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The *Exiguobacterium* genus: biodiversity and biogeography

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Abstract Bacteria of the genus *Exiguobacterium* are low G + C, Gram-positive facultative anaerobes that have been repeatedly isolated from ancient Siberian permafrost. In addition, *Exiguobacterium* spp. have been isolated from markedly diverse sources, including Greenland glacial ice, hot springs at Yellowstone National Park, the rhizosphere of plants, and the environment of food processing plants. Strains of this hereto little known bacterium that have been retrieved from such different (and often extreme) environments are worthy of attention as they are likely to be specifically adapted to such environments and to carry variations in the genome which may correspond to psychrophilic and thermophilic adaptations. However, comparative genomic investigations of *Exiguobacterium* spp. from different sources have been limited. In this study, we employed different molecular approaches for the comparative analysis of 24 isolates from markedly diverse environments including ancient Siberian permafrost and hot springs at Yellowstone National Park. Pulsed-field gel electrophoresis (PFGE) with I-*Ceu*I (an intron-encoded endonuclease), *Asc*I and *Not*I were optimized for the determination of genomic fingerprints of nuclease-producing isolates. The application of a DNA macroarray for 82 putative stress-response genes yielded strain-specific hybridization profiles. Cluster analyses of 16S rRNA gene sequence data, PFGE I-*Ceu*I restriction patterns and hybridization profiles suggested that *Exiguobacterium* strains formed two distinct divisions that generally agreed with temperature ranges for growth. With few exceptions (e.g., Greenland ice isolate GIC31), psychrotrophic and thermophilic isolates belonged to different divisions.

Keywords *Exiguobacterium* · PFGE · Macroarray · Psychrophilic and thermophilic adaptations

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Introduction

The genus *Exiguobacterium* was first described in 1983 by Collins et al. (1983) with characterization of the type species *Exiguobacterium aurantiacum*. In 1994, Farrow et al. included the species formerly identified as *Brevibacterium acetylicum incertae sedis* into the genus *Exiguobacterium*, as *E. acetylicum* (Farrow et al. 1994). Since then, 11 new species have been added to the genus (Chaturvedi et al. 2008; Chaturvedi and Shivaji 2006; Crapart et al. 2007; Fruhling et al. 2002; Kim et al. 2005; Lopez-Cortes et al. 2006; Rodrigues et al. 2006; Yumoto et al. 2004).

In addition to the type strains, *Exiguobacterium* spp. have been isolated from, or molecularly detected in, a wide range of habitats including cold and hot environments with

temperature range from −12 to 55°C. *Exiguobacterium* spp. have been detected in Siberian permafrost, temperate and tropical soils by multilocus real-time PCR (Rodrigues and Tiedje 2007). The *Exiguobacterium* genus comprises psychrotrophic, mesophilic, and moderate thermophilic species and strains (Vishnivetskaya et al. 2005), with pronounced morphological diversity (ovoid, rods, double rods, and chains) depending on species, strain, and environmental conditions (Vishnivetskaya et al. 2007). Currently, the NCBI database contains 439 *Exiguobacterium* entries, including 158 uncultured *Exiguobacterium* spp.

Several *Exiguobacterium* strains possess unique properties of interest for applications in biotechnology, bioremediation, industry and agriculture. *Exiguobacterium* strain Z8 was capable of neutralizing highly alkaline textile industry wastewater (Kumar et al. 2006); strain 2Sz showed high potential for pesticide removal (Lopez et al. 2005); strain WK6 was capable of reducing arsenate to arsenite (Anderson and Cook 2004); other *Exiguobacterium* strains could rapidly reduce Cr[VI] over a broad range of temperature, pH and salt concentrations (Okeke et al. 2007; Pattanapipitpaisal et al. 2002). A panel of mercury-resistant *Exiguobacterium* strains harbor determinants homologous to *mer* operons (Petrova et al. 2002) or mercury-resistance transposons (Bogdanova et al. 2001). Furthermore, several enzymes (alkaline protease, EKTA catalase, guanosine kinase, ATPases, dehydrogenase, esterase) with stability at a broad range of temperatures were purified from different *Exiguobacterium* strains (Hara et al. 2007; Hwang et al. 2005; Kasana and Yadav 2007; Suga and Koyama 2000; Usuda et al. 1998; Wada et al. 2004).

While reports about isolation of new *Exiguobacterium* strains continue to appear, information on genomic diversity of strains already isolated from different habitats remains quite limited. On the basis of small-subunit ribosomal RNA sequences, the species of the genus *Exiguobacterium* were clustered in proximity to *Bacillus benzoevorans*, *B. circulans*, and *B. siralis* in the order Bacillales, phylum Firmicutes (Yarza et al. 2008). The genome of *E. sibiricum* 255-15 has been sequenced in the context of the Joint Genome Institute Microbial Sequencing program (http://genome.jgi-psf.org/draft_microbes/exigu/exigu.home.html). This strain was chosen for genome sequencing on the basis of excellent survival potential after exposure to a long-term freezing at −20°C in tryptic case soy broth without addition of cryoprotectants (Ponder et al. 2005), rapid growth at temperatures as low as −6°C (Vishnivetskaya et al. 2007), and the age (2–3 million years) of the permafrost sediment from which it was derived (Vishnivetskaya et al. 2006). The genome of *E. sibiricum* 255-15 contains a 3.0 Mbp chromosome and two small plasmids of 4.9 and 1.8 kbp, respectively, with a total of 3,015 predicted protein-encoding genes and G + C content of 47.7% (Rodrigues et al. 2008). Genome sequence analysis of *E. sibiricum* 255-15 revealed that it shared 829 and 544 orthologous genes (50% similarity over 90% length) with *B. halodurans* and *B. subtilis*, respectively (Vishnivetskaya et al. 2008). Recently, the genome sequencing of a thermophilic *Exiguobacterium* isolate, strain AT1b from a Yellowstone hot spring, has also been undertaken. The draft sequence of *Exiguobacterium* sp. AT1b revealed a 2.8 Mbp genome with a G + C content of 48.3% and 3,046 candidate protein-encoding genes (http://genome.ornl.gov/microbial/exig_AT1b/).

The fact that certain strains (e.g., those from ancient permafrost) can grow at temperatures as low as −6°C whereas others (e.g., those from hot pools) have optimum growth temperatures above 45°C confers substantial interest to *Exiguobacterium* as a potential model system for the investigation of evolutionary mechanisms and genomic attributes that may correlate with adaptations of organisms to diverse thermal regimes. The objectives of the current study were to perform a comparative genomic analysis of 24 *Exiguobacterium* strains, including eight type strains, from different environments, and to evaluate the possible correlation between genotype, source (especially with regard to thermal regime), and optimal growth temperature of the organisms.

Materials and methods

Bacterial strains and growth conditions

The *Exiguobacterium* strains investigated in this study and their sources are listed in Table 1. The bacteria were routinely grown in tryptic soy broth (TSB, Difco, Sparks, MD, USA) with 0.7% yeast extract (Difco) at 24 or 30°C overnight in standing cultures, as previously described (Vishnivetskaya and Kathariou 2005). The only exception was strain GIC31 that was grown in TSB, pH 8.8 (pH adjusted with 10 M NaOH). Bacterial cultures were preserved at −70°C in TSB supplemented with 15% glycerol.

Growth temperature range determinations

Growth at different temperatures was estimated on TSB supplemented with 1.5% agar (Difco) designated here as TSA; TSA supplemented with 0.7% yeast extract; and TSA with pH 8.8. Overnight cultures (10 µl) were spotted onto plates which were then incubated at 24, 37, 42, 50 and 55°C for 24 h; at 4, 15°C for 14 days; and at −3°C for 43 days. A circle of confluent growth, or growth of only a few colonies, was considered as a positive. The growth

Table 1 Strains of the genus *Exiguobacterium* used in this study

Group	Strain	Reference	Source (age)	Geographic region	Environmental conditions	Growth temperature[a] (°C)	Nuclease activity, zone diameter[b] (mm)			Genome size[c] (kb)
							4°C	24°C	37°C	
I	*E. sibiricum*^{T} 255-15 (DSM 17290)	(Vishnivetskaya and Kathariou 2005)	Permafrost (3 M)	Russia, Siberia, Bol'shaya Chykochya River, 69°10′N, 158°4′E	43.6 m, −10°C, pH 7.3	−3 to +40	16	15	14	2546.4
	E. undae 190-11 (VKM B 2374)		Permafrost (600 K)		5.5 m, −10°C, pH 7.3	−3 to +40	10	15	15	2579.1
	E. sibiricum 7-3 (VKM B 2374)		Permafrost (30 K)	Russia, Siberia, Khomus-Yuryakh River, 68°19′N, 154°58′E	8 m,−10°C, pH 7	−3 to +40	7	16	17	2563.8
	5138					−3 to +40	12	18	18	2560.0
	E. antarcticum^T DSM14480[d]	(Fruhling et al. 2002)	Microbial mat	Antarctica, Lake Fryxell, McMurdo Dry Valleys, 77°36′S, 162°6′E	Shallow area	−3 to +42	12	15	14	2550.8
	E. undae^T DSM14481[d]		Water	Germany, Wolfenbuttel, Lower Saxony	Surface of garden pond	−3 to +42	7	14	16	2632.0
	TC38-2b	(Bogdanova et al. 1998)	Soil	Ukraine, Carpathian region	Mercury contaminated	−3 to +40	19	16	12	2590.0
	A19	(Reiter et al. 2002)	Stem	Austria, Potato plants	Infected with *Erwinia carotovora*	−3 to +40	12	14	12	2681.9
	E. oxidotolerans^T JCM12280[e]	(Yumoto et al. 2004)	Drain	Japan, Hokkaido, fish processing plant	H_2O_2	4–45	0	16	12	2729.6
	E. acetylicum^T DSM20416[d]	(Collins and Kroppenstedt 1983)	Creamery waste	UK	Unknown	15–45	13	13	15	2555.3
II	GIC31	(Miteva et al. 2004)	Ice (120 K)	Greenland, Glacier	3042.67 m, −9°C, high debris, pH 5.0	4–42	0	15	20	2380.0
	E. aurantiacum ATCC49676[f]		Unknown	Unknown	Unknown	4–42	NG	23	13	2561.9
	E. aurantiacum^T DSM6208[d]	(Collins et al. 1983)	Effluent	UK, potato processing factories	Alkaline, sodium hydroxide	4–45	NG	22	24	2464.7
	E. marinum^T DSM16307[d]	(Kim et al. 2005)	Sea water	Korea, Yellow Sea, Daepo Beach	Tidal flat	15–42	NG	0	13	2504.1
	E. aestuarii^T DSM16306[d]		Sea mud		Sea-coast with mud or slime	15–42	NG	17	20	2504.3
	810	(King et al. 2005)	Air	Hawaii, South Point of the Big Island	Air mass gusting across a sandy promontory and marine waters	4–45	NG	14	12	2568.9

Table 1 continued

Group	Strain	Reference	Source (age)	Geographic region	Environmental conditions	Growth temperature[a] (°C)	Nuclease activity, zone diameter[b] (mm)			Genome size[c] (kb)
							4°C	24°C	37°C	
	India.stream	(Knudston et al. 2001)	Water	India, Ganeshpuri, Tasa River, hot spring	37–39°C	15–45	NG	18	19	2646.1
	India.orange		Water	India, Ganeshpuri, Kunds	37°C	15–50	NG	13	20	2493.4
	Colo.Road		Water	USA, Colorado, Pinkerton hot spring	41°C	15–50	NG	16	25	2585.8
	M37	(Vishnivetskaya et al. 2005)	Water	USA, YNP, Mushroom pool runoff	Low carbonate, 37°C, pH 6.0	15–50	NG	17	26	2422.0
	AT-4		Water	USA, YNP, Angel Terrace	Slightly alkaline, highly carbonate, 50°C	15–50	NG	15	23	2419.6
	AT-1		Water			15–50	NG	14	22	2419.6
	AT-1b		Water			15–55	NG	16	26	2456.3
	NG55		Water	USA, YNP, Narrow Gauge hot spring	Bubbles H_2S, 40–55°C	15–55	NG	14	18	2567.1

[a] Growth temperature range was estimated on TSA, pH 8.8

[b] Nuclease activity was estimated as described (Jeffries et al. 1957)

[c] Genome size represents average from at least three different gels

[d] Strains were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ GmbH, Braunschweig, Germany)

[e] Strain was purchased from the Japanese Collection of Microorganisms (JCM, Japan)

[f] Strains were purchased from the American Type Culture Collection (ATCC, USA)

NG no growth, [T] type strain

determinations were done in at least two independent experiments.

Nuclease assays

Extracellular nucleases (DNases) were determined on DNase agar plates with 0.005% methyl green (Difco) as previously described (Jeffries et al. 1957). Bacteria were grown in broth cultures as described above and 10 µl of the cell suspension was spotted onto these plates which were then incubated at 24, 37, 42 and 50°C for 48 h; and at 4°C for 14 days. A clear zone around the circle of growth indicated nuclease activity. These determinations were done in at least three independent experiments.

Pulsed-field gel electrophoresis

Bacteria were grown at 24°C (Group I) and 30°C (Group II), a temperature at which (based on nuclease assays described above) nuclease activity was reduced. The cells were harvested from 6 ml of a mid-logarithmic phase culture pre-chilled on ice and resuspended in 3 ml of PIV (10 mM Tris pH 7.5; 1 M NaCl) containing 3.5% formaldehyde solution (Fisher Scientific, Pittsburgh, PA, USA) and left on ice for 1.5 h to inactivate endogenous DNase activity (Gibson et al. 1994). After washing with PIV, the cells were resuspended in 300 µl double-strength EC lysis buffer (EC buffer consisting of 6 mM Tris–HCl, pH 7.5, 1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sarcosyl) (Klaassen et al. 2002). Lysozyme (100 µl, 150 mg ml^{-1}) and proteinase K (50 µl, 20 mg ml^{-1}) (Qiagen, Valencia, CA, USA) were added and the cell suspension was immediately mixed with an equal volume of 2% SeaPlaque low-melting temperature agarose (Cambrex BioScience Rockland Inc., ME, USA). The mixture was poured into the plug mold (Bio-Rad Laboratories, Hercules, CA, USA) and allowed to solidify for 10 min at room temperature. The plugs were incubated in 3 ml of EC buffer for 2 h at 37°C with shaking, followed by an overnight incubation in 3 ml of TE buffer at 55°C in standing tubes. They were then placed in fresh TE and stored at 4°C. DNA digestions employed 10 U of I-*Ceu*I or 50 U *Asc*I for 4 h at 37°C, or 50 U *Not*I overnight at 37°C, and were performed following the instructions of the vendor of the enzymes (New England Biolabs, Waverly, MA, USA).

DNA fragments were separated in 1% pulsed-field gel electrophoresis (PFGE)-grade agarose (BioRad Laboratories, Richmond, CA, USA) in 0.5× Tris–borate–EDTA with or without an additional 50 or 100 µM thiourea for 20 h at 14°C in CHEF-DR III apparatus (BioRad Laboratories) at 6.0 V cm^{-1} with initial and final switch times of 15 and 55 s, respectively. DNA fragments were stained with 0.5 µg ml^{-1} ethidium bromide. Uncompressed digital images were captured with the EDAS 290 (Eastman Kodak, New Haven, CT, USA).

Cluster analysis of genomic fingerprints obtained by PFGE was done using GelCompar II version 4.6 (Applied Maths, Inc., Austin, TX, USA). The fingerprinting data were normalized with the reference size marker PFG lambda ladder, size range 50–1,000 kb (New England Biolabs). The Pearson correlation coefficient was applied to the densitometric curves, and the patterns were clustered by the UPGMA. The molecular weight of PFGE bands was estimated by Kodak 1D Image Analysis Software (Eastman Kodak Company, 2000).

Design of DNA targets for hybridization and DNA target hybridizations

Genome sequence data of the *E. sibiricum*^{T} 255-15 have been deposited in the NCBI database (accession number CP001022). Stress-response genes were designated as those known (in other organisms) to be upregulated in response to particular stressors and conferring stress tolerance to the organism. A panel of 82 stress-response genes were used in this study (Table 2). These 82 genes were identified in the genome of *E. sibiricum*^{T} 255-15 by a keyword search through the orthologous groups of proteins (COGs) tab-delimited data-file (http://maple.lsd.ornl.gov/cgi-bin/JGI_microbial/display_page.cgi?page=cog&org=exig&chr=20dec07). Nucleotide sequences were obtained using annotated database files and Artemis V5 (Rutherford et al. 2000). Primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi/) (Rozen and Skaletsky 2000), and were purchased from Qiagen (Valencia, CA). Primer sequences are listed in Supplementary Table S1.

Genomic DNA extraction, polymerase chain reaction, and probe construction

Bacterial cells were harvested by centrifugation of 7 ml of an overnight culture and genomic DNA was extracted as described earlier (Vishnivetskaya and Kathariou 2005). Internal fragments of each gene were amplified by polymerase chain reaction (PCR), yielding products of 300–400 bp (for primer sequences see Supplementary Table S1). For small (≤300 bp) genes, for example, *cspC*, *infA*, *relE* (198, 216, and 267 bp, respectively) an additional 100–150 bp of upstream and downstream regions was included. The desired DNA fragments were amplified with genomic DNA of *E. sibiricum*^{T} 255-15 as template, as described (Vishnivetskaya and Kathariou 2005). The PCR products were purified using QIAquick spin columns (Qiagen), eluted with 35 µl of nuclease-free water

Table 2 Location and name of the genes on the macroarray

Row	Column										
	1	2	3	4	5	6	7	8	9	10	11
A	1164, IS*605* (III)[a]	2939, ISNCY (IX)[a]	3031, *ompR*	2194, *dnaC*	1991, *phrB*	0137, anti-SF	2463, *flgM*	0664, *cspR*	0119, *infA*	2147, *tig*	2596, *desA*
B	2794, IS*605* (II)[a]	1960, *ftsQ*	1026, *recA*	2416, *uvrB*	0730, *recB*	0136, *rpoE*	1686, *cspC*	1585, *rplY*	2096, *queA*	0471, *dnaJ*	1845, *dxr*
C	1113, IS*605* (I)[a]	1826, *ftsK*	0006, *gyrA*	2098, *ruvA*	0151, *recB*	2532, *rpoE*	1683, sigma24	0047, *hsp*	1264, *relE*	2610, *dnaJ*	*L.monocytogenes proB*[b]
D	0743, IS*200* (V)[a]	2223, *uspA*	1590, *gyrA*	0961, *topA*	2075, *recD*	0832, *rpoD*	1514, *pspC*	1848, *frr*	1838, *rpl*8A	1006, *ppiB*	*L.monocytogenes* 1144[b]
E	1163, IS*200* (IV)[a]	0364, *uspA*	0005, *gyrB*	1888, *topA*	2953, *ssl2*	0065, *ctsR*	1777, *dinG*	1836, *rbfA*	0900, *efp*	2952, *osmC*	*L.monocytogenes prsF*[b]
F	2062, IS*30* (VI)[a]	No hit[e],	1589, *gyrB*	0683, *dinP*	2932, *srmB*	1840, *nusA*	2605, *ompR*	1843, *proS*	0782, *dnaJ*	0534, *osmC*	*E. sibiricum*, 16S rRNA gene[c]
G	1430, IS*3* (VII)[a]	1716, *dksA*	3033, *dnaB*	0423, *dinP*	1543, *rpoE*	0779, *hrcA*	1832, *pnp*	1837, *infB*	0781, *dnaK*	2768, *groL*	Marker II[b]
H	No hit[d], IS*3* (VIII)[a]	0299, *pleD*	2195, *dnaB*	3037, *ssb*	0164, *rpoE*	2428, *cspC*	0432, *tif*	2427, P*5rp-1*	0780, *grpE*	2769, *groS*	Empty

Microarray was constructed using the draft version of the genome. However, in the Table 2 the gene numbers and gene names are given as defined in the finished genome (http://genome.ornl.gov/microbial/exig/). Prefix Exig_ to gene number is omitted

[a] Transposase homology group number is given in parentheses as described earlier (Vishnivetskaya and Kathariou, 2005)

[b] Three genes of *Listeria monocytogenes* were used as negative control

[c] 16S rRNA gene of *E. sibiricum*[T] 255-15, and DIG-labeled Marker II were used as positive controls

[d] No hit—gene to become in frame with gene Exig_1430; the primers amplify genes Exig_1430, Exig_1678, Exig_2281

[e] No hit—gene does not get predicted in the new assembly; the primers amplify intergenic region

(Ambion, Austin, TX, USA), and evaluated for size in 1% agarose gel and for concentration using a spectrophotometer (SmartSpec 300, Bio-Rad). For negative control, we used probes specific for *rpoB*, LMO_0687, and *prsF* of *Listeria monocytogenes* F2365 (kindly provided by S. Yildirim). For positive control we used the 16S rRNA gene of *E. sibiricum* 255-15. DNA for array probes was diluted 1:1 (v/v) with DMSO (Sigma, St. Louis, MO).

Array hybridizations

Polymerase chain reaction products (probes and controls) were spotted onto positively charged nylon membranes (Osmonics Inc., Minnetonka, MN, USA) with a hand-operated 96 solid Pin Multi-Blot Replicator (V&P Scientific, San Diego, CA) that delivers 0.2 µl of specimen per pin in multiple spots for each probe. DIG-labeled DNA Molecular Weight Marker II (Roche Diagnostics Corp., IN, USA) was spotted to serve as a positive control for signal detection. Spotted membranes were denatured by incubating the membrane (printed side up) for 15 min on sheets of blotting paper soaked with 1.5 M NaCl and 0.5 N NaOH. Membranes were neutralized by dipping in two baths of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and a final bath of 1× SSC. Wet membranes were exposed face up in a UV crosslinker (Stratalinker 1800, Stratagene, La Jolla, CA) on automatic setting (1,200 µJ). Membranes were air-dried and stored at room temperature until hybridization.

Genomic DNAs (1 µg) were sheared by vigorous shaking in a vortex at maximal speed for 3 min and labeled with digoxigenin-dUTP (Roche, Indianapolis, IN) according to the manufacturer's instructions yielding 1.5 µg digoxigenin-labeled DNAs. Membranes were hybridized overnight with the digoxigenin-labeled genomic DNAs at 42°C in standard buffer (Roche) with 50% formamide. Membranes were washed as described earlier (Vishnivetskaya and Kathariou 2005). Hybridizing spots were detected by chemiluminescence using CSPD (Roche, Indianapolis, IN), following the instructions of the vendor. Genomic DNA of *E. sibiricum*[T] 255-15 served as positive control for hybridization, and genomic DNA of *Yersinia* sp. strain 1310 served as negative control.

16S rRNA gene sequence determination and analysis

The 16S rRNA genes were amplified with bacteria-specific primers 8F and 1492R, targeted to *E. coli* 16S rRNA positions 8–27 (5′-AGA GTT TGA TCC TGG CTC AG-3′), and 1,512–1,492 (5′-ACG GTT ACC TTG TTA CGA CTT-3′), respectively. The thermal PCR profile was as follows: initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, primer

annealing at 54°C for 1 min, and elongation at 72°C for 1.5 min. The final elongation step was 7 min at 72°C. The 16S rRNA genes of newly isolated strains were sequenced at the University of North Carolina at Chapel Hill (UNC-CH) Genome Analysis Facility on a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Primers 8F and 1492R (see above), and additional internal primers targeted to *E. coli* positions 342–357 (5′-CTA CGG G[A/G][G/C] GCA GCA G-3′) and 1,100–1,115 (5′-AGG GTT GCG CTC GTT G-3′) were used to obtain overlapping DNA fragments.

For 16S rRNA gene sequence-based identification of the strains isolated from hot springs at Yellowstone National Park, sequences were initially aligned with the most similar sequences in the small-subunit rRNA database using the algorithm in the Ribosomal Database Project (Maidak et al. 2001). The 16S rRNA gene sequences for other strains used in this study and additional 16S rRNA sequences from closely related bacteria were retrieved from GenBank following identification by BLAST (Altschul et al. 1990). Multiple sequence alignment was done using the program Clustal X (Higgins and Sharp 1988). Phylogenetic 16S rRNA analyses were performed by the neighbor-joining method (Saitou and Nei 1987) using the maximum composite likelihood model for estimating evolutionary distances between DNA sequences (Tamura et al. 2004). Bootstrap values were based on 1,000 replicates generated using the program Mega 4 (Tamura et al. 2007).

Nucleotide sequence accession numbers

The 16S rRNA gene sequences of *Exiguobacterium* spp. strains AT-1, AT-1b, AT-4, M37, NG55, India.stream, India.orange, A19, GIC31, TC38-2b, ATCC49676, and 810 were deposited in GenBank under accession numbers DQ407712, DQ302410, DQ407713, DQ407714, DQ407715, DQ407720, DQ407721, EU282457, EU282458, EU282459, EU282460, EU315251, respectively.

Results

Phylogeny and heterogeneity of 16S rRNA genes

To date 13 species of the genus *Exiguobacterium* have been identified, with type strains *E. acetylicum* DSM20416, *E. indicum* IAM15368, *E. artemiae* DSM16484, *E. sibiricum* DSM17290, *E. oxidotolerans* JCM12280, *E. antarcticum* DSM14480, *E. soli* JCM14376, *E. undae* DSM14481, *E. profundum* DSM17289, *E. aestuarii* DSM16306, *E. marinum* DSM16307, *E. mexicanum* DSM16483, and *E. aurantiacum* DSM6208. Phylogenetic analysis of the 16S rRNA gene sequences from the isolates in our collection, the type strains and other *Exiguobacterium* spp. obtained from the NCBI database revealed the presence of two distinct major clusters with 100% bootstrap. Interestingly, each of these clusters formed two independent sub-clusters, also with 100% bootstrap (Fig. 1). The first major cluster (Group I) consisted of isolates (including 7 type strains) mostly from cold or temperate habitats (surface waters, soils, glacial ice and permafrost); in contrast, the members (including 5 type strains) of the second cluster (Group II) were from hot springs or from slightly alkaline and marine environments, with the exception of strain GIC31, which was isolated from deep ice of a Greenland glacier (Fig. 1). However, strains from the same habitat could be placed in different clusters. Specifically, *E. artemiae*^{T} DSM16484 and *E. mexicanum*^{T} DSM16483 were both isolated from cysts of the brine shrimp, *Artemia franciscana* (Lopez-Cortes et al. 2006), but were placed in Groups I and II, respectively.

Growth at different temperatures

Comparison of the growth of *Exiguobacterium* strains isolated from cold and hot environments indicated that all could grow within a temperature range of 20–37°C. However, the minimum temperature permissive of growth appeared to vary noticeably (Table 1). Strains from permafrost, Lake Fryxell microbial mat, surface water of garden pond, potato plant stem, and soil of Carpathian region (but not Greenland glacial ice isolate) grew at temperatures as low as −3°C, and even at −6°C (Vishnivetskaya et al. 2005). Strains from food processing plants, Hawaiian air mass, and Greenland glacial ice grew at 4°C; the strain from creamery waste, while unable to grow at 4°C on TSA, grew on DNase agar plates at this temperature. None of the isolates from hot springs and Yellow Sea grew at temperatures below 15°C (Table 1).

Marked differences were also noted among the different strains regarding the maximum temperature permissive of growth. Strains from permafrost, stem of potato plants, and soil of Carpathian region grew at 37°C but not at 42°C, whereas all other isolates, including the Greenland glacial ice isolate and the isolate from Lake Fryxell microbial mat grew well at 42°C (but not at 45°C). Isolates from the hot springs, Hawaiian air mass, and food processing plants grew well at 45°C and several of the hot spring isolates also grew at 50–55°C (Table 1).

Extracellular nuclease activity

Findings with PFGE (described below) suggested production of DNase by certain *Exiguobacterium* strains. All the tested strains of *Exiguobacterium* displayed temperature-dependent nuclease activity (Table 1). Strains of Group I grew on DNase agar plates at 4°C and produced DNases,

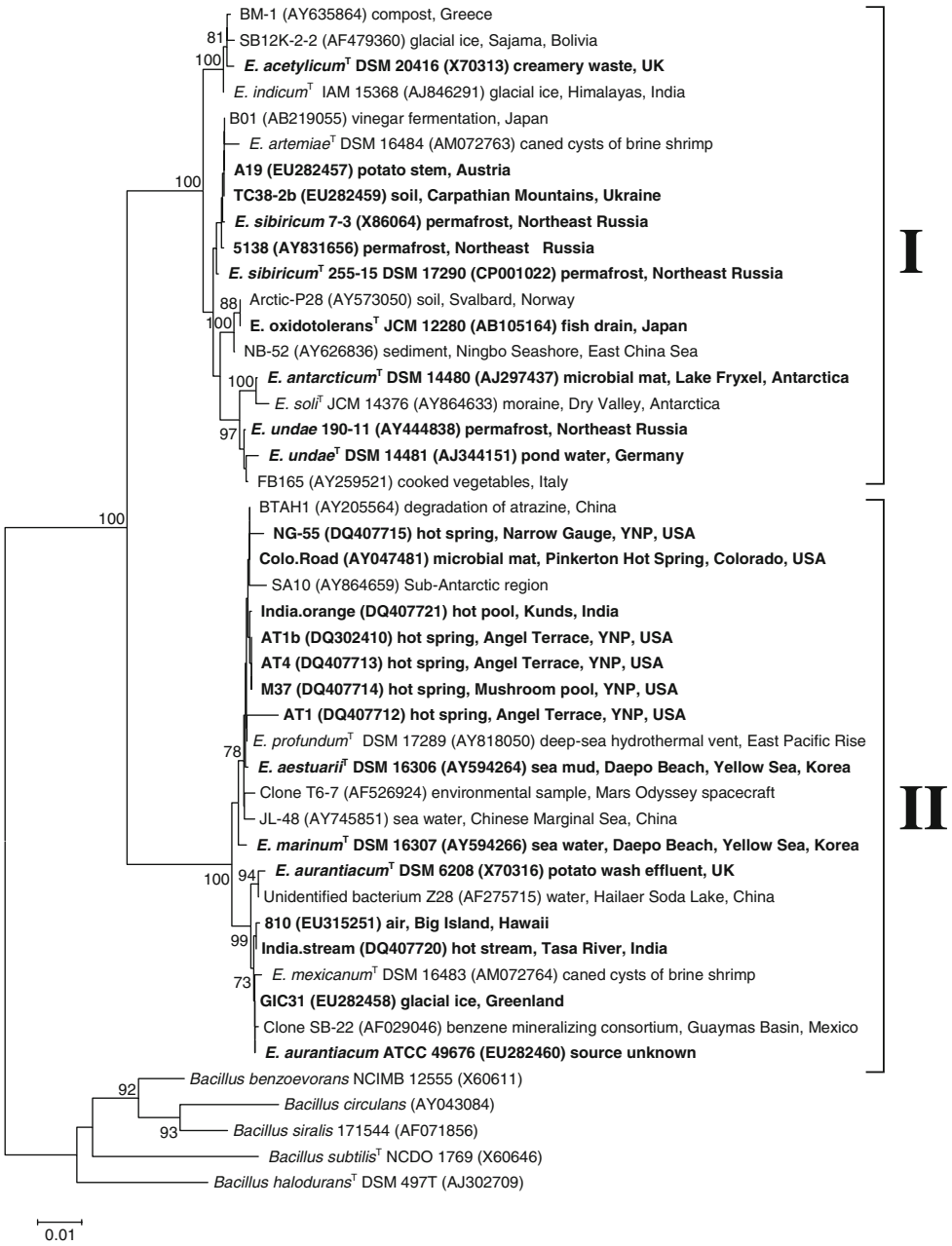

Fig. 1 Phylogenetic relationships of *Exiguobacteium* strains. Tree was produced by a neighbor-joining method. Bootstrap values were based on 1,000 replicates. The *scale bar* represents 0.01 changes per nucleotide position. ^{T} type strain. Strains used in this study are in *bold*

except for *E. oxidotolerans*^{T} JCM12280. Two strains, *E. sibiricum*^{T} 255-15 and TC38-2b, showed higher DNase activity at 4°C than at higher temperatures (Table 1). DNase activity of Group I strains varied slightly following growth at 24 and 37°C. Two strains, *E. undae*^{T} DSM14481 and *E. acetylicum*^{T} DSM20416, produced more DNase at 37°C than at 24°C.

Strains of Group II, except for strain GIC31, failed to grow on DNase agar plates at 4°C, and strain GIC31, while able to grow on DNase agar at 4°C, did not provide evidence for DNase activity. The majority (12/14) of Group II strains produced more DNase at 37°C than at lower temperatures. DNase production by strains India.orange, AT1, AT1b, AT4, and M37 was 55.2–67.7% higher at 37°C than at lower temperatures. Lower DNase production at 37°C than at lower temperature was only observed for two Group II strains (strain 810 and *E. aurantiacum*^{T} ATCC49676). Because of high DNase activity, the bacteria from Group II were considered to be PFGE-untypeable by standard approaches (data not shown).

DNase inactivation by different in situ DNA preparation methods

Several efforts were made to obtain cultures with reduced DNase activity. These included lowering growth

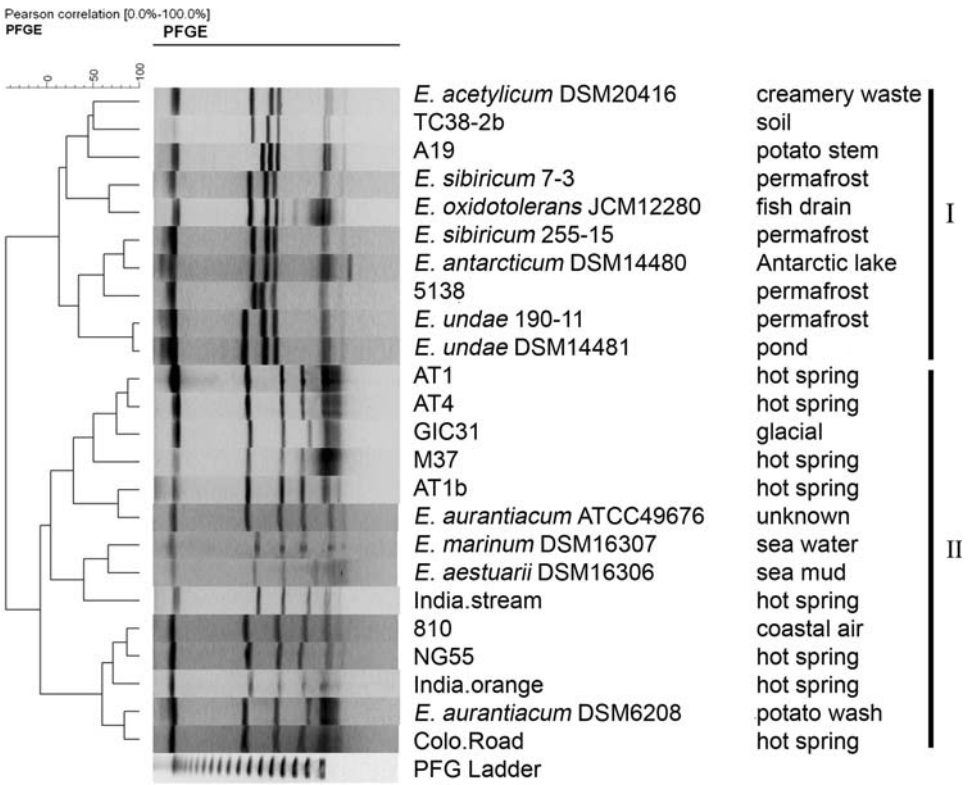

Fig. 2 Analysis of PFGE fingerprints obtained after digestion with I-*Ceu*I, Pearson correlation, UPGMA, GelCompar II software, version 4.6, applied math

temperature to 30°C for the moderate thermophilic strains and 24°C for all other strains; harvesting cells during the logarithmic phase, concentrating the cells up to 10^{10}–10^{11} cell ml^{-1}, and fixing the cells with 3% formaldehyde. This resulted in improved PFGE restriction patterns for some strains (data not shown). However, unambiguous restriction fragments separable with PFGE were only obtained when 50 μM thiourea was added to the electrophoresis buffer and gel, as described for other DNase-producing bacteria (Corkill et al. 2000). Presence of thiourea in the running buffer but not in the gel did not prevent DNA degradation (data not shown).

PFGE with *Asc*I revealed stability of the PFGE profiles following growth at 4 and 24°C for eight selected strains (data not shown). UPGMA-based analysis of the PFGE patterns generated after digestion with I-*Ceu*I divided *Exiguobacterium* strains into two groups (Fig. 2). Since I-*Ceu*I is an intron-encoded endonuclease whose recognition site is located in the 23S rRNA gene (Liu et al. 1999; Newnham et al. 1996), it can be used both for subtyping and for determination of the number of *rrn* operons. Eight bands were observed after I-*Ceu*I digestion in all tested strains (Fig. 2). Genome analysis of the *E. sibiricum*^{T} 255-15 indicates the presence of 9 *rrn* operons. The smallest I-*Ceu*I fragment with expected molecular weight of 6.1 kb was too small to be detectable by PFGE, thus accounting for the discrepancy in number of *rrn* operons. Southern hybridization of *Eco*RI digested genomic DNAs of the *Exiguobacterium* strains using as probe a DIG-labeled fragment of small-subunit 16S rRNA gene yielded 9 bands for all strains except strains 5138, India.orange (8 bands each) and strains A19, GIC31 (7 bands each) (data not shown). In the latter strains, the lower number of hybridization bands might be due to 16S rRNA genes located on *Eco*RI fragments of similar size, or the presence of two 16S rRNA genes on the same *Eco*RI fragment. Genome sizes estimated from PFGE data ranged from 2,380 kb for strain GIC31 to 2,730 kb for strain *E. oxidotolerans*^{T} JCM12280 (Table 1). The estimated genome size for *E. sibiricum*^{T} 255-15 was 2,546 kb, relatively closer to 3,000 kb determined through the genome sequencing of this strain (accession number CP001022).

Hybridization patterns of *E. sibiricum*^{T} 255-15-derived genomic fragments with genomes of different *Exiguobacterium* strains

In order to assess conservation of genomic content between *E. sibiricum*^{T} 255-15 and other *Exiguobacterium* strains, we hybridized a targeted macroarray of 82 stress-response genes (Table 2) with whole genome probes of the 24 *Exiguobacterium* strains listed in Table 1. These dot blot hybridizations yielded a distinct pattern for each strain (data not shown). Assuming that the detection threshold for the hybridization corresponded to 70% identity at the nucleic acid level, the number of homologous or conserved

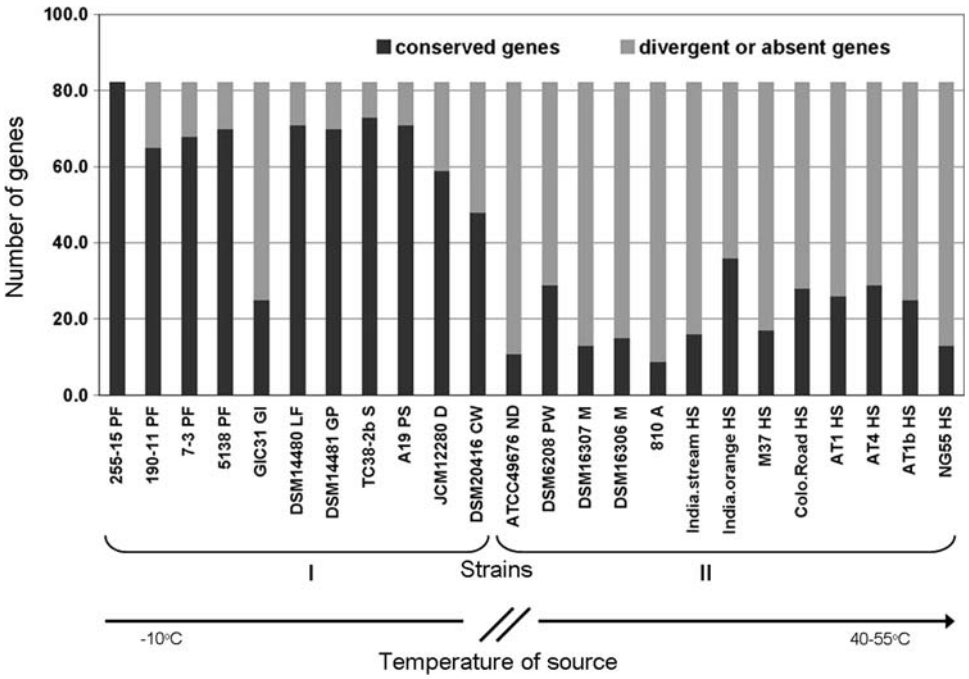

Fig. 3 Macroarray hybridization results divided *Exiguobacterium* strains into 2 groups. Strains of Group I have >50% homologous genes, but strains of Group II have <50% homologous genes. *PF* permafrost, *GI* Greenland ice, *LF* Lake Fryxell, Antarctica, *GP* garden pond, *S* soil, *PS* potato plant stem, *D* drain, *CW* creamery waste, *ND* unknown, *PW* potato wash, *M* marine, *A* air, *HS* hot spring

(>70% identity) and divergent or absent (<70% identity) genes was calculated for each strain (Fig. 3). According to the hybridization patterns, the *Exiguobacterium* strains formed two groups: permafrost strains and strains from cold or temperate habitats grouped together, whereas strains isolated from hot springs grouped together with isolates from marine and alkaline environments. One exception was strain GIC31 (Greenland ice) which fell into the second group. According to the number of shared hybridization signals, isolates from Group I had detectable homology with *E. sibiricum*^{T} 255-15 in ≥58.5% of the genes tested, whereas only 10.9–43.9% of the stress-response genes of Group II isolates had detectable homology with *E. sibiricum*^{T} 255-15 (Fig. 3).

Seven genes (encoding putative DNA gyrase, translation initiation factors *infB* and *infA*, chaperonins *groL* and *dnaK*, ribosome-associated protein Y PSrp-1, and the fatty acid desaturase *desA*) had detectable homologs in the genomes of all strains (Table 3), even though the hybridization signals between *desA* and the genomes of strains *E. marinum*^{T} DSM16307 and *E. aestuarii*^{T} DSM16306, both isolated from Yellow Sea, were at the detection limit (data not shown). All chosen *E. sibiricum*^{T} 255-15 genes hybridized with the genomes of more than one of the strains, with the exception of *ftsK* (encoding the putative DNA segregation ATPase *FtsK/Spo*IIIE) which was detected with a low hybridization signal only in the soil-derived strain TC38-2b. Ten genes, encoding putative DNA gyrase, helicases, anti-sigma factors, DNA/RNA polymerases, heat shock protein, trigger factor, and universal stress protein, were detected in all strains of Group I, but not in Group II (Table 3), suggesting that these may be group-specific. Nineteen other genes were detected in all strains of Group I but were variably detected among Group II strains. On the other hand, 21 genes were variably detected among Group I strains but were absent from strains of Group II. Hybridizations with the negative controls (*Listeria*, *Yersinia*) were largely negative, with the exception of weak hybridization signals produced by five strains (*E. acetylicum*^{T} DSM20416, AT1, AT4, India.-stream, and India.orange) with *proB* of *L. monocytogenes* F2365.

Putative transposase genes in the genome of the different *Exiguobacterium* strains

Transposases, which catalyze site-specific DNA rearrangements and horizontal gene transfer in bacteria, may play a role in the evolutionary process. In order to identify the presence and distribution of homologous transposases among *Exiguobacterium* strains we performed Southern blots with probes designed for putative transposases identified in the annotated genome of *E. sibiricum*^{T} 255-15 (Vishnivetskaya and Kathariou 2005). The putative transposases identified in the genome of *E. sibiricum*^{T} 255-15 were located in the chromosome as evidenced from the genome sequence; the only strain shown to harbor the putative class II transposon on plasmid is *Exiguobacterium* TC38-2b (Bogdanova et al. 1998). The plasmid content of the other *Exiguobacterium* strains is unknown. All 24 *Exiguobacterium* strains, irrespective of source or species, harbored genes encoding putative transposase(s) that

Table 3 Conservative genes in the genome of *Exiguobacterium* strains

Cellular function	Gene ID[a]	COG	Gene	Description	Role in cold adaptation	Detectable homologous	
						Group I	Group II
Replication and repair	Exig_0006	COG0187	*gyrA*	DNA gyrase/topo II topoisomerase IV, A subunit	Control of DNA supercoiling, reduction of linking number	+	−
	Exig_0005	COG0188	*gyrB*	DNA gyrase/topo II topoisomerase IV, B subunit		+	+
	Exig_2932	COG0513	*srmB*	DEAD-box RNA helicase	Ribosome assembly	+	−
	Exig_3033	COG0305	*dnaB*	Replicative DNA helicase	Replication initiation, regulation of its synchrony	+	−
Transcription	Exig_2463	COG2747	*flgM*	Negative regulator of flagellin synthesis (anti-sigma28 factor)	Control of transcription initiation	+	−
	Exig_0137	COG5662		Predicted transmembrane transcriptional regulator (anti-sigma factor)	Regulation of transcription	+	−
	Exig_0136	COG1595	*rpoE*	DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog	Extracytoplasmic stress response, regulation of energy homeostasis	+	−
	Exig_2532	COG1595	*rpoE*	DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog		+	−
Translation and biogenesis	Exig_0119	COG0361	*infA*	Translation initiation factor 1 (IF-1)	Preferential translation of cold-shock mRNAs, overlapping cellular function(s) with *cspC*, *cspB*	+	+
	Exig_1837	COG0532	*infB*	Translation initiation factor 2 (IF-2; GTPase)		+	+
	Exig_0047	COG1188		Ribosome-associated heat shock protein (S4 paralog)		+	−
	Exig_2427	COG1544		Ribosome-associated protein Y (PSrp-1)	Ribosome stabilization, regulation of transcription and translation	+	+
Posttranslational modification, chaperones	Exig_2147	COG0544	*tig*	FKBP-type peptidyl-prolyl cis-trans isomerase (trigger factor)	Facilitation of proper protein folding	+	−
	Exig_0781	COG0443	*dnaK*	Molecular chaperone		+	+
	Exig_2768	COG0459	*groL*	Chaperonin GroEL (HSP60 family)		+	+
Signal transduction	Exig_0364	COG0589	*uspA*	Universal stress protein	Resistance to DNA damaging agents	+	−
Lipid metabolism	Exig_2596	COG3239	*desA*	Fatty acid desaturase	Regulation of membrane fluidity	+	+

[a] Gene IDs are given as defined in http://genome.ornl.gov/microbial/exig/

hybridized with at least one of the transposase probes derived from strain 255-15 (Fig. 4). The genomic DNA of *E. antarcticum*^{T} DSM14480 hybridized with eight of the nine probes, whereas strains AT1b, AT4, M37, 810, and *E. marinum*^{T} DSM16307 hybridized with only one transposase probe, derived from IS*605* orfB (either gene Exig_1113 or gene Exig_2794). *Exiguobacterium* sp. AT1b yielded four hybridization bands with probe for gene Exig_2794 and the draft genome sequence confirmed existence of the four copies of the putative transposase IS*605 orf*B in this strain. No other transposases were found in the genome of *Exiguobacterium* sp. AT1b. We did not detect any correlation between environmental source or habitat of the strain and presence of specific transposase genes.

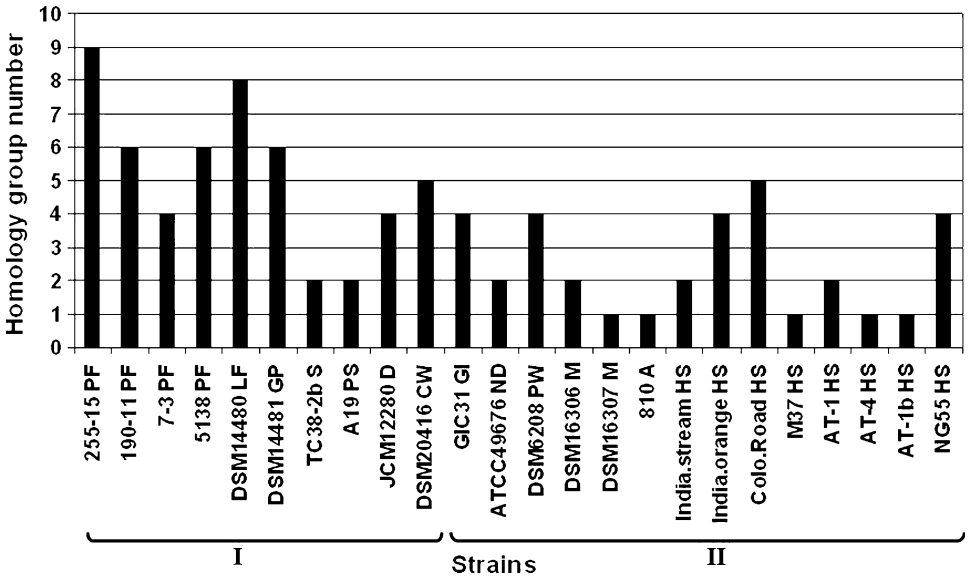

Fig. 4 Distribution of the homologous transposases among *Exiguobacteriun* strains. *PF* permafrost, *GI* Greenland ice, *LF* Lake Fryxell, Antarctica, *GP* garden pond, *S* soil, *PS* potato plant stem, *D* drain, *CW* creamery waste, *ND* unknown, *PW* potato wash, *M* marine, *A* air, *HS* hot spring

Discussion

In this study, investigations of *Exiguobacterium* spp. from different sources using 16S RNA gene sequence analysis and comparative genomic analysis with a targeted gene array unambiguously revealed two distinct groups, each consisting of organisms from environments with different prevailing temperatures: Group I included strains from permafrost and other low-temperature environments, whereas strains of Group II tended to be from high-temperature habitats. A noticeable exception was strain GIC31 which was isolated from ancient Greenland ice but was a member of Group II. However, strain GIC31 may represent an airborne microbe blown onto snow and compacted into glacial ice which may have acted as a good preserver but may well not have been the original habitat for this microorganism.

The comparative macroarray hybridization analyses did not include *E. mexicanum*^{T}, *E. artemiae*^{T}, *E. profundum*^{T}, *E. indicum*^{T}, and *E. soli*^{T} as these strains were not available during the implementation of the study. However, 16S rRNA gene sequence analysis suggested that *E. artemiae*^{T} (canned shrimp), *E. indicum*^{T} (Hamta glacier) and *E. soli*^{T} (Dry Valley, Antarctica) fell in the same cluster as strains from low-temperature environments, while *E. mexicanum*^{T} (canned shrimp) and *E. profundum*^{T} (deep sea hydrothermal vent) grouped together with other marine and hot spring isolates.

To date 13 species or 15 strains (10 strains of Group I and 5 strains of Group II) have been characterized at the genomic or metabolic level. Interestingly, comparisons of their phenotypic and metabolic characteristics indicated that nitrate reductase and oxidase were largely group-specific. Nitrate reductase was negative for all tested Group I strains, except for *E. indicum*^{T}, and positive for Group II. Furthermore, strains of Group I were oxidase-positive, whereas strains of Group II, with the exception of *E. mexicanum*^{T}, were oxidase-negative (Chaturvedi et al. 2008; Chaturvedi and Shivaji 2006; Crapart et al. 2007; Fruhling et al. 2002; Kim et al. 2005; Lopez-Cortes et al. 2006; Rodrigues et al. 2006; Yumoto et al. 2004). *Exiguobacterium* species are facultative anaerobes expected to survive in either oxygenated (low temperature) or deoxygenated (high temperature) environments. The presence of nitrate reductase in the Group II bacteria suggests that they are able to produce energy by reduction of nitrate (NO_3^-) to nitrile (NO_2^-) using anaerobic respiration. The genome analyses (http://genome.ornl.gov/microbial/exig/ and http://genome.ornl.gov/microbial/exig_AT1b/) revealed the presence of four nitrate reductase genes *narI* (2 genes for gamma subunit), *narG* (alpha subunit), *narY* (beta subunit) in the genome of thermophilic *Exiguobacterium* sp. AT1b (Group II) but not in the genome of psychrotrophic *E. sibiricum*^{T} 255-15 (Group I). On the other hand Group I oxidase-positive bacteria contain cytochrome *c* oxidase and can therefore utilize oxygen for energy production with an electron transfer chain. As evidenced from the genome sequence, both strains *E. sibiricum*^{T} 255-15 and *Exiguobacterium* sp. AT1b contain cbb_3-type cytochrome oxidase genes which have $\leq$83% nucleotide sequence identity. Such findings suggest that the partitioning of the genus into the two groups has been accompanied by specific physiological attributes.

Even though the two groups varied in the growth temperature ranges of their constituent strains, the underlying mechanisms are currently unclear. An important question is whether the psychrotrophic lifestyle of certain *Exiguobacterium* spp. may be conferred by a unique set of genes

that are absent in thermophilic members of the genus. Ten of the genes in the targeted array panel (DNA gyrase, DNA helicase, anti-sigma factors, polymerase, heat shock protein, trigger factor, and universal stress protein) were detected in all strains of Group I (cold or temperate environments), but not in Group II (moderately hot, alkaline and marine environments). These conserved genes may be mediating adaptations beneficial to life at low temperatures. Supportive evidence for possible associations of these genes with low temperature adaptations exists from other bacterial systems. For instance, a DNA-dependent RNA polymerase from the Antarctic psychrotrophic bacterium *Pseudomonas syringae* exhibited significant and consistent transcriptional activity at low temperatures (Uma et al. 1999). Trigger factor interacts with nascent polypeptides to ensure correct folding of newly translated cytosolic proteins (Ito 2005), and in the psychrotrophic bacterium *Shewanella* sp. strain SIB1 a protein in the same protein family (FKBP) exhibited higher activity at 10°C compared to 20°C (Suzuki et al. 2004). In the permafrost bacterium *Psychrobacter arcticus* 273-4, a putative FKBP-type peptidyl–prolyl isomerase was over-expressed at low temperature and elevated salinity (Zhng et al. 2007). DNA/RNA helicases have recently been implicated in enabling bacteria to survive cold-shock and to grow at low temperatures. In the Antarctic bacterium *Pseudomonas syringae* Lz4W, inactivation of the helicase encoded by *recD* resulted in a cold-sensitive phenotype (Regha et al. 2005). The universal stress protein (UspA) is a conserved protein that is found in bacteria, archaea, and eukaryotes. In the case of *E. sibiricum*^{T} 255-15, upregulation of DNA topoisomerases was observed at growth temperature extremes: gyrase B was up-regulated at −2.5°C and 39°C but gyrase A only at −2.5°C (Rodrigues et al. 2008). Proteomic analysis of cold (4°C)-grown cells of *E. sibiricum*^{T} 255-15 in comparison to 25°C-grown cells identified several cold-induced proteins, including trigger factor and a sigma factor (sigma 24 homolog) (Qiu et al. 2006). Further transcriptome and proteomic studies will be invaluable in determining whether strains of Group I share a core of cold-inducible proteins absent or repressed in Group II, and similarly whether strains of Group II, adapted to higher temperature regimes, may consistently differ in their transcriptomic and proteomic profiles from those of Group I.

To gain insight into the evolutionary strategies for temperature adaptations and to reveal the variety of metabolic capabilities and roles in carbon and nutrient cycling associated with psychrotrophic versus thermophilic lifestyles, the genome sequencing of the moderate thermophilic *Exiguobacterium* sp. AT1b has been undertaken (http://genome.ornl.gov/microbial/exig_AT1b/). Upon completion of the genome of *Exiguobacterium* sp. AT1b and its integration into the Integrated Microbial Genomes (IMG) system the comprehensive genome analysis of the *Exiguobacterium* genomes with the available genomes of psychrophilic and thermophilic bacteria would allow us to more accurately assess genome diversity among strains, and the possible correlations of such diversity with temperature-related adaptations.

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