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ORIGINAL PAPER

Comparison of bacterial diversity in proglacial soil from Kafni Glacier, Himalayan Mountain ranges, India, with the bacterial diversity of other glaciers in the world

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Abstract Two 16S rRNA gene clone libraries (KF and KS) were constructed using two soil samples (K7s and K8s) collected near Kafni Glacier, Himalayas. The two libraries yielded a total of 648 clones. Phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi Firmicutes*, *Proteobacteria*, *Spirochaetae*, *Tenericutes* and *Verrucomicrobia* were common to the two libraries. Phyla *Acidobacteria*, *Chlamydiae* and *Nitrospirae* were present only in KF library, whereas *Lentisphaerae* and TM7 were detected only in KS. In the two libraries, clones belonging to phyla *Bacteroidetes* and *Proteobacteria* were the most predominant. Principal component analysis (PCA) revealed that KF and KS were different and arsenic content influenced the differences in the percentage of OTUs. PCA indicated that high water content in the K8s sample results in high total bacterial count. PCA also indicated that bacterial diversity of KF and KS was similar to soils from the Pindari Glacier, Himalayas; Samoylov Island, Siberia; Schrimacher Oasis, Antarctica and Siberian tundra. The eleven bacterial strains isolated from the above two soil samples were phylogenetically related to six different genera. All the isolates were psychro-, halo- and alkalitolerant. Amylase, lipase and urease activities were detected in the majority of the strains. Long chain, saturated, unsaturated and branched fatty acids were predominant in the psychrotolerant bacteria.

Keywords Himalayan Mountain ranges · Kafni Glacier · 16S rRNA gene clone library · Psychrophilic enzymes · Cellular fatty acids

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Introduction

Investigating microbial populations with respect to various habitats is of enormous importance in establishing the role of microorganisms in these ecosystems. Further, the advent of molecular approaches such as cloning of the 16S rRNA genes and sequencing (Pace et al. 1986; Woese 1987) DGGE and T-RFLP have indeed enhanced our understanding of the dynamics of microbial populations in various habitats (Wani et al. 2006; Pradhan et al. 2010; Shivaji et al. 2011a, b) and have provided compelling evidence for the existence of many novel types of microorganisms in the environment (Giovannoni et al. 1990; Ward et al. 1990; Hugenholtz et al. 1998a; Shivaji et al. 2005, 2011a, b; Prabagaran et al. 2007) including entirely new prokaryotic lineages (Torsvik et al. 1996; Head et al. 1998; Hugenholtz et al. 1998b).

Bacterial diversity of various cold habitats (water, soil, ice, etc.) from cold regions such as Arctic, Antarctica and other temperate regions based on the rRNA approach is limited (Torsvik et al. 1996; Brambilla et al. 2001; Shivaji et al. 2005, 2011a). Studies on glaciers are even more scarce (Christner et al. 2001, 2003; Zhang et al. 2002;

Miteva et al. 2004; Miteva and Brenchley 2005; Skidmore et al. 2005; Shivaji et al. 2005, 2011b; Bhatia et al. 2006; Cheng and Foght 2007; Prabagaran et al. 2007; Nemergut et al. 2007; Pradhan et al. 2010). The few studies of bacterial diversity in glaciers have clearly revealed that the diversity of glacial water, sediment and basal ice are distinct from microorganisms in the supraglacial (top of the glacier) and proglacial (in front of the glacier) environments, thus implying that each of the habitats needs to be analysed separately.

In this study, both culture-independent and culture-dependent approaches were used to establish the bacterial diversity of soil samples collected near the Kafni Glacier located in the Himalayan Mountain ranges, India. In addition, the culturable bacteria were screened for cold-active enzymes and fatty acid profiles. The primary objectives of the study were to establish the bacterial diversity of a Himalayan glacier, to compare the diversity with other Himalayan, alpine and polar glaciers and other cold habitats and to biopropspect for cold-active enzymes.

Materials and methods

Sampling site and sample collection

The Kafni Glacier (30°13′05″ N 80°03′20″ E) is located in the Kumoan Himalayas of Uttaranchal, a state in India, at an altitude of about 3,853 m above sea level. Two soil samples (K7s and K8s) were collected near the glacier (Supplementary Fig. S1) in the month of September 2005, at altitudes of 3,300 and 3,500 m, respectively. Ice-free soil samples (K7s and K8s) 2 and 1 km away from the glacial snout were collected in sterile polythene bags. The surface soil (about 1 cm) was removed with a sterile spatula and using another sterile spatula the soil was collected. The soil samples were transported to the laboratory under sterile conditions and stored at −20°C until use. It was not possible to reach the snout of the glacier.

Soil analysis

Soil samples were thawed, dried, ground and allowed to pass through a 2-mm sieve. Soil pH (Rhoades 1982), water content (Blakemore et al. 1987) and the chemical characteristics (Jackson 1967) were determined using standard methods. The total element content in the soil was determined after acid digestion. Micronutrients/elements (Li, Be, Al, V, Cr, Mn, Fe, Co, Ni, Zn, Ga, As, Se, Sr, Cd, In, Cs and U) were determined using an atomic absorption spectrophotometer (Perkin Elmer Aanalyst 100 model). Each sample was analysed in triplicate and the mean values were calculated.

Bacterial count

*Bac*Light™ Bacterial Viability Kit (Invitrogen) was used for the determination of the total bacterial count (TBC) in the sediment samples as per the instructions given in the kit. Bacteria were counted in a Petroff-Hausser Counter using a fluorescent microscope (Axioplan 2, Zeiss).

Isolation and characterisation of bacterial strains

Bacterial colonies were isolated on Luria-Bertani (LB) agar plates [tryptone (1.0% w/v), yeast extract (0.5% w/v), NaCl (1.0% w/v) and agar (2.0% w/v)] and 1/10 of LB was then incubated at 4°C for 15 days (Reddy et al. 2008a). For this purpose, 1 g of the soil sample was suspended in 0.9% (w/v) NaCl solution and shaken for 2 h at 15°C. Subsequently, 100 µl of the water sample was plated on LB agar plates, colony counts recorded, and different morphotypes purified and maintained on LB agar medium (Shivaji et al. 1989).

The growth of the bacterial strains at different temperatures, pH and various salt concentrations was checked using ABM [peptone (3% w/v), yeast extract (2% w/v) and agar (2% w/v)] plates (Shivaji et al. 1992, 2011b). Extracellular enzymatic activities of amylase (Priest 1977), lipase (Booth 1978), protease (Priest 1977) and urease were checked by streaking the cultures on ABM agar plates supplemented with 0.2% soluble starch, 1% Tween-60 along with 0.01% $CaCl_2$, 0.3% casein and 2% urea (filter sterilised), respectively, and incubating the plates at 4 and 18°C for 5–10 days. For fatty acid analysis, all the isolates were grown on trypticase soy agar plates at 18°C for 2 days. Fatty acid methyl esters were prepared and analysed as previously described (Shivaji et al. 2007). DNA was isolated from all the cultures and the 16S rRNA gene was amplified and sequenced, and phylogenetic analysis was carried out as described earlier (Reddy et al. 2000, 2009; Srinivas et al. 2009; Shivaji et al. 2011b).

Extraction of total DNA from soil and PCR amplification of the 16S rRNA gene

Total DNA was isolated from the soil samples essentially according to the methods described earlier (Shivaji et al. 2004; Tsai and Olson 1991). Primers 16S3 (5′-TCC TAC GGG AGG CAG CAG-3′) and 16S4 (5′-GGC GGT GTG TAC AAG GCC C-3′) corresponding to positions 339–356 and 1,402–1,384, respectively, of the *Escherichia coli* 16S rRNA gene (Lane 1991) were used to amplify about 1.0-kb fragment of the total 1.5-kb 16S rRNA gene. Amplification was done as described earlier (Reddy et al. 2000; Shivaji et al. 2004, 2011a). The PCR product was purified with the Quiaquick PCR purification kit (Qiagen Inc.) according to the instructions provided.

Cloning and library construction of soil 16S rRNA gene sequences

The purified PCR product was cloned into pMOS Blue Blunt End vector system (Amersham Biosciences) following the instructions of the manual. Transformants were selected on an LB agar plate containing 20 μg/ml of X-gal and 60 μg/ml of ampicillin and incubated at 37°C overnight. Clones were maintained on LB agar plates containing X-gal and ampicillin.

PCR amplification, sequencing of the 16S rRNA clone libraries and phylogenetic analysis

The 16S rRNA gene was amplified from the transformants by colony PCR using the vector-targeted M13 forward (5′-GTA AAA CGA CGG CCA GT-3′) and M13 reverse (5′-GGA AAC AGC TAT GAC CAT G-3′) primers, respectively, and sequenced using the primers M13 forward, M13 reverse, pD (5′-CAG CAG CCG CGG TAA TAC-3′) and pF* (5′-ACG AGC TGA CGA CAG CCA TG-3′) (Pradhan et al. 2010). Vector and chimeric sequences were eliminated using Gene Tool version 2 (www.biotools.com). Sequences were then subjected to BLAST to identify the nearest taxa and aligned with sequences belonging to the nearest taxa (downloaded from NCBI database http://www.ncbi.nlm.nih.gov/) using CLUSTAL X. Phylogenetic trees were constructed using neighbour joining and maximum likelihood methods (Pradhan et al. 2010; Shivaji et al. 2011b). Bootstrap analysis, based on 1,000 replicate data sets, was performed to assess stability among the clades.

Statistical analyses of the cloned libraries

To compare the bacterial diversity within the two samples, 16S rRNA gene sequences of of the clones from the two libraries showing ≥97% sequence similarity were grouped into the same OTU (phylotype). Shannon–Wiener Diversity Index (http://www.changbioscience.com/genetics/shannon.html) was used to calculate Shannon Index (H′), evenness (Lefauconnier et al. 1994) and the Simpson's index (D) (Magurran 1996). Rarefaction analysis was done using the site Online Calculation (http://biome.sdsu.edu/fastgroup/cal_tools.htm). Coverage of 16S rRNA gene clone libraries was calculated as described previously (Good 1954). Rarefaction curves were generated to compare the relative diversity and coverage of each library.

The two 16S rRNA gene clone libraries from this study (KF and KS) were compared using a JAVA-based software Comm Cluster (Hur and Chun 2004) with three different cutoff values (97, 90 and 80%) and an ordination analysis was performed using principle component analysis (PCA). PCA was performed using the SPSS statistical computing package (version 16.0; SPSS Inc.) to group or separate samples based on the biogeochemical parameters (altitude, total bacterial count, water content, pH value and micronutrients/elements such as Li, Be, Al, V, Cr, Mn, Fe, Co, Ni, Zn, Ga, As, Se, Sr, Cd, In, Cs and U) and the percentages of OTUs in each sample (higher taxa ≥1% in the libraries were considered). Phylotypes (showing ≥97% similarity in 16S rRNA gene sequences) from KF and KS libraries were also compared with 16S rRNA gene clone libraries of 14 other glacier samples from different regions of the world [RKS1 and RKS6, soil samples from Roopkund Glacier Lake, India (Pradhan et al. 2010); RKS7, soil sample from Roopkund Glacier, India (Pradhan et al. 2010); P1S, P4S and P8S, soil samples, Pindari Glacier, India (Shivaji et al. 2011b); TPG, Tibetan Plateau Glacier (Liu et al. 2009), MLME, moraine Lakes, Mount Everest (Liu et al. 2006); GMWME, glacial meltwater, Mount Everest (Liu et al. 2006); JEGC, John Evans Glacier, Canada (Cheng and Foght 2007); BGA, Bench Glacier, Alaska (Skidmore et al. 2005); SOA, soil sample, Schirmacher Oasis, Antarctica (Shivaji et al. 2004); ST, soil sample, Siberian tundra; SIS, Samoylov Island, Siberia (Wagner et al. 2009)]. PCA was performed on the number of phylotypes in each of the libraries.

Nucleotide sequence accession numbers

All the sequences of the 16S rRNA gene clone libraries were deposited in GenBank with accession numbers EF421990 to EF421997; EF421999 to EF422011; EF42 2013 to EF422015; EF422018 to EF422022; EF422026 to EF422033; EF422035; EF422036; EF422038 to EF422 044; EF422046; EF422047; EF422049 to EF422063; EF422065; EF422066; EF434184 to EF434196; EF434198 to EF434202; EF434204 to EF434221; EF434223 to EF43 4225; EF434227 to EF434262; EF445432 to EF445444; EF445446 to EF445457; EF445459 to EF445478; EF44 5480 to EF445493; EF445495 to EF445503; EF445507 to EF445515; EF445517 to EF445523; EF445525 to EF445 531; EF445533 to EF445535; EF445537 to EF445544; and EU809512 to EU809923.

Results

Bacterial abundance and the physicochemical characteristics of the soil samples collected near Kafni Glacier

In the two soil samples (K7s and K8s) collected near the Kafni Glacier at an altitude of 3,300 and 3,500 m, respectively, it was observed that the pH, water content,

soil texture and elemental chemical characteristics were different (Table 1). The total bacterial count in K7s and K8s was also different (6.25 ± 0.25 and 30.71 ± 0.23 × 10^8 bacteria g^{-1} of soil respectively) (Table 1).

Culture-independent bacterial diversity from soil samples collected near the Kafni Glacier

16S rRNA gene clone libraries

The soil samples (K7s and K8s) yielded about 50 and 60 μg DNA g^{-1} of soil. About 200 ng of the DNA from K7s and K8s was used for the construction of two 16S rRNA gene libraries KF and KS, respectively. The number of clones in KF and KS were 237 and 411 clones, respectively, with an insert size of approximately 1 kb. BLAST sequence similarity analysis of the two clone libraries indicated that clones in KF and KS were affiliated to 11 and 10 phyla, respectively. Clones affiliated to eight phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Proteobacteria*, *Spirochaetae*, *Tenericutes* and *Verrucomicrobia* were common to both libraries. Phyla *Acidobacteria*, *Chlamydiae* and *Nitrospirae* were present only in the KF library and clones belonging to the phylum *Lentisphaerae* and candidate phylum TM7 were present only in the KS library (Fig. 1; Supplementary Table 1). Based on the BLAST analysis, the 237 clones from KF belong to 106 bacterial taxa and the 411 clones from KS belong to 93 bacterial taxa (Supplementary Table 1). In the two libraries (KF and KS), clones belonging to phyla *Bacteroidetes* (23.2 and 34.8%) and *Proteobacteria* (51.5 and 51.3%) were the most predominant (representing >10%) of the total clones (Supplementary Table 1).

Table 1 Physicochemical characteristics and the total bacterial count of two soil samples (K7s and K8s) collected near the Kafni Glacier, Himalayas, India

Soil parameters	K7s	K8s
Altitude (m)	3,300.0	3,500.0
Soil texture	Fine, dark brown, sandy with small stones	Fine, cream to brown, sandy with small stones
pH	7.5	7.1
Water content (%)	8.0	25.0
Ni (ppm)	30.4	12.7
In (ppb)	28.7	6.9
Cd (ppb)	282.2	54.1
Zn (ppm)	121.9	35.8
Co (ppm)	13.3	5.1
Cr (ppm)	51.5	19.9
As (ppm)	5.5	40.0
Fe (ppm)	17,662.9	9,082.9
Be (ppm)	1.1	0.6
Al (ppm)	5,229.7	2,969.7
Li (ppm)	28.1	17.3
Cs (ppb)	6,492.9	3,328.9
Mn (ppm)	659.8	407.8
U (ppb)	1,914.4	1,895.4
V (ppm)	55.0	22.2
Se (ppb)	830.9	573.3
Sr (ppm)	42.6	32.9
Ga (ppm)	22.4	4.2
Total bacterial count/ of soil (10^8) [SD]	6.3 (0.25)	30.7 (0.23)
16S rRNA gene clone library	KF	KS

Proteobacteria

Clones affiliated to all classes of the phylum *Proteobacteria namely Alpha-, Beta-*, *Gamma-*, *Delta-* and *Epsilonproteobaceria* were present in libraries KF and KS (122 and 211 clones) (Supplementary Table 1). Phylogenetic analysis indicated that 108 out of the 122 clones in KF and 161 out of the 211 clones in KS that were affiliated to the phylum *Proteobacteria* clustered with their nearest phylogenetic neighbour (Figs. 2a, 3a) (Supplementary Table 1). Some of the clones (13 and 46 clones in KF and KS, respectively) did not form a cluster with the nearest phylogenetic neighbour, but formed a cluster within the same class of phylum *Proteobacteria.* In contrast, clones KF-225, KS-106, KS-365, KS-504 and KS-202 did not cluster within the same class (Figs. 2a, 3a).

Bacteroidetes

Clones affiliated to three classes of the phylum *Bacteroidetes*, namely *Bacteroidia, Flavobacteria* and *Sphingobacteria* were present in both the libraries (KF and KS) (Supplementary Table 1). The majority of the clones, 51 out of the 55 clones in KF and 126 out of the 143 clones, clustered with their nearest phylogenetic neighbour (Figs. 2b, 3b). A few of the clones (KS-177 and KS-228) clustered outside of the phylum (Fig. 3b).

Firmicutes

Clones affiliated to four classes of the phylum *Firmicutes,* namely *Bacilli*, *Clostridia*, *Erysipelotrichia* and *Negativicutes*, were present in KF, whereas clones related to the class *Negativicutes* were absent in the KS library (Supplementary Table 1). All clones which belonged to the phylum *Firmicutes* clustered with their nearest phylogenetic neighbour in the KF library (Fig. 2b), but only 17 out of the 26 clones clustered with their nearest phylogenetic neighbour in the

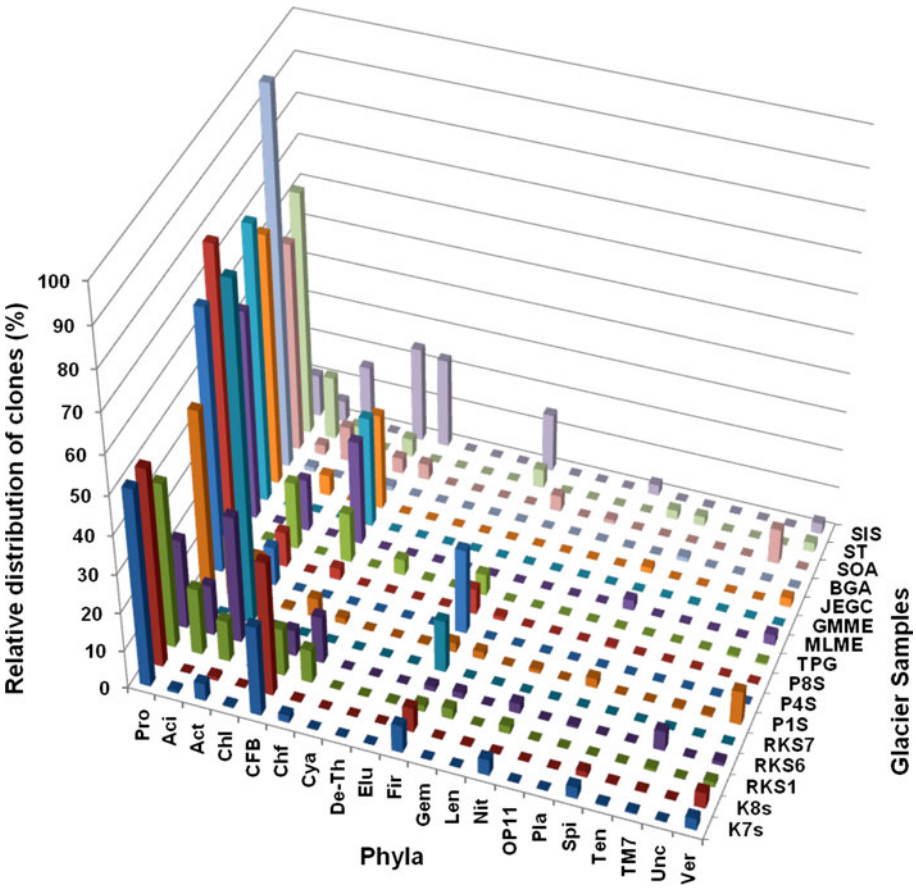


Fig. 1 Comparison of the bacterial diversity in different cold habitats as determined by 16S rRNA gene clone libraries. K7s and K8s, soil samples, Kafni Glacier, India (present study); RKS1, RKS6 and RKS7 Roopkund Glacier; P1S, P4S and P8S, Pindari Glacier; TPG, Tibetan Plateau Glacier; MLME, moraine lakes, Mount Everest; GMWME, glacial meltwater, Mount Everest; JEGC, John Evans Glacier, Canada; BGA, Bench Glacier, Alaska; SOA, Schirmacher Oasis, Antarctica and ST, Siberian tundra; SIS, Samoylov Island, Siberia. Pro, *Proteobacteria*, Aci, *Acidobacteria*; Act, *Actinobacteria*; CFB, *Cytophaga–Flavobacterium–Bacteroides*; Chf, *Chloroflexi*; Cya, *Cyanobacteria*; De-Th, *Deinococcus and Themomicrobia*; Elu, *Elusimicrobia;* Fir, *Firmicutes*; Gem, *Gemmatimonadetes*; Len, *Lentosphaerae*; Nit, *Nitrospirae*; OP11, candidate divisions OP11; Pla, *Planctomycetes*; Spi, *Spirochaetae*; Ten, *Tenericutes*; TM7, TM7_s TM7a (candidate phylum); Unc, unculturables; and Ver, *Verrucomicrobia*. Values represent the percentage of clones

KS library (Fig. 3b). Clones KS-437, KS-159, KS-327, KS-420, KS-470 and KS-483 clustered with the phylum *Bacteroidetes* instead of in *Firmicutes* (Fig. 3b) clade 5.

Actinobacteria

Clones affiliated to *Actinobacteria* in KF and KS (12 and 4 clones) (Supplementary Table 1) clustered with the related sequences (Figs. 2b, 3b). One clone KS-78 appeared to be phylogenetically affiliated to *Cryobacterium roopkundense* RuGl7^{T} (which was earlier isolated by us from the same habitat (Reddy et al. 2010) (Fig. 3b).

Verrucomicrobia

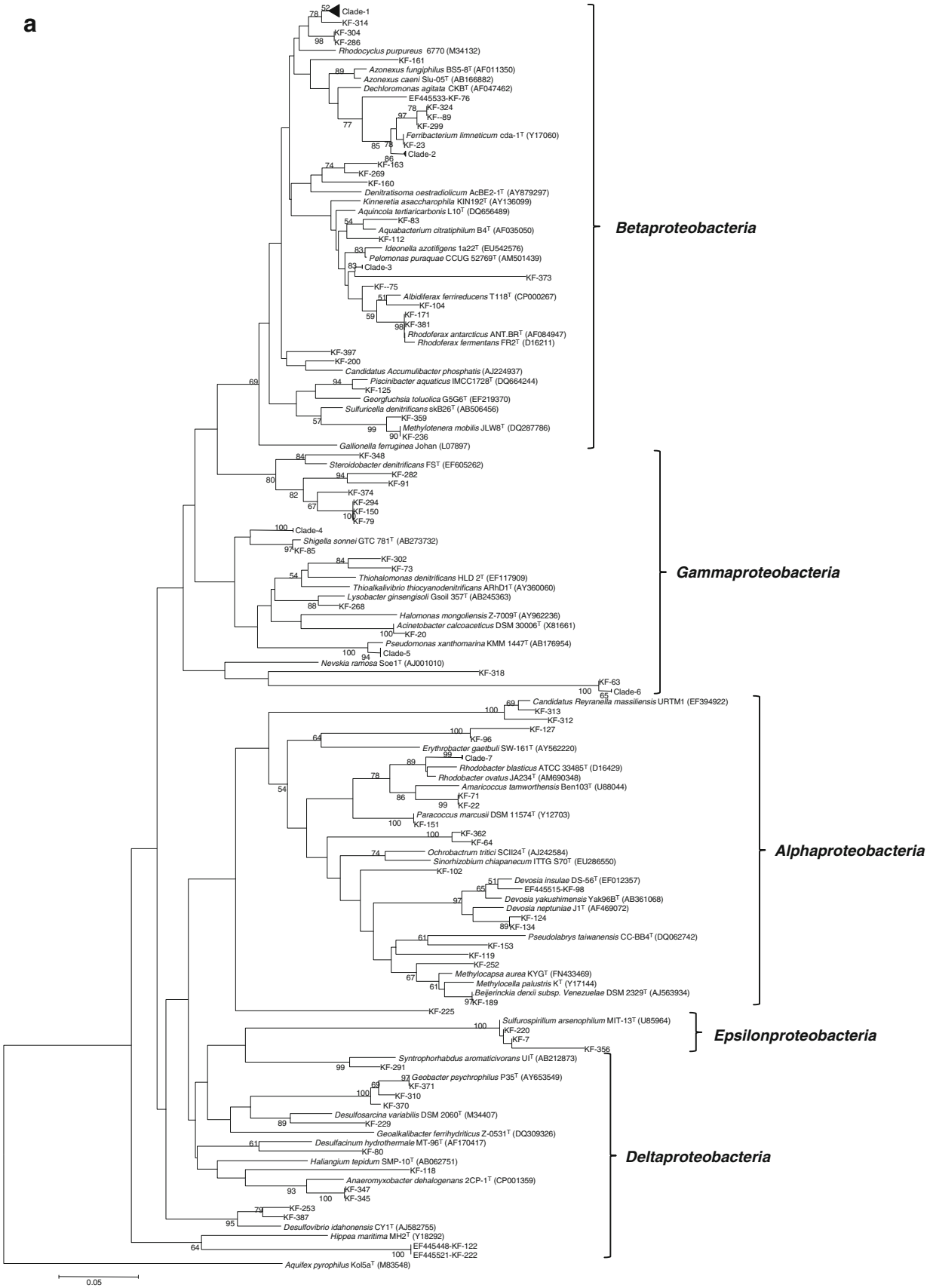
Clones affiliated to two classes of the phylum *Verrucomicrobia*, namely *Opitutae* and *Verrucomicrobiae*, were present in KF, but in KS clones related to class *Opitutae* were absent (Supplementary Table 1). KF-288 clustered with the phylum *Chlamydiae* (Fig. 2b).

Nitrospirae and Spirochaetae

Clones affiliated to *Nitrospirae* were present in only the KF library (9 clones), whereas those affiliated to *Spirochaetae* were present in both KF and KS (8 and 6 clones) (Supplementary Table 1). All the clones of *Nitrospirae* and *Spirochaetae* clustered with the nearest phylogenetic neighbours (Figs. 2b, 3b).

Acidobacteria, Chlamydiae, Chloroflexi, Lentisphaerae, Tenericutes and candidate phylum TM7_s TM7a

Clones affiliated to the phyla *Chloroflexi* and *Tenericutes* were present in both KF and KS. Clones affiliated to the phyla *Acidobacteria* and *Chlamydiae* were specific only to


a
Clade-1
KF-314
KF-304
KF-286
Rhodocyclus purpureus 6770 (M34132)
KF-161
Azonexus fungiphilus BS5-8T (AF011350)
Azonexus caeni Slu-05T (AB166882)
Dechloromonas agitata CKBT (AF047462)
EF445533-KF-76
KF-324
KF--89
KF-299
Ferribacterium limneticum cda-1T (Y17060)
KF-23
Clade-2
KF-163
KF-269
KF-160
Denitratisoma oestradiolicum AcBE2-1T (AY879297)
Kinneretia asaccharophila KIN192T (AY136099)
Aquincola tertiaricarbonis L10T (DQ656489)
KF-83
Aquabacterium citratiphilum B4T (AF035050)
KF-112
Ideonella azotifigens 1a22T (EU542576)
Pelomonas puraquae CCUG 52769T (AM501439)
Clade-3
KF-373
KF--75
Albidiferax ferrireducens T118T (CP000267)
KF-104
KF-171
KF-381
Rhodoferax antarcticus ANT.BRT (AF084947)
Rhodoferax fermentans FR2T (D16211)
KF-397
KF-200
Candidatus Accumulibacter phosphatis (AJ224937)
Piscinibacter aquaticus IMCC1728T (DQ664244)
KF-125
Georgfuchsia toluolica G5G6T (EF219370)
Sulfuricella denitrificans skB26T (AB506456)
KF-359
Methylotenera mobilis JLW8T (DQ287786)
KF-236
Gallionella ferruginea Johan (L07897)
Betaproteobacteria
KF-348
Steroidobacter denitrificans FST (EF605262)
KF-282
KF-91
KF-374
KF-294
KF-150
KF-79
Clade-4
Shigella sonnei GTC 781T (AB273732)
KF-85
KF-302
KF-73
Thiohalomonas denitrificans HLD 2T (EF117909)
Thioalkalivibrio thiocyanodenitrificans ARhD1T (AY360060)
Lysobacter ginsengisoli Gsoil 357T (AB245363)
KF-268
Halomonas mongoliensis Z-7009T (AY962236)
Acinetobacter calcoaceticus DSM 30006T (X81661)
KF-20
Pseudomonas xanthomarina KMM 1447T (AB176954)
Clade-5
Nevskia ramosa Soe1T (AJ001010)
KF-318
KF-63
Clade-6
Gammaproteobacteria
Candidatus Reyranella massiliensis URTM1 (EF394922)
KF-313
KF-312
KF-127
KF-96
Erythrobacter gaetbuli SW-161T (AY562220)
Clade-7
Rhodobacter blasticus ATCC 33485T (D16429)
Rhodobacter ovatus JA234T (AM690348)
Amaricoccus tamworthensis Ben103T (U88044)
KF-71
KF-22
Paracoccus marcusii DSM 11574T (Y12703)
KF-151
KF-362
KF-64
Ochrobactrum tritici SCII24T (AJ242584)
Sinorhizobium chiapanecum ITTG S70T (EU286550)
KF-102
Devosia insulae DS-56T (EF012357)
EF445515-KF-98
Devosia yakushimensis Yak96BT (AB361068)
Devosia neptuniae J1T (AF469072)
KF-124
KF-134
Pseudolabrys taiwanensis CC-BB4T (DQ062742)
KF-153
KF-119
KF-252
Methylocapsa aurea KYGT (FN433469)
Methylocella palustris KT (Y17144)
Beijerinckia derxii subsp. Venezuelae DSM 2329T (AJ563934)
KF-189
Alphaproteobacteria
KF-225
Sulfurospirillum arsenophilum MIT-13T (U85964)
KF-220
KF-7
Epsilonproteobacteria
KF-356
Syntrophorhabdus aromaticivorans UIT (AB212873)
KF-291
Geobacter psychrophilus P35T (AY653549)
KF-371
KF-310
KF-370
Desulfosarcina variabilis DSM 2060T (M34407)
KF-229
Geoalkalibacter ferrihydriticus Z-0531T (DQ309326)
Desulfacinum hydrothermale MT-96T (AF170417)
KF-80
Haliangium tepidum SMP-10T (AB062751)
KF-118
Anaeromyxobacter dehalogenans 2CP-1T (CP001359)
KF-347
KF-345
KF-253
KF-387
Desulfovibrio idahonensis CY1T (AJ582755)
Hippea maritima MH2T (Y18292)
EF445448-KF-122
EF445521-KF-222
Deltaproteobacteria
Aquifex pyrophilus Kol5aT (M83548)
0.05

◀ **Fig. 2** Neighbour joining phylogenetic tree of 16S rRNA gene clones from KF library, showing the phylogenetic relationship of clones affiliated to *Proteobacteria* (**a**) and clones affiliated to phyla *Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chloroflexi*, *Firmicutes*, *Nitrospirae, Spirochaetae*, *Tenericutes* and *Verrucomicrobia* (**b**). *Aquifex pyrophilus* Kol5a^{T} was taken as an outgroup. Numbers at the nodes are bootstrap values. The bar represents 0.05 substitutions per alignment position in **a** and **b**

KF; those affiliated to *Lentisphaerae* and candidate phylum TM7_s TM7a were specific only to the KS library and appeared at a very low frequency (Supplementary Table 1). Phylogenetic analysis indicated that clones affiliated to the above phyla clustered with their respective phylogenetic neighbours (Figs. 2b, 3b), except KS-330 which was clustered within the phylum *Bacteroidetes* (Fig. 3b).

Statistical analysis

The 16S rRNA gene clones from the 2 soil samples (K7s and K8s) based on 16S rRNA gene sequence similarity criteria of ≥97% could be categorised into 125 and 238 phylotypes (Supplementary Fig. S2; Table 2). The common phylotypes in KF and KS libraries are shown in Supplementary Table 1. Species richness at 97% cutoff was 125 and 238, and the diversity coverage 47.3 and 42.1% for KF and KS libraries, respectively. Reducing the cutoff from 97 to 90% and from 97 to 80% reduced the species richness to 12 and 11 and increased the coverage to 94.9 and 97.3%, respectively (Table 2). The rarefaction curves indicated that at 80% cutoff, both the curves in K7s and K8s soil samples plateaued, while only K7s soil sample plateaued when 90% cutoff was considered (Supplementary Fig. S2). The rarefaction curve analysis implied that these were likely to be minimal estimates of diversity. These observations are supported by bacterial diversity parameters, such as Shannon–Wiener Diversity Index, Shannon entropy, Simpson's Index, coverage, Chao1 and evenness (Table 2). Further based on principal component analysis, K7s and K8s clustered distantly at 97% cutoff value, but came closer at both 90 and 80% cutoff values (Fig. 4a, b, c).

The result of PCA based on percentage of a specific OTU (Phyla with ≥1% clones were considered) in the two libraries (KF and KS) is shown in Fig. 5a, and it appears that the principal component factors 1 and 2 (PC1-72.419 and PC2-27.581%) explain 100% of the total variances. The phyla *Acidobacteria*, *Chlamydiae* and *Nitrospirae*, which were present only in the KF library, clustered together, whereas phyla *Lentisphaerae* and candidate phylum TM7, which were present only in the KS library, clustered together. The phyla *Chloroflexi*, *Actinobacteria*, *Spirochaetae* and *Tenericutes*, which were dominant in the KF library, clusterd together close to the right side horizontal line of PCA plot. The other phyla *Firmicutes*, *Proteobacteria*, *Verrucomicrobia* and *Bacteroidetes*, which were nearly equal in both the libraries or higher in the KS library, clustered together away from the other phyla.

The result of PCA based on the biogeochemical properties is shown in Fig. 5b and the principal component factors 1 and 2 (PC1, 89.464 and PC2, 10.535%) explained 100% of the total variances. The PCA plot indicated that arsenic and water content which are higher in the K8s soil sample grouped closely (Fig. 5b; Table 1). All other parameters which are numerically high in K7s, such as Ga, In, Cd, Zn, V, Co, Ni, Cr, Al, Cs, Be, Li, Se, Mn, Sr, U, altitude and pH, clustered together (Fig. 5b). Thus, it may imply that As and water content are the key parameters that influence the observed differences in the percentage of specific OTUs in the two 16S rRNA gene clone libraries. The high bacterial content in K8s may also be influenced by water content (Fig. 5b). Thus, PCA does discriminate the two soil samples.

The result of PCA based on phylotypes with 97% cutoff value of 16 clone libraries, 2 from the present study (KF and KS libraries) and 14 16S rRNA gene clone libraries from other glaciers, is shown in Fig. 6. The 16 libraries grouped into 5 clusters represented by cluster 1 (KF and KS from the Kafni Glacier; P1s and P8s from Pindari Glacier; SOA from Schirmacher Oasis, Antarctica; ST from Siberian tundra and and SIS from Samoylov Island, Siberia), cluster 2 (P4s sample from Pindari Glacier; TPG from Tibetan Plateau Glacier, Tibet and MLME from moraine lakes of Mount Everest) cluster 3 (BGA from Bench Glacier, Alaska; GMWME from glacial meltwater of Mount Everest and JEGC from John Evans Glacier, Canada) cluster 4 (RUGL1 and RUGL6 from Roopkund Glacier, Himalayas, India) and cluster 5 (RUPGL7 also from Roopkund Glacier which clustered between the RUGL1, RUGL6 and KF and KS libraries). Cluster 2 also appeared to be close to cluster 1 and 3.

Characterisation of the bacterial strains isolated from the soil samples collected near the Kafni Glacier

A total of 11 bacterial strains were isolated from the 2 soil samples (Table 3). The 11 isolates were psychro-, halo- and alkalitolerant (Table 3). Amylase, lipase and urease activities were detected in the majority of the strains (7, 9 and 11, respectively) at 4 and 18°C (Table 3), but protease activity was not detected at 4 and 18°C.

Fatty acid profiles of the isolated strains from soil samples collected near the Kafni Glacier

The fatty acid composition in the 11 isolates was different and not even a single fatty acid was common to all the isolates. Some fatty acids such as iso-$C_{10:0,}$ $C_{12:0}$, $C_{14:0}$,

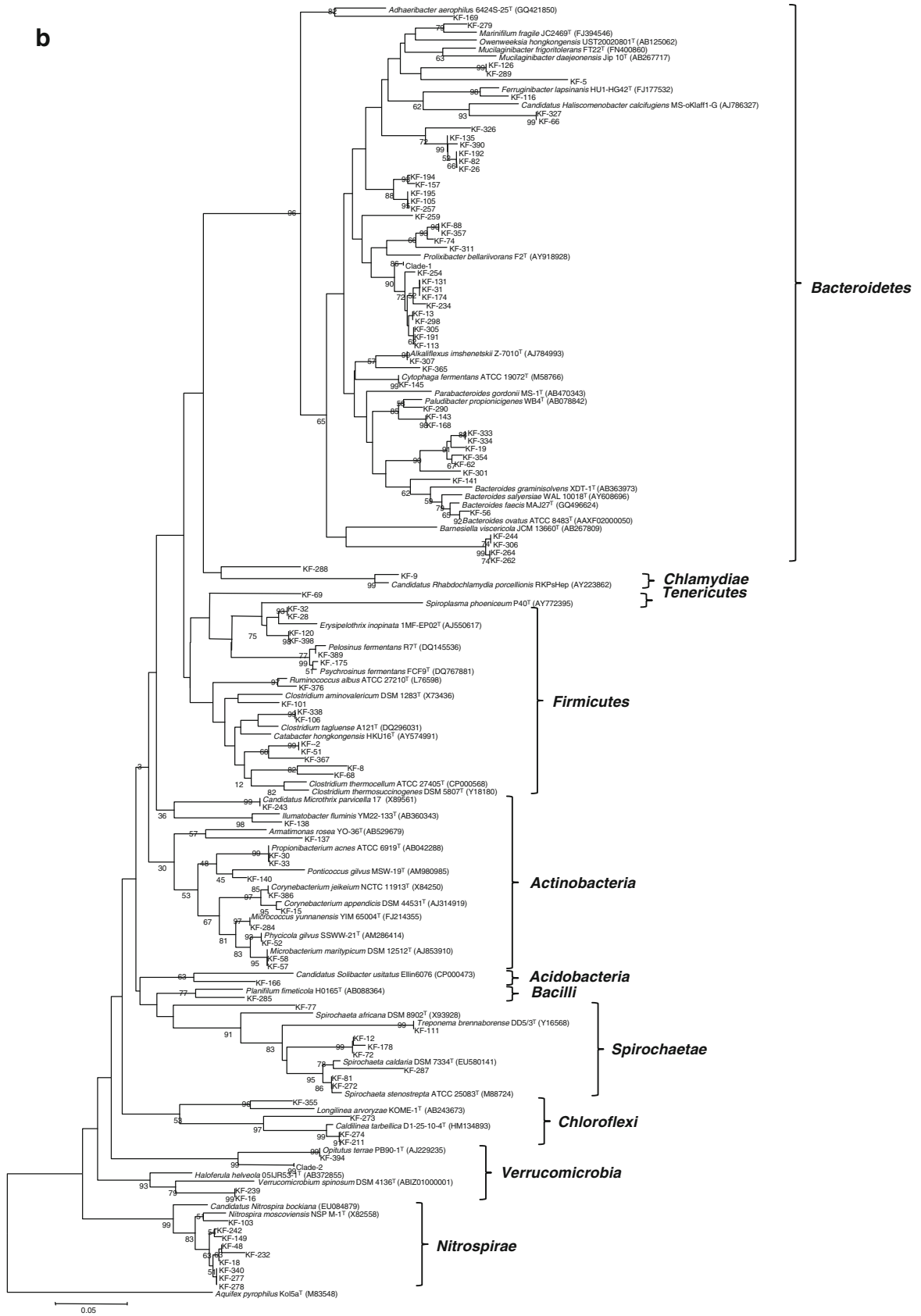


Fig. 2 continued

iso-$C_{15:0}$, anteiso-$C_{15:0}$, anteiso-$C_{15:1}$, iso-$C_{16:0}$, $C_{16:0}$, anteiso-$C_{17:1}$, $C_{18:0}$, $C_{18:1\ \omega9c}$ and summed feature 3 were present in the majority of the isolates (>6 out of 11 isolates) (Supplementary Table 2). The results indicate that in the psychrotolerent bacteria, saturated, iso-, anteiso-, hydroxyl- and unsaturated fatty acids are predominant and together constitute a significant proportion of the total fatty acid composition. The composition of iso-fatty acids ranged from 0 to 29.0%, antesio-fatty acids from 0 to 66.2%, hydroxyl-fatty acids from 0 to 6.8%, saturated fatty acids from 0 to 19.8% and unsaturated fatty acids from 0 to 50.0% (Supplementary Table 2). Out of the 11 strains, only one strain (K7SC-13) contained 1 different polyunsaturated fatty acid ($C_{18:3\ \omega6,\ 9,12c}$) with 0.2% (Supplementary Table 2).

Identification of bacterial strains isolated from soil samples collected near the Kafni Glacier

Taxonomic analysis of all the 11 strains of different morphotypes, indicated that 4 strains were gram-negative and 7 were gram-positive. BLAST sequence similarity search based on 16S rRNA gene sequence indicated that 2 strains belonged to the genus *Acinetobacter*, 3 strains each to the genera *Bacillus* and *Viridibacillus*, and 1 strain each to the genera *Lysinibacillus*, *Pseudomonas* and *Psychrobacter*, respectively (Table 3). Phylogenetic analysis confirmed the affiliation of all the 11 isolates with their nearest phylogenetic neighbour (Supplementary Fig. S3; Table 3).

Discussion

In the present study, bacterial diversity was studied using two soil samples K7s and K8s collected from the viccinity of the Kafni Glacier using viable isolates and 16S rRNA gene clones from two libraries (KF and KS) constructed using the soil DNA.

The majority of the clone sequences, 92% in the KF and 81.5% in the KS library, could be assigned to the nearest phylogenetic neighbour. A few of the clones exhibited <97% similarity with already known species at the 16S rRNA gene level and did not cluster with the nearest phylogenetic neighbour (for example KF-225, KS-106, KS-365, KS-504 and KS-202). Such bacteria need to be isolated and characterised.

In the present study, clones belonging to phyla *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Proteobacteria*, *Spirochaetae*, *Tenericutes* and *Verrucomicrobia* were common to KF and KS (Fig. 1; Supplementary Table 1). Other clones belonging to the phyla *Acidobacteria, Chlamydiae*, *Lentisphaerae*, *Nitrospirae*, and candidate phylum TM7s TM7a were present only in one of the libraries (0.2–3.7%; Supplementary Table 1). In fact, except for the phyla affiliated to *Bacteroidetes* and *Proteobacteria*, all the other phyla common to both the libraries were present in low frequency (<10%). These observations are in agreement with earlier studies, which had also indicated that clones affiliated to *Verrucomicrobia* (Cheng and Foght 2007; Liu et al. 2006, 2009; Pradhan et al. 2010; Wagner et al. 2009; Zhou et al. 1997), *Chlamydiae* (Shivaji et al. 2004), *Chloroflexi* (Costello and Schmidt 2006; Li et al. 2008; Liu et al. 2006, 2008, 2009; Lysnes et al. 2004; Pradhan et al. 2010; Reed et al. 2006; Shivaji et al. 2004; Wagner et al. 2009), *Nitrospirae* (Pradhan et al. 2010; Shivaji et al. 2004) and candidate phylum TM7 s TM7a (Liu et al. 2009; Pradhan et al. 2010; Shivaji et al. 2011b) are present in other cold habitats (Fig. 1) at a low frequency. Phyla *Lentisphaerae* and *Tenericutes* are represented only by a single clone, and reports on the presence of these bacteria in cold habitats is lacking. This study also differs with the above studies in the absence of *Cyanobacteria* (alpine tundra wet meadow soil, alpine dry meadows soil, unvegetated deglaciated soil and periglacial soil), *Deinococcus* (unvegetated deglaciated soil), *Elusimicrobia* (glacial lake soil), *Gemmatimonadetes* (glacial lake soil and periglacial soil), *Planctomycetes* (glacial lake soil, periglacial soil, alpine tundra wet meadow soil and alpine dry meadows soil), *Themomicrobia* (permafrost-affected soil) and candidate divisions like OS-K and OP10 (alpine tundra wet meadow soil).

The current data supports the earlier data that *Proteobacteria*, *Cytophaga–Flavobacterium–Bacteroides* (CFB) and high G + C gram-positive bacteria are common in most cold habitats (Cheng and Foght 2007; Steven et al. 2007; Shivaji et al. 2011a, b; Srinivas et al. 2009; Reddy et al. 2009). In both the libraries, the most predominant bacteria observed were gram-negative belonging to phyla *Proteobacteria* (51.5 and 51.3%) and *Bacteroidetes* (23.2 and 34.8%). The frequency of gram-positive bacteria belonging to the phyla *Actinobacteria* (5.1 and 1.0%) and *Firmicutes* (6.7 and 6.3%) was significantly low compared with gram-negative bacterial diversity in these samples. These results are in agreement with the earlier studies from similar habitats such as from Roopkund Glacier Lake, India (Pradhan et al. 2010); Pindari Glacier, India (Shivaji et al. 2011b); Tibetan Plateau Glacier (Liu et al. 2009), moraine lakes and glacial meltwaters of Mount Everest (Liu et al. 2006); John Evans Glacier, Canada (Cheng and Foght 2007); Bench Glacier, Alaska (Skidmore et al. 2005); Schirmacher Oasis, Antarctica (Shivaji et al. 2004); Siberian tundra and Samoylov Island, Siberia (Wagner et al. 2009), which had also indicated that gram-negative bacteria predominated over gram-positive bacteria.

The diversity of bacteria in the two soil samples near the Kafni Glacier are comparable with that reported (Figs. 1,


a
Deltaproteobacteria
Alphaproteobacteria
Epsilonproteobacteria
Betaproteobacteria
Gammaproteobacteria
Clade-1
Clade-2
Clade-3
Clade-4
Clade-5
Clade-6
0.02

◄ **Fig. 3** Neighbour joining phylogenetic tree of 16S rRNA gene clones from KS library, showing the phylogenetic relationship of clones affiliated to *Proteobacteria* (**a**) and to *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Firmicutes*, *Lentosphaerae*, *Spirochaetae*, *Tenericutes*, TM7_s TM7a (candidate phylum) and *Verrucomicrobia* (**b**). *Aquifex pyrophilus* Kol5a^T was taken as an out-group. Numbers at nodes are bootstrap values. The *bar* represents 0.02 and 0.05 substitutions per alignment position in **a** and **b**, respectively

6) for the ice core from the Muztag Ata Glacier (Xiang et al. 2005), solid ice, firn and snow of the Kuytun 51 Glacier (Xiang et al. 2009), soil from the western Himalayas (Gangwar et al. 2009) and Puruogangri ice (Zhang et al. 2006, 2008) with respect to the presence of *Actinobacteria, Proteobacteria* and *Acidobacteria*. Xiang et al. (2004) and Liu et al. (2006, 2009) reported only *Proteobacteria* and *Cytophaga–Flavobacterium–Bacteroides* from the Malan ice core, Tibetan Plateau and glacial meltwater from the Mount Everest, which are cold deserts. In Roopkund glacial soil, the predominant phyla were *Actinobacteria* and *Firmicutes* (Pradhan et al. 2010) and in the Pindari glacial soil the predominant phyla were *Acidobacteria*, *Actinobacteria*, *Firmicutes* and *Proteobacteria* (Shivaji et al. 2011b). Similarly, Skidmore et al. (2005) reported only four phyla *Acidobacteria*, *Cytophaga–Flavobacterium–Bacteroides*, *Proteobacteria* and *Spirochaetes* from the Bench Glacier, Alaska. These studies indicate that though the number of dominant phyla is limited, the phyla are not identical.

Comparison of the biodiversity of the Kafni Glacier soil samples with those of two other geographically close glaciers, namely Pindari and Roopkund glaciers, at species level indicated that out of 174 species or taxa (including candidate taxa and uncultured clones belonging to different taxa) from the present study, only 16 were common to Pindari Glacier samples (*Arenimonas oryziterrae*, Candidatus *Solibacter usitatus*, *Cryobacterium roopkundense*, *Desulfacinum hydrothermale*, *Devosia insulae*, *Geoalkalibacter ferrihydriticus*, *Georgfuchsia toluolica*, *Haliangium tepidum*, *Ilumatobacter fluminis*, *Lysobacter ginsengisoli*, *Methylotenera mobilis*, *Nitrospira moscoviensis*, *Propionibacterium acnes*, *Pseudolabrys taiwanensis*, *Steroidobacter denitrificans* and *Sulfuricella denitrificans*), 28 taxa were common to Roopkund Glacier samples (*Alkaliflexus imshenetskii*, *Anaeromyxobacter dehalogenans*, *Azospira oryzae*, *Bellilinea caldifistulae*, *Cryobacterium roopkundense*, *Cytophaga fermentans*, *Dechloromonas hortensis*, *Devosia neptuniae*, *Geoalkalibacter ferrihydriticus*, *Geobacter psychrophilus*, *Haliangium tepidum*, *Haloferula helveola*, *Ilumatobacter fluminis*, *Longilinea arvoryzae*, *Nitrospira moscoviensis*, *Owenweeksia hongkongensis*, *Prolixibacter bellariivorans*, *Propionivibrio limicola*, *Pseudolabrys taiwanensis*, *Rhodobacter ovatus*, *Rhodoferax antarcticus*, *Sphingomonas jaspsi*, *Steroidobacter denitrificans*, *Syntrophus gentianae*, *Thiohalomonas denitrificans*, *Variovorax boronicumulans*, *Verrucomicrobium spinosum* and *Zoogloea caeni*) and only seven taxa were common to all three samples (*Cryobacterium roopkundense*, *Geoalkalibacter ferrihydriticus*, *Haliangium tepidum*, *Ilumatobacter fluminis*, *Nitrospira moscoviensis*, *Pseudolabrys taiwanensis* and *Steroidobacter denitrificans*) representing 9.2, 16.1 and 4%, respectively (Pradhan et al. 2010; Shivaji et al. 2011b). Thus, this implies that bacterial diversity associated with glaciers or in the vicinity of glaciers is likely to be influenced not only by temperature and water content, but also by other physicochemical characteristics of the soil.

The observed difference in the bacterial diversity in the two libraries KF and KS (Supplementary Table 1) may reflect the inherent changes observed in the physicochemical characteristics of the two samples. PCA analysis indicated that As (7.3 times more in K8s sample) was the key parameter that influenced the observed diversity (Fig. 5b), thus confirming an earlier finding that increased As content resulted in low diversity (Shivaji et al. 2011b). Clones related to *Sulfurospirillum arsenophilum*, *Clostridium, Rhodoferax* and *Bacillus*, which are supposed to utilise arsenate, were also detected in the present libraries. Further, in addition to As, factors such as pH (Eichorst et al. 2007), water content (Treves et al. 2003) and texture of soil are also known to influence microbial community composition and diversity (Zhang et al. 2008). The texture was very similar. Therefore, the high bacterial count in the K8s soil sample (30.71×10^8) compared to the K7s soil sample (6.25×10^8) may be attributed to the high water content in K8s. In fact, the total bacterial counts from the Kafni Glacier were higher than in most of the glaciers worldwide (Liu et al. 2009; Miteva et al. 2004; Pradhan et al. 2010; Shivaji et al. 2011b; Skidmore et al. 2005; Xiang et al. 2006, 2009; Zhang et al. 2007). This could be attributed to the fact that the Kafni Glacier is located near human settlements and influenced by anthropogenic activities (Zhang et al. 2007).

Comparison of the bacterial diversity of the Kafni Glacier soil with that of other nonpolar cold habitats such as alpine tundra wet meadow soil (Costello and Schmidt 2006; Zhou et al. 1997), surface snow (Segawa et al. 2005), alpine dry meadows soil (Lipson and Schmidt 2004), unvegetated deglaciated soil (Nemergut et al. 2007), periglacial soil (Schmidt et al. 2009) and permafrost-affected soil (Wagner et al. 2009) indicated that *Betaproteobacteria, Bacteroidetes, Chloroflexi*, *Spirochaetes Verrucomicrobia*, *Actinobacteria* and *Acidobacteria* were present in most of these habitats, though one or more of these taxa were absent in some of the habitats as in the permafrost-affected soil (Wagner et al. 2009). Further clones belonging to *Proteobacteria, Acidobacteria, Actinobacteria* and *Cytophaga–Flavobacterium–Bacteroides* have also been

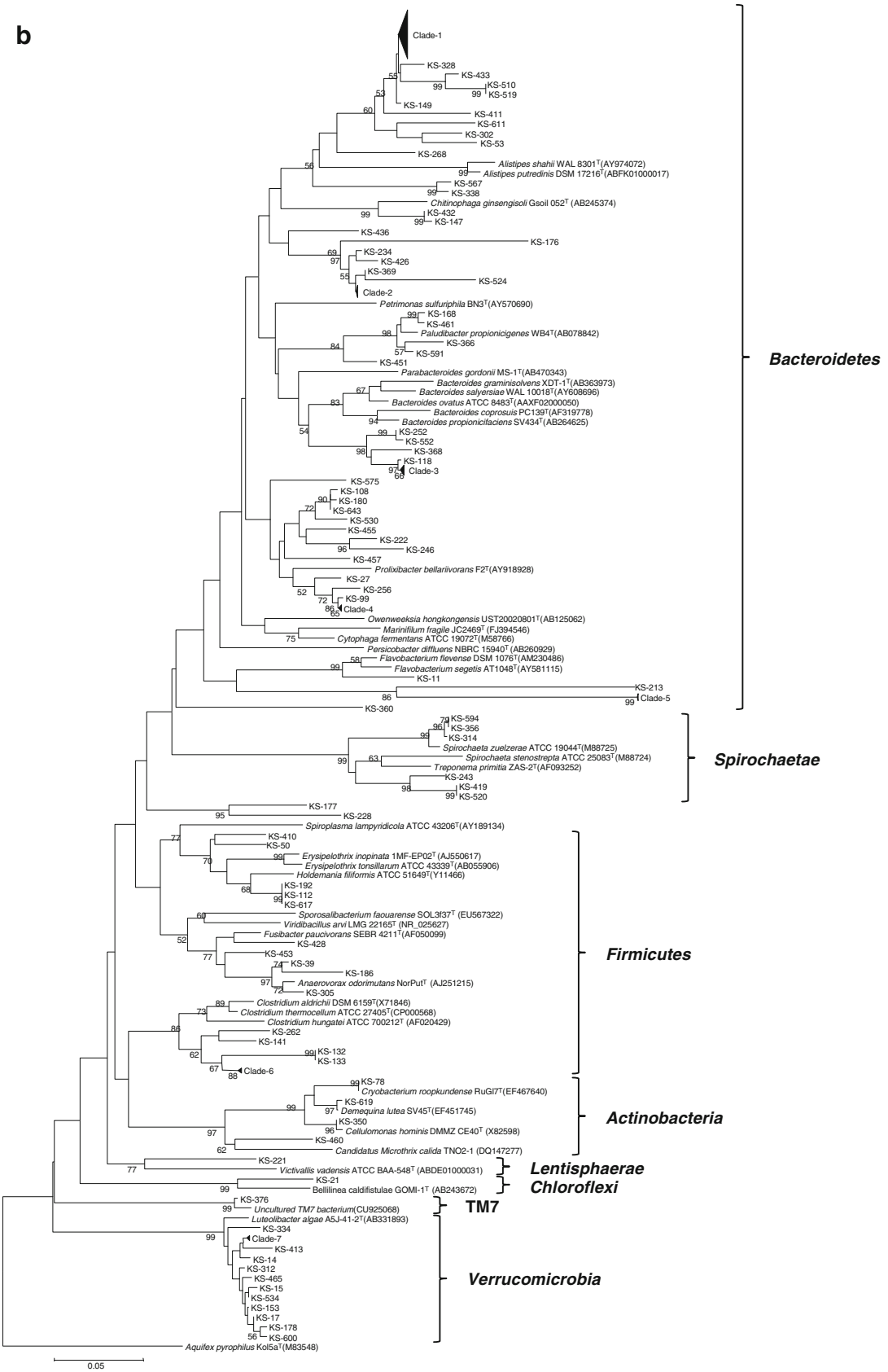


Fig. 3 continued

Table 2 Estimated numbers of phylotypes using three different cutoff values (80, 90 and 97%) in the two 16S rRNA gene clone libraries (KF and KS) constructed using two soil samples (K7s and K8s) collected near the Kafni Glacier, Himalayas, India, using different diversity indices

	KF (80%)	KS (80%)	KF (90%)	KS (90%)	KF (97%)	KS (97%)
Number of clones	237.00	411.00	237.00	411.00	237.00	411.00
Species richness (*S*)	12.00	11.00	73.00	236.00	125.00	238.00
Coverage (%)	94.90	97.30	69.20	42.60	47.30	42.10
Evenness	0.62	0.67	0.87	0.86	0.93	0.87
Shannon–Wiener Diversity Index	1.53	1.62	3.75	4.69	4.48	4.78
Shannon entropy	2.21	2.33	5.41	6.77	6.46	6.90
Simpson's (D)	0.28	0.24	0.05	0.03	0.02	0.02
Chao1	18.25	20.50	328.57	12,173.00	476.08	3,654.79

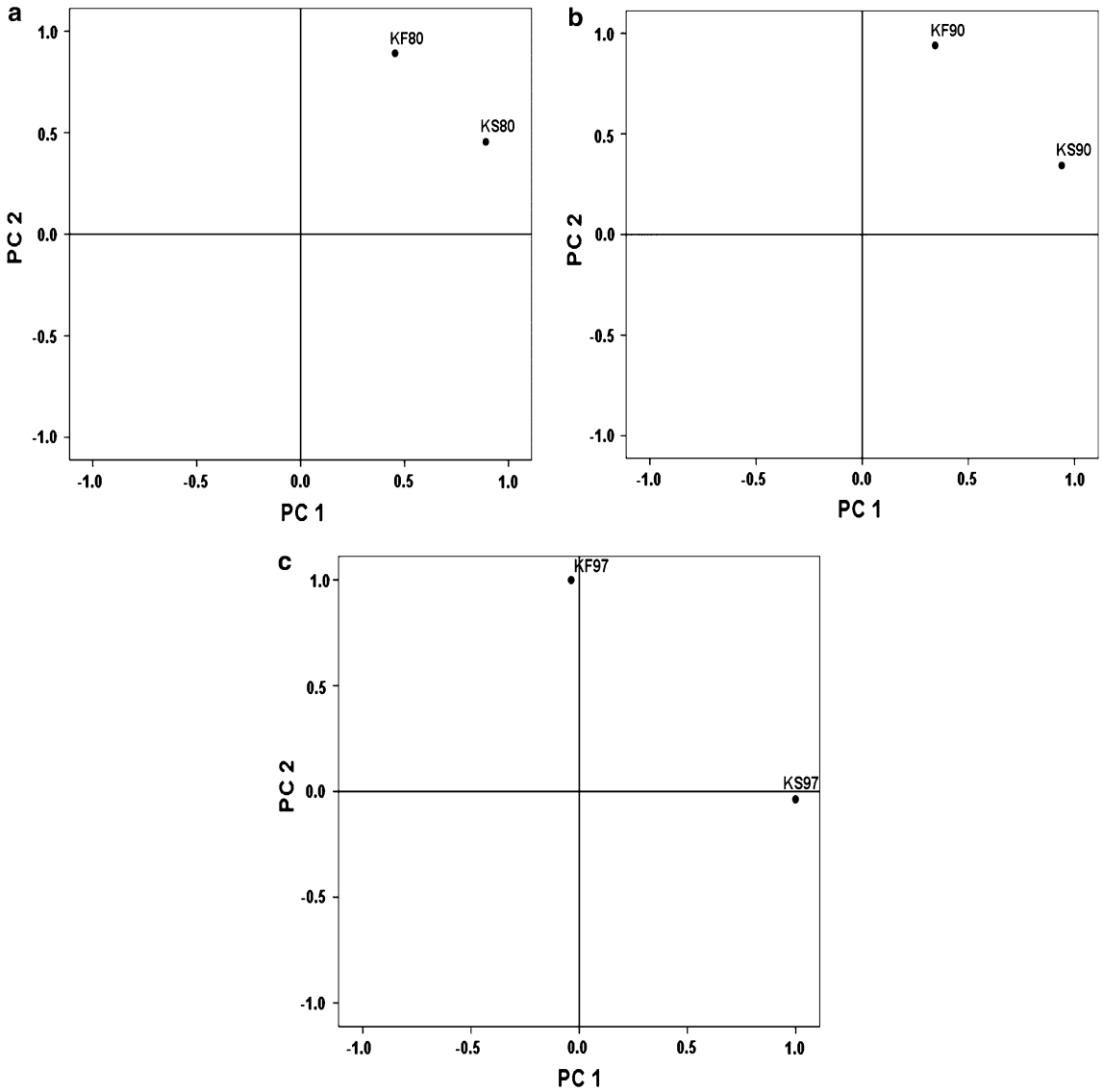


Fig. 4 Ordination diagrams based on principal component analysis using frequency tables obtained from the two 16S rRNA gene clone libraries (KF and KS) defined at different cutoff similarity values of 97% (**a**), 90% (**b**) and 80% (**c**) using Comm Cluster Software. PC 1, principal component analysis factor 1; PC 2, principal component analysis factor 2

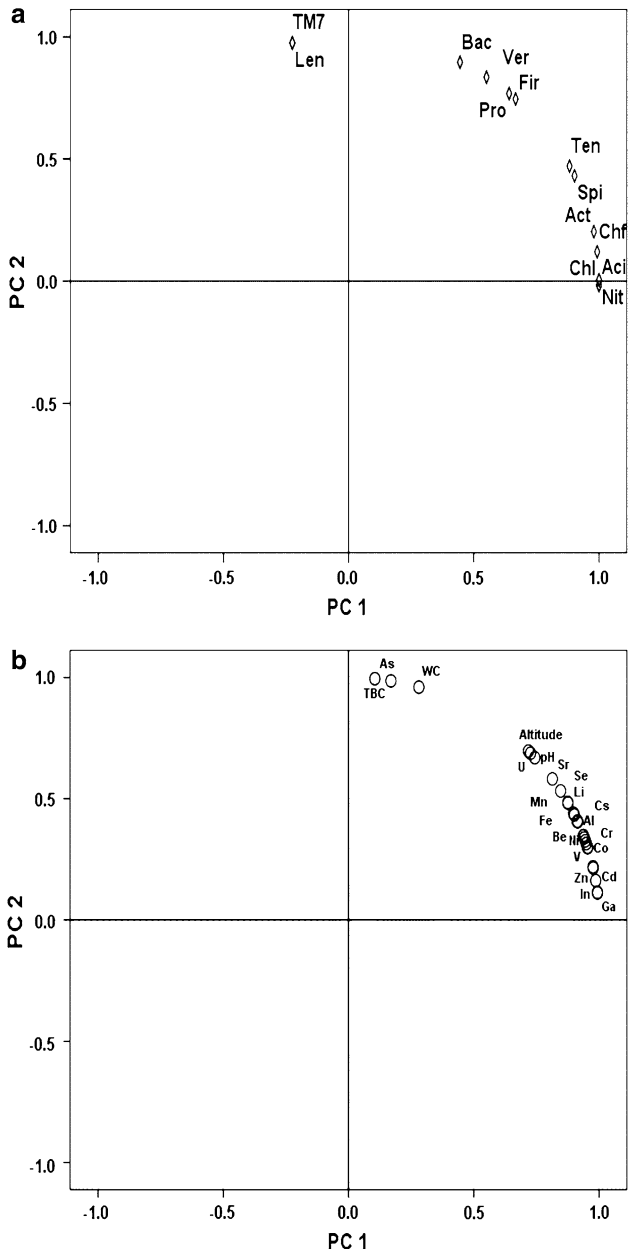


Fig. 5 **a** Principal component analysis based on percentage of specific OTUs from two 16S rRNA gene clone libraries (PC1, 72.419% and PC2, 27.581%). **b** PCA plot based on biogeochemical properties (altitude, total bacterial count, pH value and micronutrients Li, Be, Al, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, As, Se, Rb, Sr, Cd, In, Cs, Pb, Bi and U) of two soil samples (PC1, 89.464% and PC2, 10.535%)

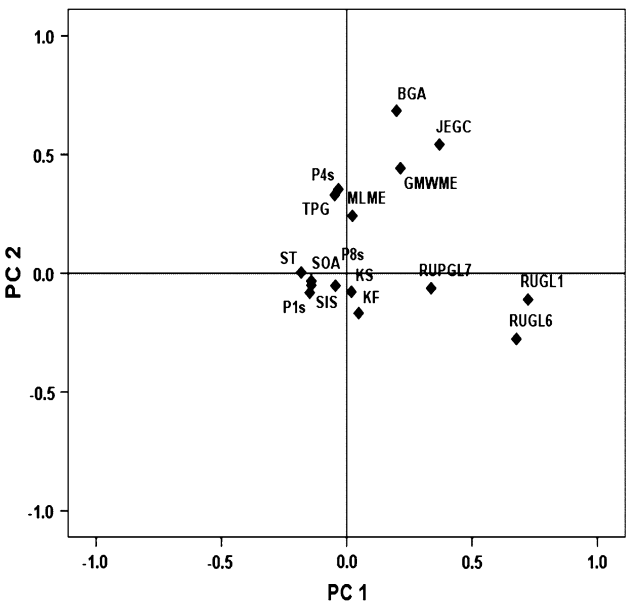


Fig. 6 Principal component analysis based on phylotypes with 97% cutoff value from sixteen 16S rRNA gene clone libraries KF and KS, Kafni Glacier, India; P1s, P4s and P8s, Pindari Glacier, India; RUGL1 and RUGL6, Roopkund Glacier Lake, India; RUPGL7, Roopkund Glacier, India; TPG, Tibetan Plateau Glaciers; MLME, moraine lakes, Mount Everest; GMWME, glacial meltwater, Mount Everest; JEGC, John Evans Glacier, Canada; BGA, Bench Glacier, Alaska; SOA, Schirmacher Oasis, Antarctica; ST, Siberian tundra; and SIS, Samoylov Island, Siberia

reported from: the Tibetan Plateau Glacier, China; Mount Everest, Nepal; John Evans Glacier, Canada; Bench Glacier, Alaska; Schirmacher Oasis soil, Antarctica and Siberian tundra soil samples (Fig. 1). The occurrence of identical phyla in geographically diverse cold environments is indicative of the ability of microorganisms to adapt to similar strategies to survive freezing and remain active at low temperatures (Abyzov et al. 1998; Priscu and Christner 2003). The bacterial diversity of K7s and K8s was very different compared to polar habitats. Bacterial taxa such as *Arthrobacter*, *Bacillus*, *Cryobacterium*, *Hymenobacter*, *Janthinobacterium*, *Micrococcus*, *Myxococcus*, *Planococcus*, *Pseudomonas*, *Psychrobacter* and *Sphingobacterium* (Alam et al. 2003; Bowman et al. 1996; Shivaji et al. 1992, 2004; Reddy et al. 2009) were predominant in polar habitats and this variation in diversity could be attributed to the pristine environment and severe climatic conditions in the polar regions.

The 11 bacterial strains isolated from the soil samples were psychro-, halo- and alkalitolerant. The majority of the strains resembled the nearest phylogenetic neighbour in these characteristics. For instance, strains K7Sc-3b, K7Sc-2, K7Sc-4, K8Sc-3, K7Sc-8a, K7Sc-11a and K7Sc-13 were similar to their nearest phylogenetic neighbours *Lysinibacillus parviboronicapiens* BAM-582^T (Miwa et al. 2009), *Viridibacillus arvi* LMG 22165^T (Albert et al. 2007), *Acinetobacter lwoffii* (Reddy et al. 2009) and *Psychrobacter nivimaris* (Heuchert et al. 2004; Srinivas et al. 2009). The isolates belonging to the genera *Bacillus*, *Lysinibacillus* and *Psychrobacter* were not observed in the clone library analysis. A few strains such as those affiliated to *Bacillus simplex* and *Pseudomonas lutea* have been reported to be mesophilic, and psychrotolerant strains have not been

Table 3 Identification of the 11 bacterial strains isolated from the two soil samples (K7s and K8s) collected near the Kafni Glacier, Himalayas, India, based on BLAST analysis of the 16S rRNA gene sequences and growth characteristics and activities of amylase, lipase, protease and urease

Sl. No.	Culture ID	Nearest phylogenetic neighbour (NCBI-BLAST) based on 16S rRNA gene sequence similarity (%)	Growth characteristics			Enzymatic activities			
			Temp range (°C)	NaCl tolerance (M)	pH range	Amylase	Lipase	Protease	Urease
Firmicutes									
1	K7Sc-7a	*Bacillus simplex* NBRC 15720^T AB363738 (99.7)	4–37	1.00	6–9	+	+	–	+
2	K7Sc-9b	*Bacillus simplex* NBRC 15720^T AB363738 (99.7)	4–37	1.00	6–9	+	+	–	+
3	K8Sc-2	*Bacillus simplex* NBRC 15720^T AB363738 (99.8)	4–37	1.00	6–9	+	+	–	+
4	K7Sc-3b	*Lysinibacillus parviboronicapiens* BAM-582^T AB300598 (98.9)	4–37	0.50	6–9	+	+	–	+
5	K7Sc-2	*Viridibacillus arvi* LMG 22165^T AJ627211 (99.1)	4–30	0.50	6–9	–	–	–	+
6	K7Sc-4	*Viridibacillus arvi* LMG 22165^T AJ627211 (99.1)	4–30	0.50	6–9	+	–	–	+
7	K8Sc-3	*Viridibacillus arvi* LMG 22165^T AJ627211 (98.9)	4–30	0.50	6–9	–	+	–	+
Proteobacteria									
8	K7Sc-8a	*Acinetobacter lwoffii* DSM 2403^T X81665 (98.9)	4–37	0.15	6–9	+	+	–	+
9	K7Sc-11a	*Acinetobacter lwoffii* DSM 2403^T X81665 (99.0)	4–30	0.15	6–9	+	+	–	+
10	K7Sc-3a	*Pseudomonas lutea* OK2^T AY364537 (98.5)	4–37	0.50	6–9	–	+	–	+
11	K7Sc-13	*Psychrobacter nivimaris* 88/2-7^T AJ313425 (99.3)	4–30	1.00	6–9	–	+	–	+

reported. Noticeably, neither enteric nor pathogenic bacteria were detected in this study, though enteric bacteria were isolated from glacial ice on the Ellesmere Island (Dancer et al. 1997) and surface snow of the Tateyama Mountains in Japan (Segawa et al. 2005). In addition when the culturable bacterial diversity of the Kafni Glacier soil samples was compared with that of the Pindari Glacier, only one taxa *Viridibacillus arvi* was common to both (Shivaji et al. 2011b). As of now, only one new species of *Bacillus* has been described from a Himalayan Glacier (Reddy et al. 2008b).

In cold habitats, psychrophilic bacteria release hydrolytic enzymes, which can degrade complex biomolecules into smaller and easily accessible compounds. Strain K7Sc-3a was similar to its phylogenetic neighbour *Pseudomonas lutea* with respect to amylase activity. Strains K7Sc-7a, K7Sc-9b, K8Sc-2, K7Sc-2 and K8Sc-3 were similar to their phylogenetic neighbours *Bacillus simplex* and *Viridibacillus arvi* with respect to amylase activity, but differed from the same strain with respect to protease and urease activities. Surprisingly, none of the strains produced extracellular proteases.

The synthesis of unsaturated fatty acids is very vital for survival at low temperature (Nishida and Murata 1996). In accordance with previous studies, medium chain fatty acids with 10–12 carbons, unsaturated fatty acids and branched fatty acids (including the iso- and anteiso-fatty acids) that maintain the membrane in a fluid state and which are common in psychrophilic bacteria (Chintalapati et al. 2004; Shivaji et al. 2007) were also present in the bacterial isolates from the Kafni Glacier soil samples.

Conclusions/novel findings

1. Bacterial diversity of two soil samples (K7s and K8s) collected near the Kafni Glacier based on 16S rRNA gene sequence analysis led to the identification of clones affiliated to *Actinobacteria* (5.1 and 1.0%), *Bacteroidetes* (23.2 and 34.8%), *Chloroflexi* (1.7 and 0.2%), *Firmicutes* (6.7 and 6.3%), *Proteobacteria* (51.5 and 51.3%), *Spirochaetae* (3.4 and 1.5%), *Tenericutes* (0.4 and 0.2%) and *Verrucomicrobia* (3.4 and 4.1%) as the common bacterial phyla. Clones affiliated

to the phyla *Lentisphaerae* and *Tenericutes* have so far only been reported from mesophilic habitats.

2. A total of 106 and 93 bacterial taxa were identified from K7s and K8s soil samples, respectively. Arsenic content is probably the key parameter that influences the heterogeneity observed in the bacterial diversity in K8s.
3. Clones related to bacterial taxa that have a role in arsenic cycle, such as *Sulfurospirillum arsenophilum*, *Clostridium* and *Rhodoferax*, and strains related to the genus *Bacillus* were identified from both the libraries. Clones related to *Sulfurospirillum arsenophilum* were predominant in the KS library (42) compared to the KF library (3).
4. Comparison of the bacterial diversity of the Kafni Glacier with the diversity in other Himalayan cold habitats, other non-polar high-altitude habitats and various polar habitats indicated that it was different in terms of distribution and abundance of the taxa.
5. PCA indicated that bacterial diversity of KF and KS libraries were closely affiliated to the diversity in the Pindari (P8s), SIS (Samoylov Island, Siberia), SOA (Schirmacher Oasis, Antarctica) and ST (Siberian tundra) libraries.
6. All the bacterial isolates were alkali-, halo- and psychrotolerant. Cold-active enzymes and fatty acids isolated from bacterial strains of the Kafni Glacier might offer novel opportunities for biotechnological exploitation.

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