this strain within *Exiguobacterium*. Phylogenetic trees constructed based on *gyrB* (Figure 3), *citC* (Figure 4), *rpoB* (Figure 5), and *hsp*70 (Figure 6) sequences of the following genes. Analysis of the *16S rRNA* gene sequences indicates that sp. HA2 had a high level of similarity to sp. SH3 and *undae*. Also, the alignment and comparison sequence of *gyrB*, *citC*, *rpoB*, and *hsp*70 of sp. HA2 showed highest similarity to *Exiguobacterium undae*. Analysis of the gene sequences of sp. HA2 with other members of the genus *Exiguobacterium* indicated that similarities with *Exiguobacterium undae*.

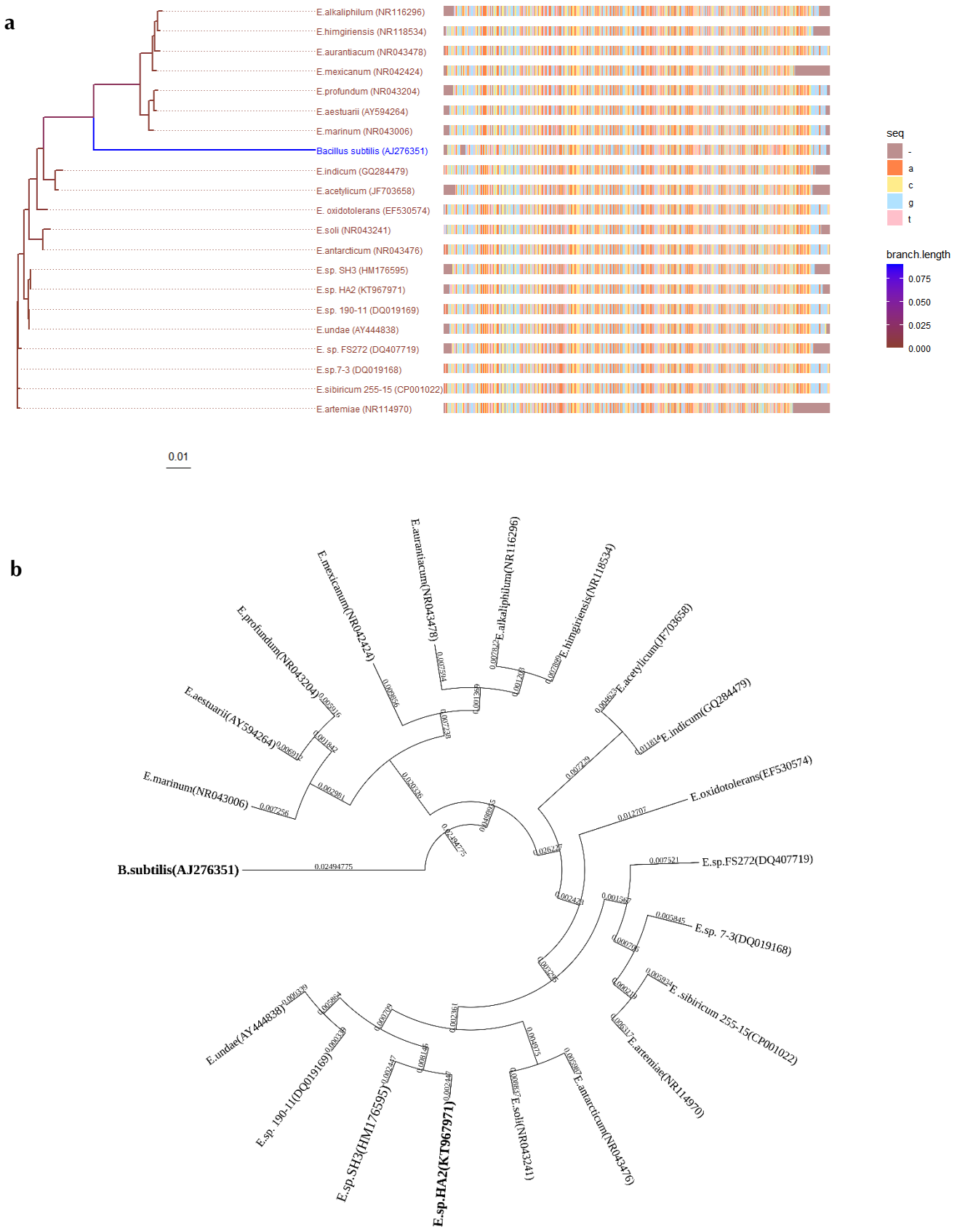


Figure 2. a) Phylogenetic Tree Based on *16S rRNA* Gene Sequence. Numbers at the nodes indicate the bootstrap values on neighbour joining analysis. **b**) Colorized Phylogenetic Tree Representing Populations. The distance scale has shown 0.6% genetic variation per nucleotide substitution.

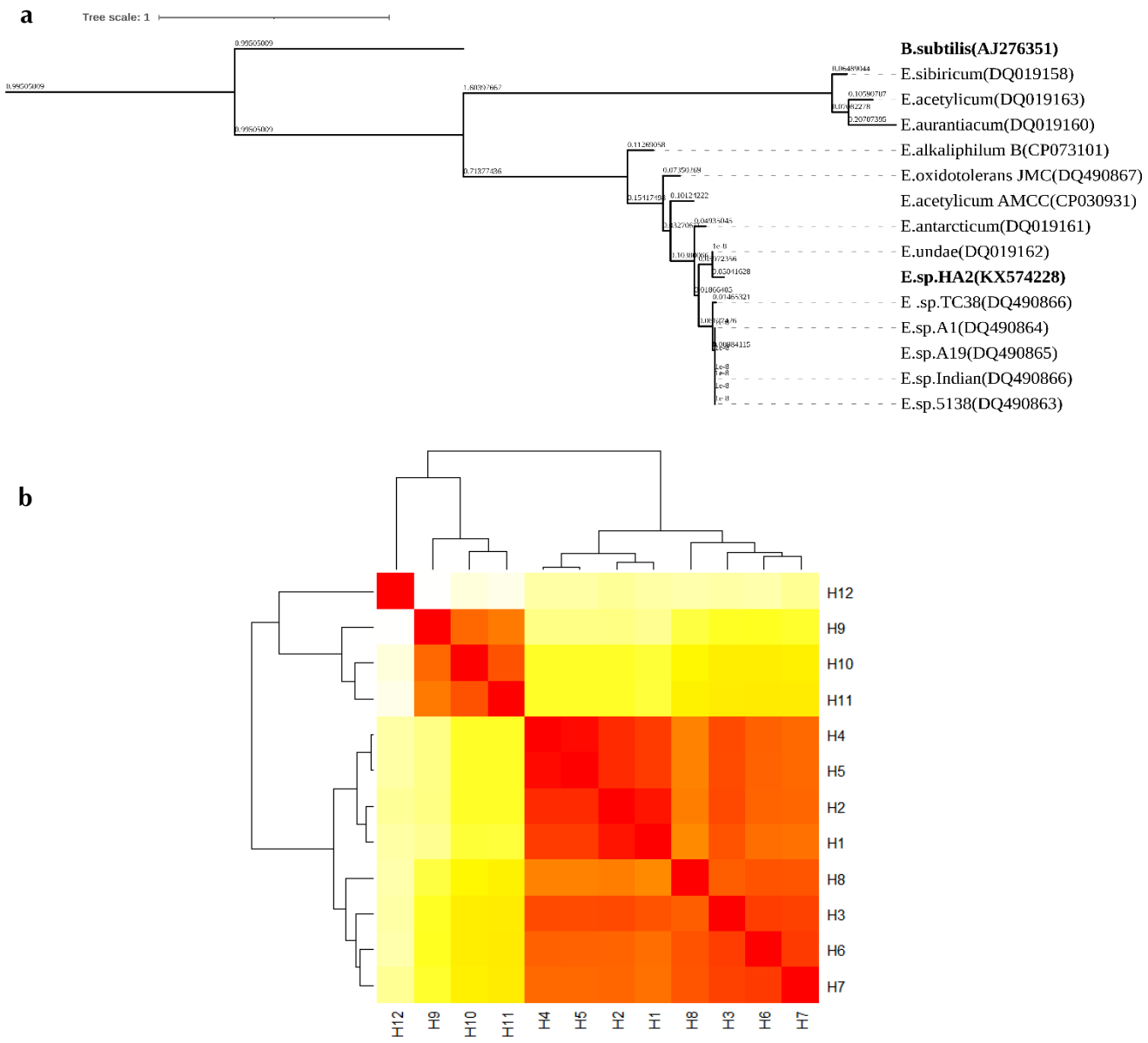
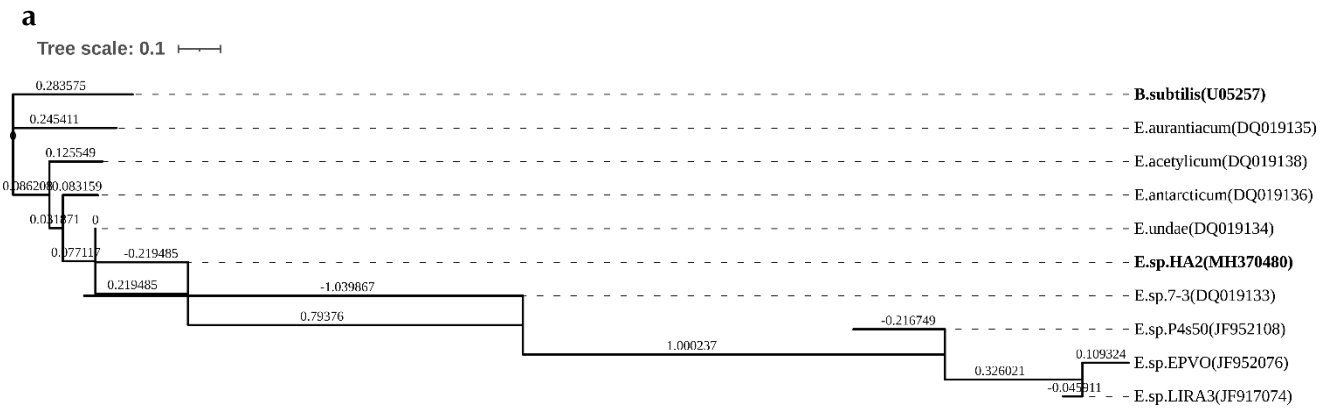


Figure 3. a) Phylogenetic Tree Based on *gyrB* Gene Sequence. Numbers at the nodes indicate the bootstrap values on neighbour joining analysis. **b**) Heat Map Based on the Number of Nucleotide Differences Between the Haplotypes.
1: *E.* sp.HA2 (KX574228), 2: *E. undae* (DQ019162), 3: *E. antarcticum* (DQ019161), 4: *E.* sp. A1 (DQ490864), 5: *E.* sp.5138 (DQ490863), 6: *E.* sp. A19 (DQ490865), 7: *E.* sp. Indian (DQ490866), 8: *E.* sp.TC38 (DQ490866) 9: *E. oxidotolerans* JMC 12880 (DQ490867), 10: *E. acetylicum* AMCC 101217 (CP030931), 11: *E. alkaliphilum* B-3531D (CP073101), 12*: E. sibiricum* (DQ019158), 13: *E. acetylicum* DSM 20416 (DQ019163), 14: *E. aurantiacum* DSM 6208 (DQ019160), 15*: B. subtilis* (AJ276351.1).



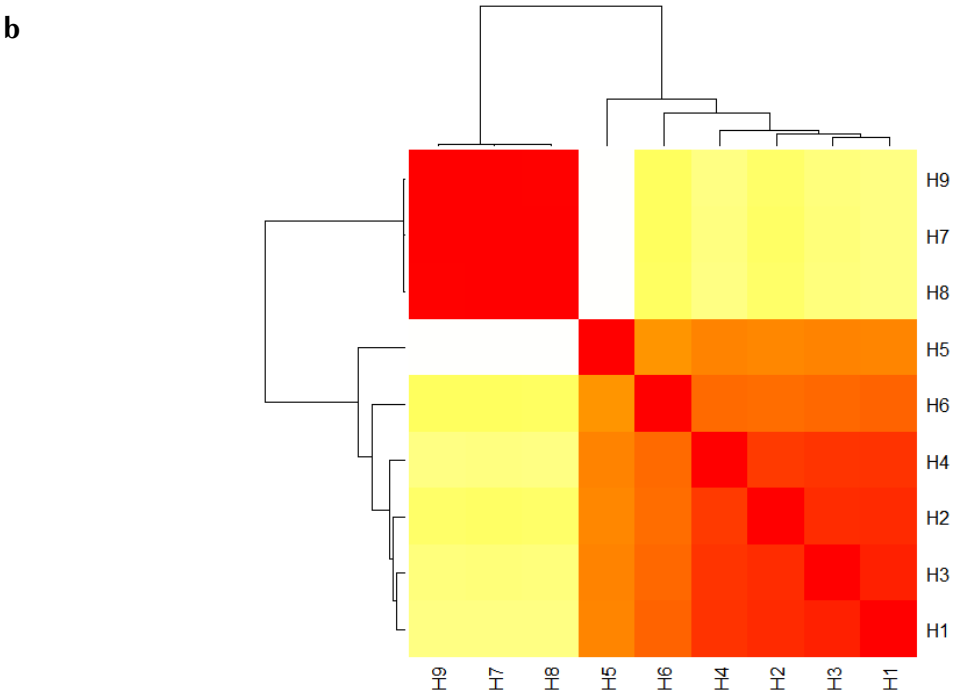
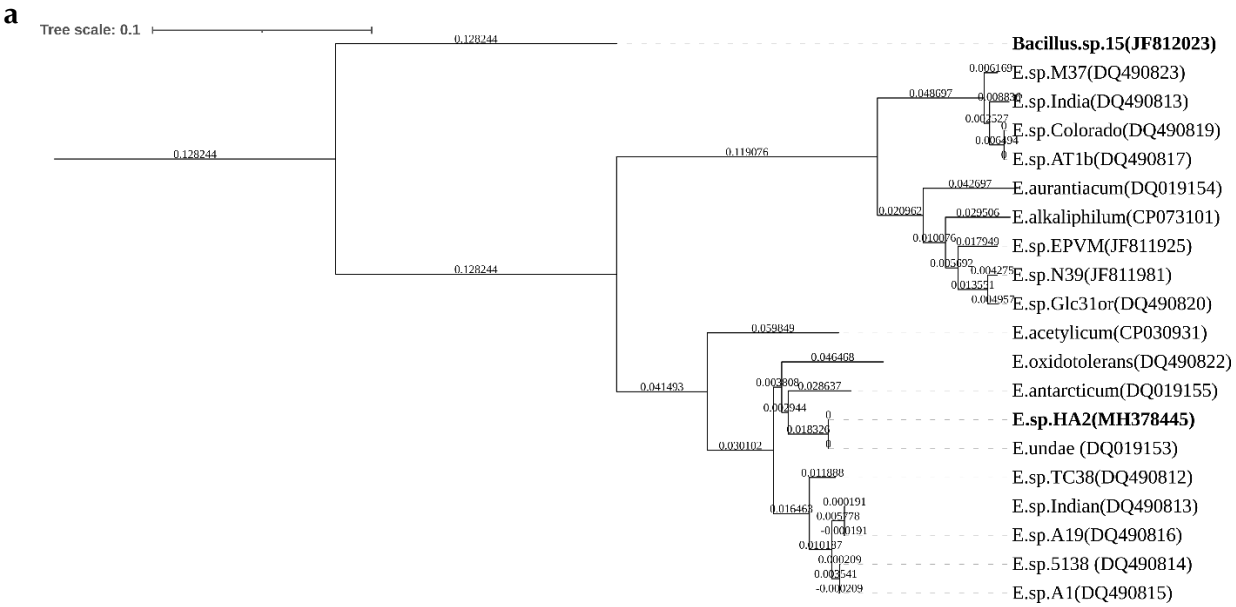


Figure 4. a) Phylogenetic Tree Based on *citC* gene sequence. Numbers at the nodes indicate the bootstrap values on neighbour joining analysis. **b)** Heat Map Based on the Number of Nucleotide Differences Between the Haplotypes.
1: *E.* sp. HA2 (MH370480.1), 2: *E. undae* (DQ019134.1), 3: *E.* sp.7-3 (DQ019133.1), 4: *E. antarcticum* (DQ019136.1) 5: *E. acetylicum* (DQ019138.1), 6: *B. subtilis* (U05257.1), 7: *E. aurantiacum* (DQ019135.1), 8: *E.* sp. LIRA3 (JF917074.1), 9: *E.* sp. P4s50 (JF952108.1), 10: *E.* sp. EPVO (JF952076.1).



Influence of Cold Stress on E. sp. HA2 Gene Regulation

According to previous studies, six classes of the main cold-shock proteins (CSP) are found in the *Exiguobacterium.* All of CSP have conserved 201 bp domain and act as mRNA chaperone at low temperature. The results of our study show that the expression level of *cspC1* was significantly increased at 4 °C compared to 25 °C. (p<0.05, $\log_2$ = 3.21). Previous studies have shown that transcription elongation protein NusA is involved in the process of synthesis of RNA and protein at low temperatures. Our study shows that the level of *nusA* was significantly increased at 4 °C compared to 25 °C. (p<0.05, $\log_2$ = 2.05). DnaJ was reported to be transcriptionally repressed in the response to low temperature and overexpression in the high temperature. Analysis of our data shows that *dnaJ* expression is significantly reduced at 4 °C compared to 25 °C. (p<0.05, $\log_2$ = -1.75). All data related to gene expression in *Exiguobacterium* sp. HA2 are presented in Figure 7.

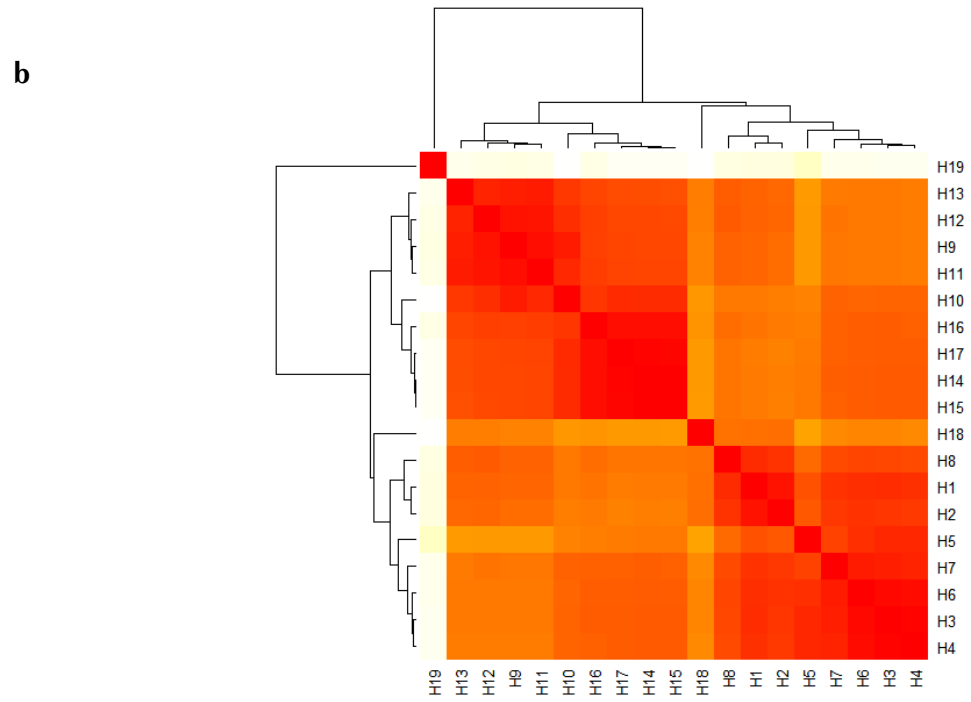
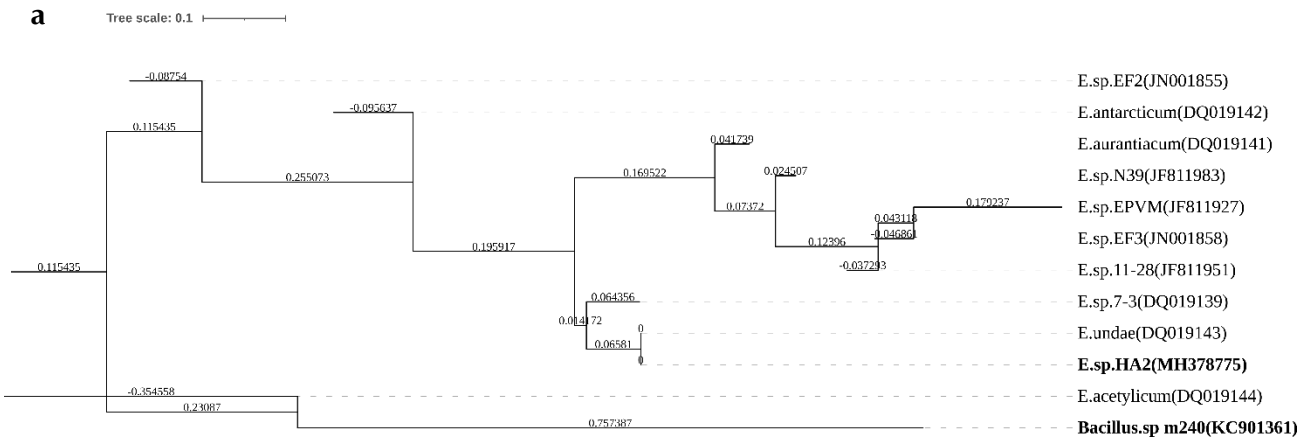


Figure 5. a) Phylogenetic Tree Based on *rpoB* Gene Sequence. Numbers at the nodes indicate the bootstrap values on neighbour joining analysis. **b)** Heat Map Based on the Number of Nucleotide Differences Between the Haplotypes.
1: *E.* sp. HA2 (MH378445), 2: E. undae (DQ019153), 3: E. antarcticum (DQ019155), 4: *E.* sp. 5138(DQ490814), 5: *E.* sp. Indian (DQ490813) 6: *E.* sp. A1 (DQ490815), 7: *E.* sp. TC38 (DQ490812), 8: *E.* oxidotolerans JMC 12880 (DQ490822), 9: *E. acetylicum* AMCC (CP030931), 10: *E.* sp. N39 (JF811981), 11: *E.* sp. Glc31or (DQ490820), 12: E. sp. EPVM (JF811925), 13: *E. alkaliphilum* B-3531D (CP073101), 14: *E. aurantiacum* DSM 620 (DQ019154), 15: *E.* sp. Colorado Road (DQ490819)16: *E.* sp. AT1b (DQ490817), 17: *E.* sp. India Orange (DQ490813), 18: *E.* sp. M37 (DQ490823), 19: *Bacillus* sp. 15.4 (JF812023), 20: *E. sibiricum* (DQ019152).



Discussion

Exiguobacterium genus has been isolated from an impressive range of enormous habitats include microbialites, biofilm, ocean, Saline and alkaline environment and Arctic permafrost. In this study, investigations genotype and phylogeny studies were performed to determine whether the new strain isolates from soil around mountain Ilam are different from other reference strains. For this purpose, using *16S rRNA* gene and six other genes sequence belongs to the *Exiguobacterium* genus. This bacterium had the closest relation with *Exiguobacterium undae*, and thus, it was considered to be from the *Exiguobacter* family, and was dubbed *Exiguobacterium* sp. HA2.

In the present study, this psychrophilic bacterium was identified, stained, and observed under the microscope to find that it is an aerobic, Gram-positive, bacilli-shaped, and non-spore-forming bacterium. Colonies of this bacterium are round and orange in color, and unable to survive in temperatures exceeding 30 °C. Hence, according to Morita definition, it is a psychrotrophic bacterium.[23] According to biochemical studies (Biotyping and molecular), through the sequencing of *16S rRNA* coding gene, this bacterium was

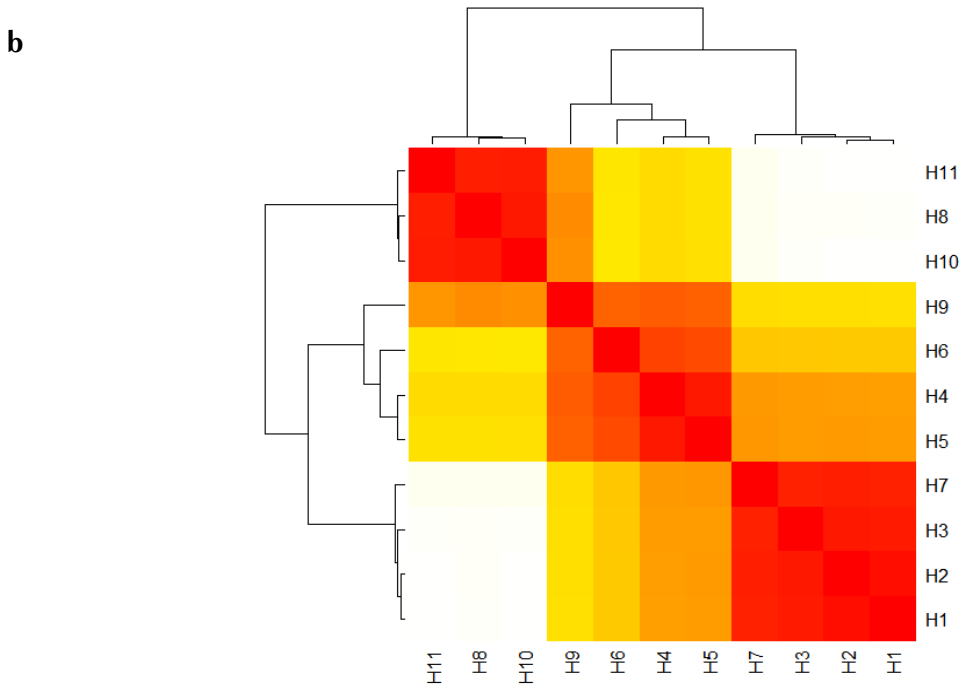


Figure 6. a) Phylogenetic Tree Based on *hsp70* Gene Sequence. Numbers at the nodes indicate the bootstrap values on neighbour joining analysis. **b**) Heat Map Based on the Number of Nucleotide Differences Between the Haplotypes.
1: *E.* sp. HA2 (MH378775), 2: *E. undae* (DQ019143), 3: *E.* sp. 7-3 (DQ019139), 4: *E. antarcticum* (DQ019142), 5: *E.* sp. EF2 (JN001855), 6: *E.* sp. EF3 (JN001858), 7: *Bacillus* sp. m240 (KC901361.1), 8: *E. acetylicum* (DQ019144), 9: *E.* sp. 11-28 (JF811951), 10: *E.* sp. EPVM (JF811927), 11: sp. N39 (JF811983), 12: *E. aurantiacum* (DQ019141)
We defined the close relationships with "darkred" color and far relationships with "white" color.

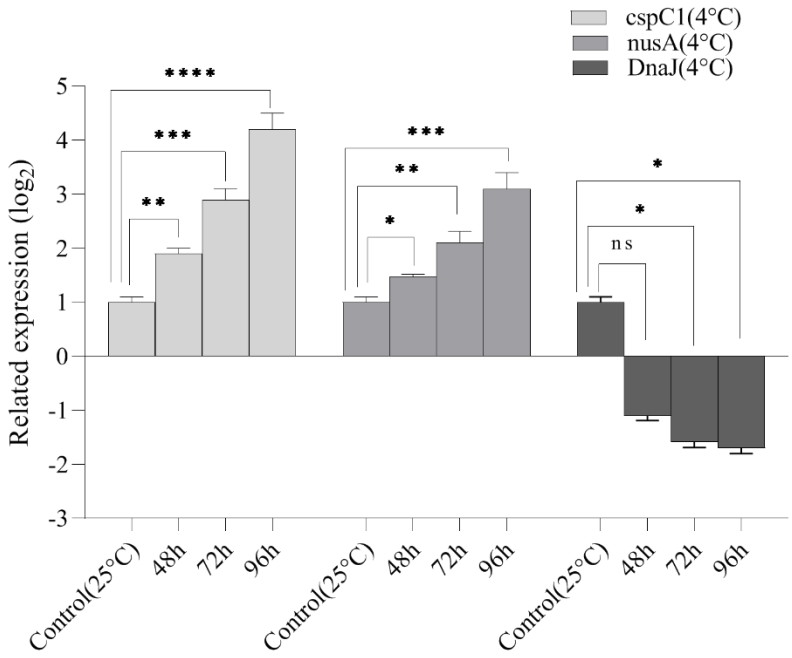


Figure 7. Effects of Low Temperature for 48-96 h on the Gene (*cspC1*, *nusA*, and *dnaJ*) Expression *in Exiguobacterium* sp. HA2. Data from three independent experiments are expressed as means ± SD (n = 3). Error bars indicate standard deviation of three samples (ns: not significant, $p<0.05$ *, $p<0.01$ * *, $p< 0.001$ * * *).

found to be *Exiguobacterium*, with the closest relation to *Exiguobacterium undae*.

In all strains, the main isoprenoid quinone was quinone with seven isoprene units (MK7), but in some strains found a minor quinone such as MK6 and MK8 which is consistent with our study results.[14] In analysis of polar lipid isolated

from Siberia strains, lipids such as phosphatidylserine or phosphatidylinositol were not found, while in some strains such as Exiguobacterium undae and Exiguobacterium sp. HA2 of these compounds was observed.[24]

The main fatty acids in our strain were iC13:0, iC15:0 and C16:0, which were similar to *E. undae*.[27] Our strains were also tested for their ability to grow at different temperatures, from 0 to 25 °C. Strains *E. sibiricum*, *E. undae* and *E. antarcticum* grew at all temperatures tested.[14,25]

Exiguobacterium sp. HA2 has 85% similarity of carbon source utilization with *E. undae* than with the other strains. In addition, *E. antarcticum*, *E. sibiricum* were clustered together and had more similar carbon utilization patterns, respectively with 60% and 54% similarity to *Exiguobacterium* sp. HA2. The percentage of similarity for the *16S rRNA* gene, *gyrB*, *citC*, *rpoB*, and *hsp70* sequence of strain *Exiguobacterium* sp. HA2 with *E. undae* are 99%, 99%, 98%, 99%, 99%, and 99%, respectively. The genus is categorized to two distinct groups based on the temperature of their environment (low-temperature and high-temperature environments).

Consequently, *Exiguobacterium* was isolated from the soil samples collected from the Ilam Mountains, and its genotypic and phenotypic characteristics were assessed and identified. In previous studies, the amylase and pullulanase produced by the cold-adapted *Exiguobacterium* sp. HA2 isolated from Tehran were optimized.[1] Our experiences with other identified genes[26] also indicate that the continuation of research on the specific genes and statistical analysis,[27] optimization,[28] secretion of related protein expression,[29] and immobilization of their produced protein on bacterial spore or chitosan, promoter manipulation and mutagenesis[30] of the new identified strains will have beneficial results.[31] So, since the *Exiguobacterium* is a remarkable bacterium with several applications, continued research and further studies on *Exiguobacterium* sp. HA2 for detection and extraction of enzymes and proteins, especially cold-resistant products, and optimization of their production, are highly desirable.

Conclusion

In this study, The *Exiguobacterium* sp. HA2 can be regarded as representing considered a novel species of genus *Exiguobacterium* in Iran. Also, the *cspC1*, *nusA* and *dnaJ* genes were used to confirm adaptation to cold stress through real-time quantitative PCR. The significance of this study is highlighted by the fact that psychrophilic *Exiguobacterium* sp. HA2 have adapted to a cold stress environments and so are likely to have a wide range of commercial applications. Due to the potential of *Exiguobacterium* sp. HA2 to tolerance cold stress, further investigation of these psychrophilic is desirable for their use in the form of various biological formulations and the development of bio-fertilizers in extreme environments.

Authors' Contributions

All authors contributed equally to current study.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

Authors' Note

The GenBank/EMBL/DDBJ accession number for the *16S rRNA* gene, *gyrB*, *citC*, *rpoB* and *hsp*70, and *csp* sequence of strain *Exiguobacterium* sp. HA2 are MK272780, KX574228, MH370480, MH378445, and MH378775, respectively.

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