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Characterization of *Exiguobacterium* isolates from the Siberian permafrost. Description of *Exiguobacterium sibiricum* sp. nov.

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Abstract Three Gram-positive bacterial strains, 7-3, 255-15 and 190-11, previously isolated from Siberian permafrost, were characterized and taxonomically classified. These microorganisms are rod-shaped, facultative aerobic, motile with peritrichous flagella and their growth ranges are from −2.5 to 40°C. The chemotaxonomic markers indicated that the three strains belong to the genus *Exiguobacterium*. Their peptidoglycan type was A3α L-Lys-Gly. The predominant menaquinone detected in all three strains was MK7. The polar lipids present were phosphatidyl-glycerol, diphosphatidyl-glycerol and phosphatidyl-ethanolamine. The major fatty acids were iso-C13:0, anteiso-C13:0, iso-C15:0, C16:0 and iso-C17:0. Phylogenetic analysis based on 16S rRNA and six diverse genes, *gyr*B (gyrase subunit B), *rpo*B (DNA-directed RNA polymerase beta subunit), *rec*A (homologous recombination), *csp* (cold shock protein), *hsp*70 (ClassI-heat shock protein—chaperonin) and *cit*C (isocitrate dehydrogenase), indicated that the strains were closely related to *Exiguobacterium undae* (DSM 14481^T) and *Exiguobacterium antarcticum* (DSM 14480^T). On the basis of the phenotypic characteristics, phylogenetic data and DNA–DNA reassociation data, strain 190-11 was classified as *E. undae*, while the other two isolates, 7-3 and 255-15, comprise a novel species, for which the name *Exiguobacterium sibiricum* sp. nov. is proposed.

Keywords *Exiguobacterium* · *Exiguobacterium sibiricum* sp. nov. · Polyphasic taxonomy · Siberian permafrost

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Introduction

Temperature is a major environmental condition that affects microbial physiology and growth. Viable microorganisms in permafrost survive and perhaps metabolize in their continuously frozen habitat. Earth's permafrost is characterized by low carbon availability, low water availability and continuous exposure to gamma radiation (0.03 rad/year) originating from the soil minerals. Despite these challenging conditions, microorganisms in several microbial groups remain viable for 20,000 to 3 million years. Many of the studies demonstrating this point were performed by Russian scientists in the Kolyma Lowland, region of northeast Siberia. This area has tundra vegetation and an Arctic climate with a mean annual air temperature of −13.4°C and an annual precipitation of 229 mm (Shi et al. 1997). The ice content of the permafrost is 20–50% with 2–5% of liquid water in films adsorbed to soil particles (Zvyagintsev et al. 1985; Matsumoto et al. 1995). The permafrost in this region is formed from deposits of sediment that came from shallow lake bottoms, alluvial deposits and marine sediments during the late Pliocene and Pleistocene periods (Shi et al. 1997), and can reach a thickness of 600–800 m. While the upper tundra layer, 0.5–1 m deep, freezes and thaws every year, the strata below have remained permanently frozen since they were buried by geological events. The temperature at a depth of 14 m

below the surface is stable at −10°C (Gilichinsky et al. 2003). This region is considered to be among the oldest continuously frozen localities on Earth (McKay et al. 1991) and make this environment ideal for studies of microorganisms that are adapted to survive at subzero temperatures.

Three *Exiguobacterium* strains were previously isolated from Kolyma Lowland permafrost cores of three different ages. *Exiguobacterium* strain 255-15 was isolated from a depth of 43.6 m from a geological layer estimated to be 2–3 million years old. Strains 7-3 and 190-11 were isolated from layers estimated to be 20–30 thousand years and 200–600 thousand years old, respectively. Because of their unique physiology and habitat, we characterized these strains and established their taxonomy. Two of the strains comprise a new species.

Materials and methods

Bacterial strains

Exiguobacterium strains 255-15 and 190-11 were isolated by Vishnivetskaya et al. (2000) and strain 7-3 was isolated by Vera Soina (Moscow State University). They were compared to four reference strains obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), *Exiguobacterium acetylicum* (DSM 20416^T), *Exiguobacterium aurantiacum* (DSM 6208^T), *Exiguobacterium antarcticum* (DSM 14480^T) and *Exiguobacterium undae* (DSM 14481^T). The strains were grown and maintained on ½ Tryptic Soy Broth (TSB) and ½ Tryptic Soy Agar (TSA; Difco Laboratories, Detroit, MI, USA) at 30°C. The strains were stored at −80°C in ½ TSB supplemented with 25% glycerol.

Genotypic characterization

Chromosomal DNA isolation and purification was performed as described by Marmur (1961) from cells grown overnight at 30°C in ½ TSB. DNA concentrations were quantified by UV spectrophotometry at 260 nm (Cary 50 Bio from Varian).

The genomic diversity of the strains was determined by repetitive element-PCR (Kim et al. 2003; Seurinck et al. 2003) with BOXA1R primer as described previously (Versalovic et al. 1991). The patterns were analyzed using GelCompar II (Applied Maths Version 3.0) using the pairwise Pearson's product-moment correlation coefficient (Rademaker et al. 1998).

DNA–DNA reassociation was determined fluorometrically by the method of Ezaki et al. (1989) using photobiotin-labeled DNA probes and black MaxiSorp microplates (Nunc). Hybridizations were performed at 37°C in eight replicates for each sample. The reported DNA–DNA reassociation values are the averages following exclusion of the outliers according to the criteria established for this method (Goris et al. 1998).

The phylogeny of the new isolates was determined from sequences of 16S rRNA gene and six other genes previously used for phylogenetic classification (Yamamoto et al. 2000; Watanabe et al. 2001; Chen and Tsen 2002; Fukushima et al. 2002; Ko et al. 2002; Whitaker et al. 2003): *gyr*B (gyrase subunit B), *rpo*B (DNA-directed RNA polymerase beta subunit), *rec*A (homologous recombination), *csp* (cold shock protein), *hsp*70 (ClassI-heat shock protein—chaperonin) and *cit*C (isocitrate dehydrogenase). The primers (Table 1) were designed based on alignments of these genes found in *Exiguobacterium* 255-15 genome (http://genome.jgi-psf.org/draft_microbes/exigu/exigu.home.html) with the same genes of other Gram-positive microorganisms using ARB (Ludwig et al. 2004), with the exception of the standard 16S rRNA (Lane 1991) and *csp* primers (Francis and Stewart 1997). Amplification, for all primers, was by standard procedures (Eden et al. 1991). The annealing temperatures for each primer sets were 56°C for *csp*, 53°C for *rpo*B, 49°C for *gyr*B and *cit*C and 47°C for *rec*A and *hsp*70. The 16S rRNA amplified product was purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA, USA) and sequenced. For the other genes, the amplicons were cloned using the TOPO TA Cloning kit for sequencing (Invitrogen Life Technologies) and the clones were extracted using the Qiagen Plasmid Mini kit (Qiagen, Valencia, CA, USA). Cycle sequencing was performed on a Perkin-Elmer 9600 thermal cycler using ABI dye terminator chemistry (PE Applied Biosystems) and products were analyzed on an ABI 373a DNA sequencer. Five primers described in Table 1 were used for the 16S rRNA gene sequencing, and primers for the cloning vector (M13F and M13R) were used for sequencing the other genes. Assembled sequences were generated using the Sequencher program version 3.1.1 (Gene Codes Corporation, Ann Arbor, MI, USA). Sequences were deposited in GenBank (accession numbers DQ019127 to DQ019169). Sequences from strain 255-15 were obtained from the sequenced genome.

The consensus sequences were aligned against the most similar sequences using ARB (Ludwig et al. 2004). Phylogenetic analyses were performed by the neighbor-joining (Saitou and Nei 1987) and distance (Desoete 1983) methods from within the ARB environment and by a maximum-likelihood method using fastDNAml (Olsen et al. 1994). The robustness of the inferred tree was evaluated by applying 1,000 bootstrap re-samplings.

Phenotypic characterization

Temperature requirements were determined by growing all the *Exiguobacterium* strains on ½ TSA. Inoculated

Table 1 Primers used for PCR amplification for MLST analysis. All the primers, except for the major cold shock protein, where designed based on the *Exiguobacterium* sequences as well as other strains from the *Bacillus* genus

Gene amplified	Sequence (5′–3′)	Fragment size (bp)
DNA Gyrase—beta subunit		
*gyr*B-F	AAA CGT CCG GGT ATG TAT ATC GGA TCG AC	1,539 bp
*gyr*B-R	CGG CGG CTG SGC AAT RTA SAC GTA	
Universal major cold shock protein (adapted from Francis and Stewart 1997)		
CSPU5	CCC GAA TTC GGT AHA GTA AAA TGG TTY AAC KC	200 bp
CSPU3	CCC GGA TCC GGT TAC GTT ASC WGC TKS HGG DCC	
DNA-directed RNA polymerase beta subunit		
*rpo*B-F	CGA ACA TGC AAC GTC AGG C	1,078 bp
*rpo*B-R	ACA TCY TCY TCA CGN GCA CC	
ClassI-heat shock protein—chaperonin		
*hsp*70-F	GGT ATT GAY TTA GGA ACA ACA AAC T	1,455 bp
*hsp*70-R	CTT CTG CWT CTT TKA CCA T	
Homologous recombination protein		
*rec*A-F	GAR AAR CAA TTY GGB AAA GGT TC	869 bp
*rec*A-R	TGT TTY GMA TTT TCA CGK CCT TG	
Isocitrate dehydrogenase		
*cit*C-F	GGD GAY GGM ACW GGW CCW GAY ATT TGG	1,165 bp
*cit*C-R	AAT TCW GAA CAT TTM ACT TCT GT	
16S rRNA gene universal primers (adapted from Lane 1991)		
8F	AGA GTT TGA TCC TGG CTC AG	
787R	TAC CAG GGT ATC TAA T	
802F	ATT AGA TAC CCT GGT A	
1100R	AGG GTT GCG CTC GTT G	
1525R	AAG GAG GTG WTC CAR CC	

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

agar plates were incubated for 24–48 h at 30°C, 4–6 days at 12°C, 20 days at 0°C, 32 days at −2.5°C in order to reach a colony size of 1–2 mm of diameter. Cell morphology at different temperatures was examined by phase contrast microscopy and transmission electron microscopy (TEM) using colonies grown on the same plate. Flagellum type was observed by TEM at all temperatures. Cultural and physiological characteristics were determined by using API 50 CHE (bioMérieux) and Biolog GP2 microplate (Riley et al. 2001). Production of constitutive enzymes was assessed with API ZYM strips (bioMérieux) as recommended by the manufacturer. Cells for the fatty acid methyl ester analysis (Miller and Berger 1984) and chemotaxonomic markers were grown at 28°C on TSA and analyzed by DSMZ. The chemotaxonomic markers studied were cell wall amino acids using the methodology of Schleifer and Kandler (1972), polar lipids by Minnikin et al. (1979), peptidoglycan structure by MacKenzie (1987), Schleifer (1985) and Schleifer and Kandler (1972) and isoprenoid quinones by Collins et al. (1977) and Groth et al. (1996).

Cytochrome oxidase was tested using the Bactident Oxidase kit from Merck (1.13300) and catalase activity was tested using H_2O_2 as described by Koneman et al. (1979). Nitrate reduction was analyzed as described by Cowan and Steel (1965). The ability of the new isolates and the four reference strains to grow in ½ TSB supplemented with 5, 10, 12, 15 and 17% of NaCl was also determined. Results were only considered positive if an increased medium turbidity was observed for both duplicates.

Results and discussion

Genotypic analysis

Genotypic studies were performed on *Exiguobacterium* permafrost strains 7-3, 190-11 and 255-15 to determine their phylogenetic relationship with the most relevant set of reference strains. The rep-PCR profiles confirmed that the new isolates and reference strains were all distinct genotypes (data not shown). Sequence analysis of the 16S rRNA genes of the strains placed them in the genus *Exiguobacterium* (Fig. 1a). Strains 7-3, 190-11, 255-15, *E. undae*, *E. antarcticum*, *E. acetylicum* and *Exiguobacterium oxidotolerans* form a closely related group with a bootstrap value of 1,000, while *E. aurantiacum*, *Exiguobacterium marinum* and the two *Exiguobacterium aestuarii* strains form another closely related group. For a more robust analysis of the phylogenetic relationships of strains within *Exiguobacterium*, we analyzed the phylogeny of six protein-encoded genes (Fig. 1b–g).

Since protein-encoding genes evolve much faster than ribosomal RNAs, they provide higher resolution than 16S rRNA gene sequences (Yamamoto and Harayama 1998). Furthermore, such results were shown with *Acinetobacter* to fill the resolution gap between 16S rRNA gene sequence analysis and DNA–DNA reassociation values (Yamamoto et al. 1999).

The basic tree topologies of *rpo*B (Fig. 2b), *rec*A (Fig. 2c), *hsp*70 (Fig. 2d), *gyr*B (Fig. 2e) and *cit*C (Fig. 2f) were very similar. *E. undae* DSM 14481^T and strain 190-11 showed very similar sequences and

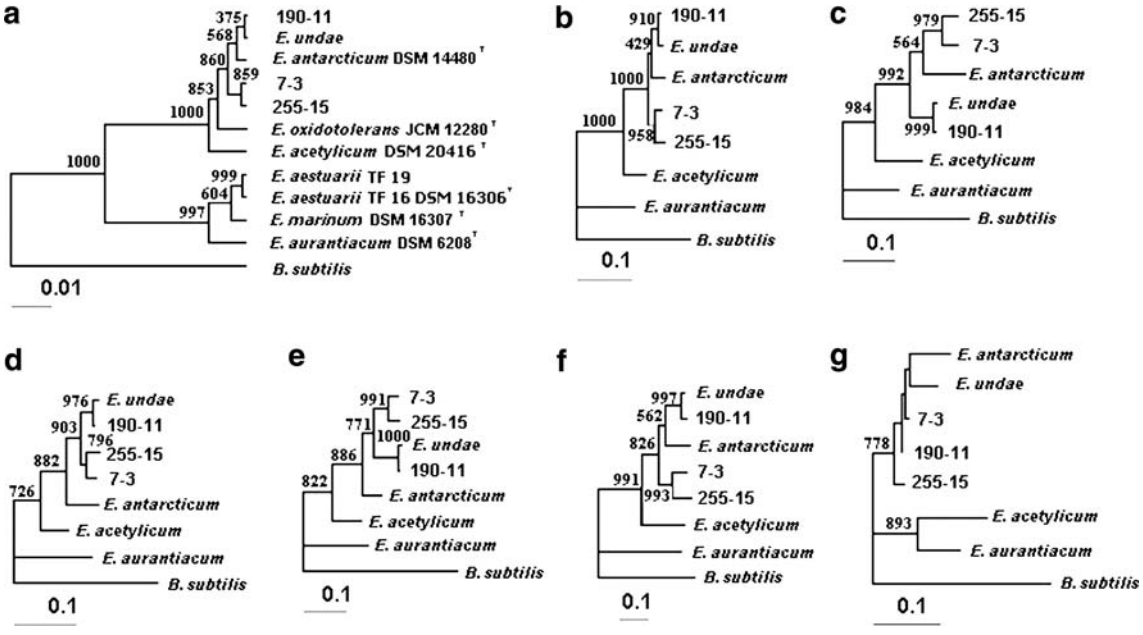

Fig. 1 Phylogenetic trees based on 16S rRNA gene (**a**), *rpo*B (**b**), *rec*A (**c**), *hsp*70 (**d**), *gyr*B (**e**), *cit*C (**f**), *csp* (**g**) sequences of the following genes of indicated *Exiguobacterium* strains. The trees were produced by a maximum-likelihood method using fastDNAml_loop and were rooted using those genes from *Bacillus subtilis*. The nodes without bootstrap values represent either branching orders that were inconsistent between the phylogenetic tree generated by the maximum-likelihood method and the consensus tree generated from 1,000 bootstraps or had bootstrap values of 40% or less. The scale bar represents 0.1 changes per nucleotide position

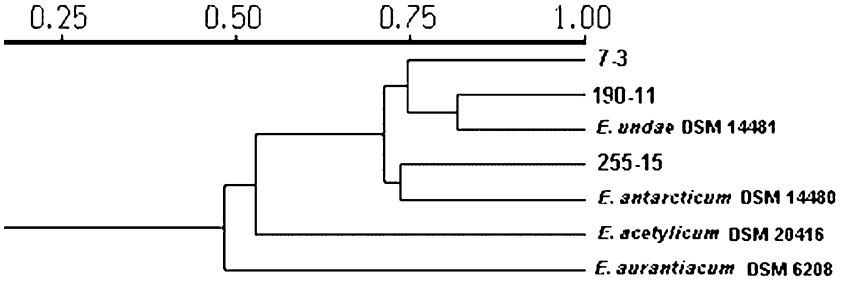

Fig. 2 Phenogram obtained from the carbon source utilization of the seven *Exiguobacterium* strains based on the UPGMA method (unweighted pair-group method using arithmetic averages) after measuring similarity/dissimilarity among the strains using the coefficient of simple matching, which considers the same weight positive and negative similarities

always clustered together with very high bootstrap values. Strains 7-3 and 255-15, also clustered together, albeit at a slightly lower sequence similarity. As found for the 16S rRNA gene sequence analysis, *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T were always clearly separated from the other *Exiguobacterium* strains by long branches. *E. antarcticum* DSM 14480^T was the only strain for which the clustering was inconsistent over the different genes, either clustering with strain 190-11 and *E. undae* DSM 14481^T (*rpo*B, *cit*C) or with strain 7-3 and 255-15 (*rec*A). In other cases (*hsp*70, *gyr*B), *E. antarcticum* DSM 14480^T was separated from these four related *Exiguobacterium* strains. Notably, the branch nodes were often not well supported by the bootstrap values, which makes the affiliation of *E. antarcticum* DSM 14480^T uncertain.

In order to unequivocally determine the species status of the new isolates, DNA–DNA hybridization experiments were performed (Table 2). All reassociation values with strains *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T were well below the threshold for species identity (14–44%). Strain 190-11 and *E. undae* DSM 14481^T showed a DNA relatedness of 91%, which indicates that these two strains belong to the same species. This is in agreement with the multi-locus sequence typing (MLST) analysis, and many of the phenotypic analyses (see below). Furthermore, strain 190-11 showed a relatively high reassociation (65%) with *E. antarcticum* DSM 14480^T.

A DNA reassociation value of 71% was found between the strains 7-3 and 255-15, suggesting that these strains maybe members of the same species. However, both 7-3 and 255-15 had a relatively high similarity

Table 2 DNA–DNA similarity of *Exiguobacterium* strains using the microplate DNA–DNA hybridization technique

Strain	190-11	*E. undae* DSM 14481^T	*E. antarcticum* DSM 14480^T	7-3	255-15	*E. acetylicum* DSM 20416^T
190-11	100					
E. undae DSM 14481^T	**91.2 ± 7.2**	100				
E. antarcticum DSM 14480^T	65.2 ± 13.4	59.9 ± 5.5	100			
7-3	62.0 ± 4.7	67.5 ± 3.6	65.3 ± 2.9	100		
255-15	56.4 ± 6.4	64.7 ± 5.8	65.3 ± 6.0	**71.2 ± 3.3**	100	
E. acetylicum DSM 20416^T	41.7 ± 9.9	43.5 ± 7.2	38.8 ± 3.7	38.3 ± 6.9	37.7 ± 7.0	100
E. aurantiacum DSM 6208^T	13.7 ± 2.6	16.2 ± 1.8	21.0 ± 3.0	15.3 ± 3.1	15.5 ± 2.4	18.0 ± 1.4

Data greater than 70% in bold

(65–68%) with the type strains *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T (Table 2). The DNA–DNA reassociation values are borderline for species delineation, making it difficult to classify the new strains as belonging to one or to the other species. Those two type strains are clearly distinct based on their phenotypic differences and DNA–DNA reassociation values of 51% by Fruhling et al. (2002), and confirmed by our results (59%). Since the two isolates, 7-3 and 255-15, cannot be clearly classified as belong to either one of these type strains and have consistent MLST data supporting that they are related and distinct from the other species previously described, we suggest that they are a new species.

Phenotypic properties

Phenotypic studies were also performed to determine whether the new isolates are phenotypically distinguishable from each other and from the reference strains of *Exiguobacterium*. Allocation of the strains 7-3, 190-11 and 255-15 to the genus *Exiguobacterium* was confirmed by the presence of the peptidoglycan type A3α L-Lys-Gly, which is typically the genus (Collins et al. 1983).

The major isoprenoid quinone (Table 3) in all strains was an unsaturated menaquinone with seven isoprene units (abbreviated as MK7). Strains 7-3, 190-11, *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T contained minor but significant amounts of MK6 and MK8 quinones, while for 255-15 the major quinone was MK7 with trace amounts of MK6 and MK8, which provides a good distinction from the other closely related strains like *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T. In the analysis of polar lipids, none of the Siberian isolates (7-3, 190-11 and 255-15) contained phosphatidyl-serine or phosphatidyl-inositol as was observed in *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T (Table 3). All strains were catalase positive. *E. antarcticum* DSM 14480^T and *E. aurantiacum* DSM 6208^T were the only oxidase negative strains (Table 3). *E. aurantiacum* DSM 6208^T was also the only strain positive for nitrate reduction. The major fatty acids in the new isolates (Table 4) were iso-C13:0, anteiso-C13:0, iso-C15:0, C16:0 and iso-C17:0, which were similar to these in *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T, further supporting the assignment of the new isolates as *Exiguobacterium*.

Exiguobacterium aurantiacum DSM 6208^T was the only strain that did not express any of the 19 enzymes tested (API ZYM), and 7-3 and 190-11 were the only strains that expressed esterase lipase (C8) and β-glucosidase, respectively. All *Exiguobacterium* strains expressed α-glucosidase and β-galactosidase with the exception of *E. aurantiacum* DSM 6208^T, as mentioned above, and *E. acetylicum* DSM 20416^T which only expressed α-glucosidase. Interestingly, even though 190-11 and *E. undae* DSM 14481^T were highly similar in all genotypic tests, only 190-11 and *E. antarcticum* DSM 14480^T did not express alkaline phosphatase, while 7-3, 255-15 and *E. undae* DSM 14481^T did express this enzyme.

The Biolog and API 50 CH systems (Table 5) were used to assess the metabolic profile of *Exiguobacterium* strains. A dendrogram obtained with this data shows that 255-15 shares a slightly more similar carbon utilization pattern with *E. antarcticum* DSM 14480^T (76% similarity) than with the other strains (Fig. 2). In addition, 7-3, 190-11 and *E. undae* DSM 14481^T were clustered together and had more similar carbon utilization patterns, respectively with 73 and 82% similarity to *E. undae* DSM 14481^T. However, the higher similarity observed was between 190-11 and *E. undae* DSM 14481^T, which had 82% similarity in the carbon source utilization, confirming the genotypic data that suggests that they are the same species. In the case of 7-3 and 255-15, even though they were clustered with two different reference strains in the phenogram, when we look at the matrix (data not shown) the pairwise comparison of those two strains with the other reference strains (except for *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T) showed a similarity of 71 and 73% for 7-3 with *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T, respectively, and a similarity of 73 and 76% for 255-15 with *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T, respectively. Hence, this data is not conclusive with regard to a species assignment for these two new strains.

The strains were also tested for their ability to grow at different temperatures, from −2.5 to 30°C. The

Table 3 Phenotypic characteristics of new isolates and type strains of *Exiguobacterium*

Characteristic	1	2	3	4	5	6	7	8	9	10
Flagellum type	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Peritrichous	Single polar
Colour of colonies	Bright orange	Bright orange	Orange	Orange	Bright orange	Orange-yellow	Orange-yellow	Orange-yellow	Pale orange	Deep orange
Oxidase	+	+	+	+	–	–	+	+	–	–
Catalase	+	+	+	+	+	+	+	+	+	+
Growth in NaCl (%)	10	10	12	12	5	12	10	12	> 19	> 17
Nitrate reduction	–	–	–	–	–	+	–	–	+	+
Growth range (°C)	−2.5 to 40	−2.5 to 40	−2.5 to 40	−2.5 to 40	−2.5 to 40	12 to 30	7 to 43	4 to 36	10 to 47	10 to 43
Enzymatic activity										
Alkaline phosphatase	+	+	–	+	–	–	+	–	ND	ND
Esterase lipase (C8)	+	–	–	–	–	–	–	–	ND	ND
β-Galactosidase	+	+	+	+	+	–	–	+	ND	ND
α-Glucosidase	+	+	+	+	+	–	+	–	ND	ND
β-Glucosidase	–	–	+	–	–	–	–	–	ND	ND
Acid phosphatase	–	–	–	–	–	–	–	+	ND	ND
Isoprenoid quinines (ratio)										
MK8	8	Traces	12	15	20	–	ND	ND	ND	ND
MK7	85	100	83	75	71	Major	ND	Major	Major	Major
MK6	6	Traces	6	2	2	+	ND	ND	ND	ND
Polar lipids										
Phosphatidyl-glycerol	+	+	+	+	+	+	+	+	+	+
Diphosphatidyl-glycerol	+	+	+	+	+	+	+	+	+	+
Phosphatidyl-ethanolamine	+	+	+	+	+	+	+	+	+	+
Phosphatidyl-serine	–	–	–	+	+	–	±	+	+	+
Phosphatidyl-inositol	–	–	–	+	+	–	–	+	+	+

Strains: 1 = 7-3; 2 = 255-15; 3 = 190-11; 4 = *E. undae* DSM 14481^T (data obtained from this work and Frühling et al. 2002); 5 = *E. antarcticum* DSM 14480^T (data obtained from this work and Frühling et al. 2002); 6 = *E. aurantiacum* DSM 6208^T (data obtained from this work and Frühling et al. 2002); 7 = *E. acetylicum* DSM 20416^T (data obtained from this work, Frühling et al. 2002 and Collins et al. 1983); 8 = *E. oxidotolerans* (adapted from Yumoto et al. 2004); 9 = *E. aestuarii* (adapted from Kim et al. 2005); 10 = *E. marinum* (adapted from Kim et al. 2005). + positive, - negative, *ND* not determined

Table 4 Fatty acid composition of the Siberian permafrost isolates and the type strains of *Exiguobacterium*

	7-3	255-15	190-11	*E. undae* DSM 14481Ta	*E. antarcticum* DSM 14480Ta	*E. aurantiacum* DSM 6208Ta	*E. acetylicum* DSM 20416Ta
$iC_{11:0}$						2	
$iC_{12:0}$	2	2	2	2	3	3	
$C_{12:0}$	1		1		1	2	1
$iC_{13:0}$	**9**	**13**	**8**	**9**	**12**	**18**	**5**
$AiC_{13:0}$	**11**	**15**	**10**	**9**	**11**	**12**	**6**
$iC_{14:0}$	1	1	1	2	1		1
$C_{14:1\omega5c}$							2
$C_{14:0}$	3	1	2	2	2	3	**13**
$iC_{15:0}$	**13**	**12**	**13**	**10**	**11**	4	**8**
$aiC_{15:0}$	3	4	3	3	2		1
$C_{16:1\omega11c}$	**8**	3	**7**	**8**	**18**	**10**	**26**
$iC_{16:0}$	2	2	2	2			
$C_{16:1\omega5c}$	1		1				2
$iC_{16:1\ H}$			1				
$C_{16:1\omega7c}$	1		1	**7**	3		**13**
$C_{16:0}$	**17**	**20**	**12**	**17**	**13**	**27**	**10**
$C_{17:1\omega10c}$	2	2	3	2	3		1
$aiC_{17:1\omega9c}$			1				
$C_{17:0}$		1					
$iC_{17:0}$	**9**	**12**	**8**	**7**	**5**	**6**	1
$aiC_{17:0}$	3	3	3	2			
$C_{18:1\omega9c}$	2	1	2	3	**6**	2	**5**
$C_{18:1\omega7c}$	2	1	3	3			2
$C_{18:1\omega5c}$			1				
$C_{18:0}$	4	**5**	3	**6**	**5**	**5**	1

Only values >1% are indicated; values ≥5% are given in bold
[a]Data obtained from Frühling et al. (2002)

reference strains *E. acetylicum* DSM 20416^T and *E. aurantiacum* DSM 6208^T were unable to grow at 4°C or below, but were able to grow at 30 and 12°C. Strains 7-3, 190-11, *E. undae* DSM 14481^T and *E. antarcticum* DSM 14480^T grew at all temperatures tested. The cell pigmentation remained unaltered at low growth temperatures, except for *E. acetylicum* DSM 20416^T, which at 12°C, became much more orange and therefore more similar to the other *Exiguobacterium* strains. These data confirm results obtained with the *csp* gene (Fig. 2g), i.e., that these two strains unable to grow below 12°C cluster together, while the others that are able to grow at lower temperatures formed a separate cluster. This observation suggests that *csp* primers may be able to discriminate strains that grow at lower temperatures from the ones that cannot.

In regard to growth at different salt concentrations, 7-3 and 255-15 were able to grow in up to 10% NaCl, as was the case for *E. acetylicum* DSM 20416^T. Strain 190-11 was able to grow at salinities of up to 12% NaCl, like *E. undae* DSM 14481^T, *E. oxidotolerans* and *E. aurantiacum* DSM 6208^T (Table 3). These data show that *E. undae* DSM 14481^T and 190-11 have another phenotype in common. The same is observed for the strains 7-3 and 255-15 that also exhibit the same tolerance to salt.

Colonies of all the new isolates and the reference strains (except for DSM 20416^T, which was yellow colored) were orange-colored, smooth, circular, convex and shiny. The orange pigment did not diffuse into the medium. The isolates also showed genus-specific characters such as rod-shape morphology, motility, peritrichous flagella and absence of spore formation at all temperatures tested (Table 3). Strains were inoculated on ½ TSA plates and cell morphology was observed in different temperatures with phase contrast microscopy and transmission electron microscope after negative staining. The cell shape and slime secretion of 190-11 and 255-15 strains did not change with temperature and were very similar to *E. antarcticum* DSM 14480^T and *E. undae* DSM 14481^T. However, inclusions were observed inside the cells of strain 255-15 at 0°C, which could be carbon storage polymer (Fig. 4). Strain 7-3 was the only strain that showed temperature-dependent cell morphology. For temperatures from 0 to 12°C, the cells became much more elongated in comparison to all the other strains and in comparison to its growth at 30°C (Figs. 3, 4). This elongation state was not permanent, as cell size returned to normal when incubated at 30°C. Interestingly, at −2.5°C the cell size of 7-3 was similar to the other strains.

The phenotypic analyses confirmed that the new isolates are *Exiguobacterium*, that 190-11 and *E. undae* DSM 14481^T share similar properties and hence are the same species, consistent with the genotypic analyses; and that 255-15 and 7-3 have some phenotypic properties that are not consistent with any current species. The genotypic results were clearer cut for 7-3 and 255-15 classification, in that they had high

Table 5 Phenotype properties that differentiate the novel isolates from *Exiguobacterium* type strains using Biolog and API 50 CH analyses

Substrates	7-3	255-15	190-11	*E. undae* DSM 14481	*E. antarcticum* DSM 14480	*E. aurantiacum* DSM 6208	*E. acetylicum* DSM 20416
2,3-Butanediol	–	±	–	–	–	–	–
3-Methyl-D-glucose	±	+	+	+	+	–	+
Acetic acid	+	+	+	+	–	–	±
a-Cyclodextrin	+	–	–	±	–	+	–
Adenosine-5′-monophosphate	+	+	±	+	+	+	–
a-D-Glucose-1-phosphate	–	–	–	–	–	–	+
a-Methyl-D-glucoside	+	+	–	–	–	–	+
Amidon (starch)	+	+	+	+	+	+	–
b-Cyclodextrin	±	–	–	–	–	±	–
b-Hydroxybutyric acid	+	±	+	±	–	–	–
D-Galactose	+	+	+	+	+	–	–
D-Melibiose	–	±	+	+	–	–	–
D-Alanine	±	–	–	–	–	–	–
D-Fructose-6-phosphate	–	–	–	–	–	–	+
D-Glucose-6-phosphate	–	–	–	–	–	–	+
D-Lactose (bovine origin)	+	–	–	–	–	–	–
D-L-a-Glycerol phosphate	–	–	–	–	–	–	+
D-Mannitol	+	+	+	+	±	–	+
D-Mannose	+	–	+	+	–	–	–
D-Melezitose	–	±	–	+	–	–	–
D-Melibiose	–	±	±	+	–	–	–
D-Raffinose	+	+	+	+	+	–	–
D-Ribose	+	+	+	+	+	+	–
d-Sorbitol	+	–	+	+	+	–	+
D-Xylose	+	±	–	±	+	–	–
Esculin ferric citrate	+	–	+	+	+	+	+
Glycyl-L-glutamic acid	+	±	+	+	+	–	+
L-Alanine	±	–	–	–	–	–	–
L-Alanyl-glycine	+	+	+	+	+	–	±
L-Asparagine	±	+	–	–	+	–	+
L-Glutamic acid	–	±	–	–	+	–	+
L-Lactic acid	–	–	–	–	–	+	–
L-Malic acid	–	±	–	±	–	–	–
L-Pyroglutamic acid	–	–	–	–	+	–	–
L-Rhamnose	–	±	±	–	+	–	–
L-Serine	±	±	–	±	+	–	±
Methyl-alpha-D-glucopyranoside	±	–	–	–	–	+	–
N-Acetyl-b-D-mannosamine	–	±	–	±	–	–	–
N-Acetyl-D-glucosamine	+	+	+	+	+	+	+
N-Acetyl-L-glutamic acid	–	–	–	+	–	–	–
Propionic acid	+	+	±	–	+	–	+
Pyruvic acid	+	+	+	+	+	–	+
Pyruvic acid methyl ester	+	+	+	±	+	+	–
Salicin	+	+	+	+	+	+	–
Sedoheptulosan	–	±	–	–	+	–	–
Stachyose	–	–	–	–	±	–	–
Succinic acid mono-methyl ester	+	+	+	+	+	–	+
Thymidine-5′-monophosphate	+	+	+	+	+	–	-
Turanose	±	+	±	+	+	–	+
Uridine-5′-monophosphate	+	+	±	+	+	+	–

Positive results for the following substrates: 2′-deoxy adenosine, adenosine, a-D-glucose, a-ketovaleric acid, amygdalin, arbutin, b-methyl-D-glucoside, D-mannose, D-cellobiose, dextrin, D-fructose, D-glucose, D-maltose, D-psicose, D-saccaharose (sucrose), gentiobiose, glycerol, glycogen, inosine, D-trehalose, maltose, maltotriose, thymidine, Uridine, palatinoseNegative results for the following substrates: a-D-lactose, a-hydroxybutyric acid, a-ketoglutaric acid, a-methyl-D-galactoside, a-methyl-D-mannoside, b-methyl-D-galactoside, D-arabitol, D-fucose, D-galacturonic acid, D-gluconic acid, D-lactic acid methyl ester, D-lyxose, D-malic acid, D-sorbitol, D-tagatose, D-turanose, dulcitol, g-hydroxybutyric acid, inositol, inulin, lactamide, lactulose, L-alaninamide, L-arabinose, L-arabitol, L-fucose, L-rhamnose, L-sorbose, mannan, methyl-alpha-D-mannopyranoside, p-hydroxyphenylacetic acid, potassium 2-ketogluconate, potassium 5-ketogluconate, potassium gluconate, putrescine, succinamic acid, succinic acid, Tween 40, Tween 80, xylitol

DNA–DNA reassociation and were clustered together in all but one of the MLST analyses. Hence, we conclude that 255-15 and 7-3 are members of the same species and that they belong to a different species from the reference strains described so far. The phenotypic results for all *Exiguobacterium* new and old members are not strikingly distinctive among the species, except for the most distantly related strains. Hence, the genotypic analyses are more conclusive for the species of this genus.

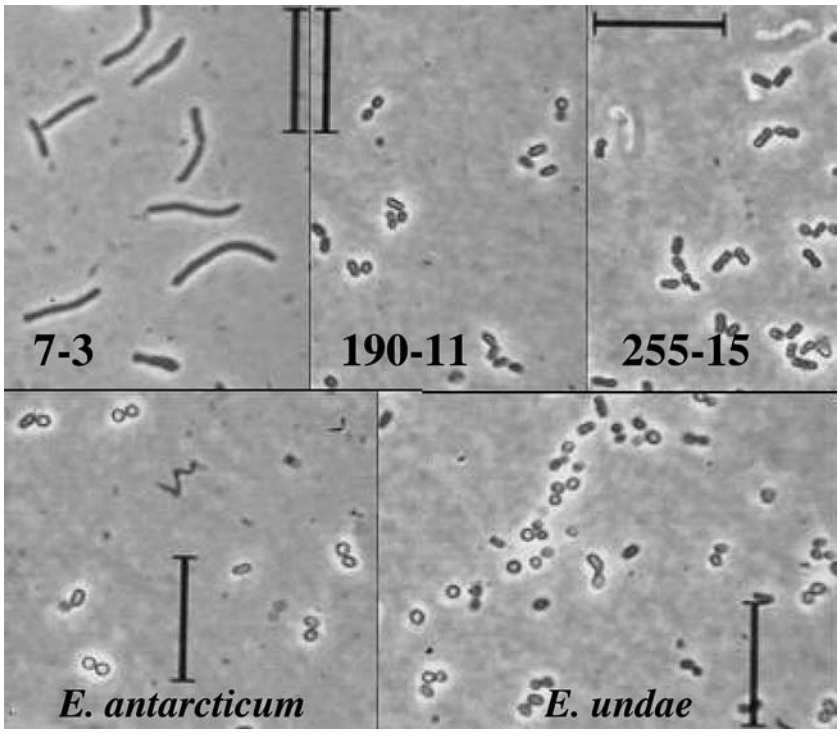

Fig. 3 Cell morphology of new isolates and reference strains of *Exiguobacterium antarcticum* DSM14480^T and *Exiguobacterium undae* DSM 14481^T grown at 0°C by phase contrast microscopy. Bar scale corresponds to 10 μm

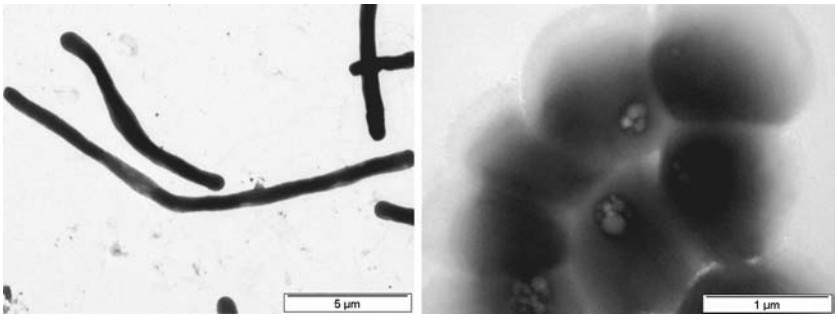

Fig. 4 Negatively stained electron micrograph of 7-3 and 255-15 grown at 0°C. Strain 7-3 presents elongated cells and 255-15 presents intracellular granules

Description of *Exiguobacterium sibiricum* sp. nov.

Exiguobacterium sibiricum (si.bi.ri.cum M. L.—Sibir' from Siberia, a Russian region)

Cells are Gram-positive and facultatively anaerobic, non-spore-forming rods, motile with peritrichous flagella. The cells can vary in shape and size depending on growth temperature. At 30°C, they can have 0.8 μm long and 0.6 μm in diameter, but can reach up to 15 μm long at temperatures between 12 and 0°C. Surface colonies on ½ TSA are 3.5–4 mm in diameter after 50 h at 30°C, bright orange, convex, entire and shiny. The orange pigment does not diffuse in the medium. Growth occurs at −2.5 to 40°C, with optimum temperature growth rate at 36°C. Growth does occur in ½ TSB with 10% NaCl, but does not occur at 12%. The peptidoglycan type is L-Lys-Gly and the predominant menaquinone is MK7; MK6 and MK8 might or might not occur. The only polar lipids are phosphatidyl-glycerol, diphosphatidyl-glycerol and phosphatidyl-ethanolamine, and the major fatty acids are iso-C13:0, anteiso-C13:0, iso-C15:0, C16:0 and iso-C17:0. The G + C content of DNA of the type strain is 47.7 mol% and its genome size is 3 Mb. The type strain is strain 255-15 (= DSM 17290^T).

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