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Bacterial community characterization of Batura Glacier in the Karakoram Range of Pakistan

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Abstract

High-altitude cold habitats of the Karakoram are rarely explored for their bacterial community characterization and metabolite productions. In the present study, bacterial communities in ice, water, and sediments of Batura Glacier were investigated using culture-dependent and culture-independent methods. Twenty-seven cold-adapted bacterial strains (mostly psychrotrophic) were isolated using R2A, Tryptic Soy Agar (TSA), and Luria-Bertani (LB) media, at 4 °C and 15 °C. Most of the isolates exhibited growth at a wide range of temperature (4–35 °C), pH (5–12), and salinity (1–6%). Among the bacterial isolates, 52% were identified as Gram-positive and the remaining 48% represented as Gram-negative. The results of phylogenetic analysis indicated that all the culturable bacteria belonged to 3 major phylogenetic groups, i.e., Actinobacteria (48%), Bacteroidetes (26%), and Proteobacteria (22%), while *Flavobacterium* (26%), *Arthrobacter* (22%), and *Pseudomonas* (19%) were represented as the dominant genera. Similarly, Illumina amplicon sequencing of 16S rRNA genes after PCR amplification of DNA from the whole community revealed dominance of the same phylogenetic groups, Proteobacteria, Actinobacteria, and Bacteroidetes, while *Arthrobacter*, *Mycoplana*, *Ochrobactrum*, *Kaistobacter*, *Janthinobacterium*, and *Flavobacterium* were found as the dominant genera. Among the culturable isolates, 70% demonstrated activity for cellulases, 48% lipases, 41% proteases, 41% DNases, and only 7% for amylases. Most of the glacial isolates demonstrated antimicrobial activity against other microorganisms including the multiple-drug-resistant strains of *Candida albicans*, *Klebsiella pneumoniae*, *Acinetobacter* sp., and *Bacillus* sp. 67% of Gram-negative while 46% of Gram-positive glacial bacteria were resistant to trimethoprim/sulfamethoxazole. Resistance against methicillin and vancomycin among the Gram-positive isolates was 23% and 15%, respectively, while 11% of the Gram-negative isolates exhibited resistance against both colistin sulfate and nalidixic acid.

Keywords Batura Glacier · Karakoram · Cold-adapted bacteria · Psychrotrophs · Metagenomics

Introduction

More than 80% of the biosphere is constantly at temperatures under 5 °C. The deep sea represents a major fraction of these cold environments as the oceans cover 71% of the planet and 90% of their volume is below 5 °C (Casanueva et al. 2010; Rodrigues and Tiedje 2008). Additionally, glaciers account for 10% of the land surface (Russell 1990; Margesin and

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Miteva 2011). The cold biosphere harbors a diverse group of cold-adapted microorganisms called psychrophiles or psychrotrophs, inhabiting terrestrial and aquatic environments in the Arctic and Antarctic polar regions as well as the deep ocean, high mountains of alpine regions, upper atmosphere, and the cave systems. Cold-adapted bacteria have successfully colonized these environments through a vast array of adaptation strategies including maintenance of membrane fluidity and structural flexibility of their enzymes, expression of the cold shock proteins, and production of antifreeze proteins and compatible solutes (Collins and Margesin 2019; Tribelli and López 2018; Barria et al. 2013).

The concept of glacial ecology has gained the interest of researchers in recent years, mainly focusing on the two key glacier ecosystems: the supraglacial and subglacial (Hodson et al. 2008). The supraglacial ecosystems represent the outer surface of the glaciers and were inhabited by a group of complex microbial

communities including algae, bacteria, phytoflagellates, viruses, archaea, fungi, and rotifers (Hodson et al. 2008). The subglacial ecosystems represent the ice-bed interface and are dominated by the heterotrophs including bacteria and fungi (Hodson et al. 2008). Cold-adapted microorganisms are involved in key ecological functions in freezing environments where photoautotrophy is the basis of food webs in the proglacial and chemoautotrophy dominates the subglacier environments (Boetius et al. 2015). Glacier ice with extremely low temperature is considered one of the harsh environments for living organisms on the biosphere. Apart from the extremely low temperatures, other limitations include low nutrient and water availability, frequent freeze-thaw events, and increased darkness (Margesin and Miteva 2011). However, despite all these limitations, it is estimated that glaciers and ice sheets around the world contain as many as 10^{29} microbial cells (Irvine-Fynn and Edwards 2014). Studies on the structure of microbial communities and their possible ecological role in cold environments have been reported from various glaciers of the polar and non-polar regions (Segawa et al. 2011; Shivaji et al. 2011; Miteva et al. 2004).

Extremophiles are organisms that can thrive in environments that are hostile or inhabitable to other forms of life (Horikoshi et al. 2010). Psychrophiles represent the most abundant group of extremophiles in terms of distribution, diversity, and biomass (D'Amico et al. 2006). Apart from their ecological role, cold-adapted microorganisms have shown tremendous potential for biotechnological applications ranging from enzymes and antimicrobial productions to environmental bioremediations (Cavicchioli et al. 2011; Sánchez et al. 2009; Gratia et al. 2009). Nature is considered the best reservoir for the discovery of industrially important metabolites. The less explored extreme environments are seen as new avenues for the bioprospecting of novel bioactive molecules including enzymes and antimicrobial compounds (Sánchez et al. 2009; Cavicchioli et al. 2002).

The north-most territories of Pakistan consist of the greatest mountain ranges in the world including the western Himalayas, Karakoram, and Hindukush. These ranges host at least 5000 glaciers in the Pakistani geographical region (Rasul et al. 2011). Some of the notable glaciers of the Karakoram includes Siachen Glacier (~ 75 km long), Biafo Glacier (~ 68 km), Baltoro Glacier (~ 62 km), and Batura Glacier (~ 59 km) (Hewitt 2014). Interestingly, glaciers of the Karakoram are stable or even growing contrary to the ice bodies worldwide that are receding due to global warming (Rankl et al. 2014). This unusual behavior of the Karakoram glaciers is known as the "Karakorum Anomaly" (Hewitt 2005). However, glaciers in the Karakoram are the least explored in terms of their bacterial diversity. It would be interesting to study the bacterial community of these glaciers that have exhibited resilience in climate change when other glaciers in the world are retreating. Previously, genome sequencing of the glacial isolate *Pseudomonas* sp. BGI-2 unveiled 11

exopolysaccharide (EPS)-producing genes, which are not present in the closely related mesophilic *Pseudomonas* species (Ali et al. 2019a). Also, BGI-2 produced high yield of a cryoprotective EPS at low temperatures and demonstrated ability to withstand freeze-thaw stress (Ali et al. 2020). Another glacial isolate BGI-11 demonstrated the ability to grow at higher salinity and utilized hexadecane as carbon source (Ali et al. 2019b).

The aim of the present study was to characterize the bacterial community of an unexplored glacier in the Karakoram Range of Pakistan and explore some of their metabolic potential for possible industrial applications. To the best of our knowledge, this is the first study where culture-independent method was used to characterize the bacterial community in any glacier of the Karakoram in the Pakistani geographical region.

Materials and methods

Sampling, bacterial count, and isolation

Description of the sampling site and collection of samples

Sampling was done in Batura Glacier located in the Passu village of the upper Hunza valley in the northern Pakistan (Fig. 1). Hunza valley is located at the base of the Karakoram Range along the Karakoram Highway. Batura Glacier (36° 32′ N, 74° 40′ E) is one of the longest glacier outside the Polar regions with the highest elevation around 7795 m in the West and the lowest nearly 2570 m in the East (Hewitt 2014). Batura Glacier basin is ~ 48% glacierized and surrounded by several major peaks with altitude > 7000 m including, Batura Muztagh ~ 7795 m (Hodson et al. 2002).

Triplicate samples of ice, water, and sediment were collected aseptically in sterile 500-mL wide-mouth polypropylene bottles. Handling of samples was performed according to standard microbiological techniques. Sterilized tools were used for sampling of glacial ice and sediments. Surface ice and sediment up to 10–13 cm were removed and discarded. Underlying samples were collected into sterile bottles, temperature, and pH recorded at the time of sampling. The tubes were sealed, placed in an isothermal box, and transported to Applied, Environmental and Geomicrobiology Laboratory, at the Department of Microbiology, Quaid-i-Azam University Islamabad, and stored at − 20 °C. Geographic coordinates were recorded at the time of sampling using a GPS device.

Direct bacterial count using epifluorescence microscopy

Direct bacterial count from the three glacier samples performed using the method used by Zhang et al. (2008) and Chen et al. (2001) with minor modifications. About 2 to

Fig. 1 Picture of the sampling site in Batura Glacier with Passu Cathedral peak in the background, village Passu, District Hunza, Gilgit Baltistan

3 mL of glacier samples (melted ice and water) were filtered through 0.2-μm polycarbonate membrane filter (Millipore) under gentle vacuum pressure. CYBR Gold (Invitrogen) working solution (2 ×) was prepared from an original stock (10,000 ×) using TE buffer (10 mM Tris-Cl; 1 mM EDTA, pH 7.4–7.6). Bacterial cells on the filters were stained with 300 μL of the CYBR Gold solution (2 ×) for 15 min in the dark and the residual stain was removed by applying vacuum. Ten microliters of mounting solution (50% glycerol and 50% TE buffer) was placed on the slides and stained filters were mounted on top of the drop with cover slips placed over it. The samples were examined under blue-green light excitation (480–495 nm) using fluorescent microscope (Axioplan, Zeiss). The number of bacterial cells per milliliter or gram for each sample was calculated based on count of at least 20 randomly selected microscopic fields and the volume of each filtered sample.

Enumeration, isolation, and viable bacterial count

Enumeration of culturable heterotrophic bacteria was carried out through the plate count method. Three general purpose culture media including Tryptic Soy agar (Oxoid), Luria Bertani agar (Miller), and R2A agar (Difco) were used to isolate psychrophilic/psychrotrophic bacteria from the samples. About 100 μL aliquots of the three glacier samples, or a 10-fold dilution of them, was spread onto agar plates. These plates were incubated at two different temperatures (4 °C and 15 °C) for up to 4 weeks and observed daily for colony appearance. Plates were also incubated at 4 °C for an extended period of up to several months until growth of new colonies was no longer detected. Morphologically different colonies from each plate were sub-cultured on separate fresh plates to obtain pure cultures. All the viable counts were performed in replicates and the results presented are the mean of duplicate assays.

For ice samples, 100 μL of melted ice was directly plated on tryptic soy agar, LB agar, and R2A agar, and incubated at 4 °C for 9 months and 15 °C for 6 months. Enrichment was also carried out by adding 10 mL of melted ice sample to 90 mL of liquid culture media (R2A broth, TSB and LB broth). Enrichment samples were incubated in shaker incubators (150 rpm) at two different temperatures (4 °C and 15 °C). Samples were then visualized for bacterial growth (media turbidity) every day up to 2 to 3 weeks. One hundred microliters of diluted and undiluted samples from each enrichment culture was plated on their corresponding solid media for separate colonial growth. Morphologically different colonies observed were streaked on separate plates to obtain pure cultures. A similar procedure was used for enrichment of water and sediment samples for maximum recovery of bacterial isolates.

Identification of culturable bacterial strains

Biochemical identification

The bacterial isolates were differentiated and identified initially based on colony morphology (size, elevation, margin, and pigmentation), cell morphology (cocci, coccobacilli, or rods), and biochemical characteristics (sugar fermentation, H_2S production, catalase, oxidase, nitrate reduction, and citrate utilization), while later on subjected to molecular identification through 16S rRNA gene sequencing.

Identification through 16S rRNA gene sequencing and phylogenetic analysis

DNA was extracted from each isolate using a Genomic DNA Isolation Kit (Norgen Biotek Corporation, Canada). The 16S rRNA gene was amplified using universal primers 27F and 1492R. The PCR conditions for 16S rRNA gene amplification were as follows: initial denaturation at 96 °C for 5 min followed by 35 cycles of denaturation at 96 °C for 30 s, annealing at 55 °C for 30 s, and elongation at 72 °C for 30 s. The final elongation step was 10 min at 72 °C. Sequencing of 16S rRNA was done by Macrogen Inc., Seoul, Korea. The ambiguous low-quality aligned regions from sequences were removed using Chromas (version 2.6.6). 16S rRNA gene sequences for all the isolates were analyzed using EzBioCloud database (Yoon et al. 2017). 16S rRNA gene sequences for all the isolates were deposited in the NCBI GenBank database and accession numbers were obtained. A phylogenetic tree was constructed by neighbor-joining method with bootstrap value > 50 using the MEGA 7.0 software (Fig. 2) (Saitou and Nei 1987).

Characterization of isolated bacteria

Optimization of physico-chemical culture conditions

Growth of the isolates was checked at different temperatures (4, 5, 10, 15, 20, 25, 30, 35, 40, and 45 °C) and pH (3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0, and 13.0) on R2A agar plates (Zhang et al. 2013). Isolates were checked for their ability to grow under halophilic conditions by streaking them on R2A agar plates supplemented with NaCl at concentration ranges from 2 to 10% (*w/v*). Growth medium was also optimized by inoculating on to different media named R2A (Difco), tryptic soy agar (Oxoid), nutrient agar (Oxoid), LB agar (Oxoid), Sabouraud dextrose agar (Oxoid), mannitol salt agar (Oxoid), MacConkey agar (Oxoid), and Mueller Hinton agar (Oxoid). For all the above tests, the result was analyzed after incubating plates at 15 °C for 5 days in duplicates. These tests were performed according to the methods as previously described (Zhang et al. 2013).

Antibiotic resistance

Antimicrobial susceptibility was determined on R2A and Mueller-Hinton agar (MHA) plates using disc diffusion method (Bauer et al. 1966). All the isolates were grown in R2A broth at 15 °C for 48 h. Inoculums were diluted to turbidity equivalent to 0.5 McFarland standard and swabbed on the surface of agar plates (R2A and MHA) using sterile cotton swabs. Seven antibiotics representing different classes were used including methicillin (10 μg), imipenem (10 μg), ofloxacin (5 μg), vancomycin (5 μg), colistin sulfate (10 μg), nalidixic acid (30 μg), and trimethoprim sulfamethoxazole (30 μg). All the plates were incubated at 15 °C and results were interpreted after 72–96 h. The

Fig. 2 Phylogenetic tree of isolates recovered from the three glacier samples using neighbor-joining method with a bootstrap value (%) greater than 50 from 1000 replicates. Numbers in the brackets are GenBank accession numbers for the 16S rRNA sequences and *E.coli* ATCC 11775T was used as an outgroup to root the tree

susceptibility of strains to each antibiotic was determined by measuring zone of inhibition (Fig. 3a).

Extracellular enzyme activity

Isolates were screened for their ability to produce extracellular enzymes. Starch hydrolysis test was performed for amylase production (Priest 1977). Lipase activity was checked by streaking isolates on basal medium supplemented with 1% Tween-40 along with 0.001% rhodamine B and 0.01% CaCl$_2$, according to the method as previously described (Booth 1971). Cellulase activity was demonstrated on carboxymethylcellulose (CMC) agar plates according to the methods from Kasana et al. (2008). Zone of clearance around the isolates indicated a positive response for cellulase production (Fig. 3b). Protease activity was checked by the method as previously described by Priest (1977). For DNase test, isolates were spot inoculated on surface of DNase agar and plates were incubated at 15 °C for 5 days. Plates were then flooded with 1 N hydrochloric acid. Hydrolysis of DNA was indicated by a clear zone around the growth of tested organism.

Antimicrobial activity

Antimicrobial activity of all the glacial isolates was tested against a number of microorganisms including the multidrug resistance strains of *Candida albicans*, *Klebsiella pneumoniae*, *Acinetobacter* sp., and *Bacillus* sp. and two ATCC strains *Pseudomonas aeruginosa* (ATCC27853) and *Staphylococcus aureus* (ATCC6538). Antimicrobial activity was determined through spot on lawn assay (Rafiq et al. 2017). Briefly, R2A agar and Mueller-Hinton agar plates were swabbed with overnight culture of indicator organisms and isolates were spot inoculated on plates. The plates were incubated at 15 °C for 3 to 5 days and observed for a clear zone around the isolates (Fig. 3c). The multiple drug resistance strains were provided by the Medical Microbiology Laboratory, in the Department of Microbiology, Quaid-i-Azam University, Islamabad.

High-throughput sequencing of bacterial community 16S rRNA gene sequencing

Extraction of community DNA and 16S rRNA gene sequencing

DNA from ice, water, and sediment samples was extracted using DNeasy® PowerWater Kit (Qiagen GmbH,
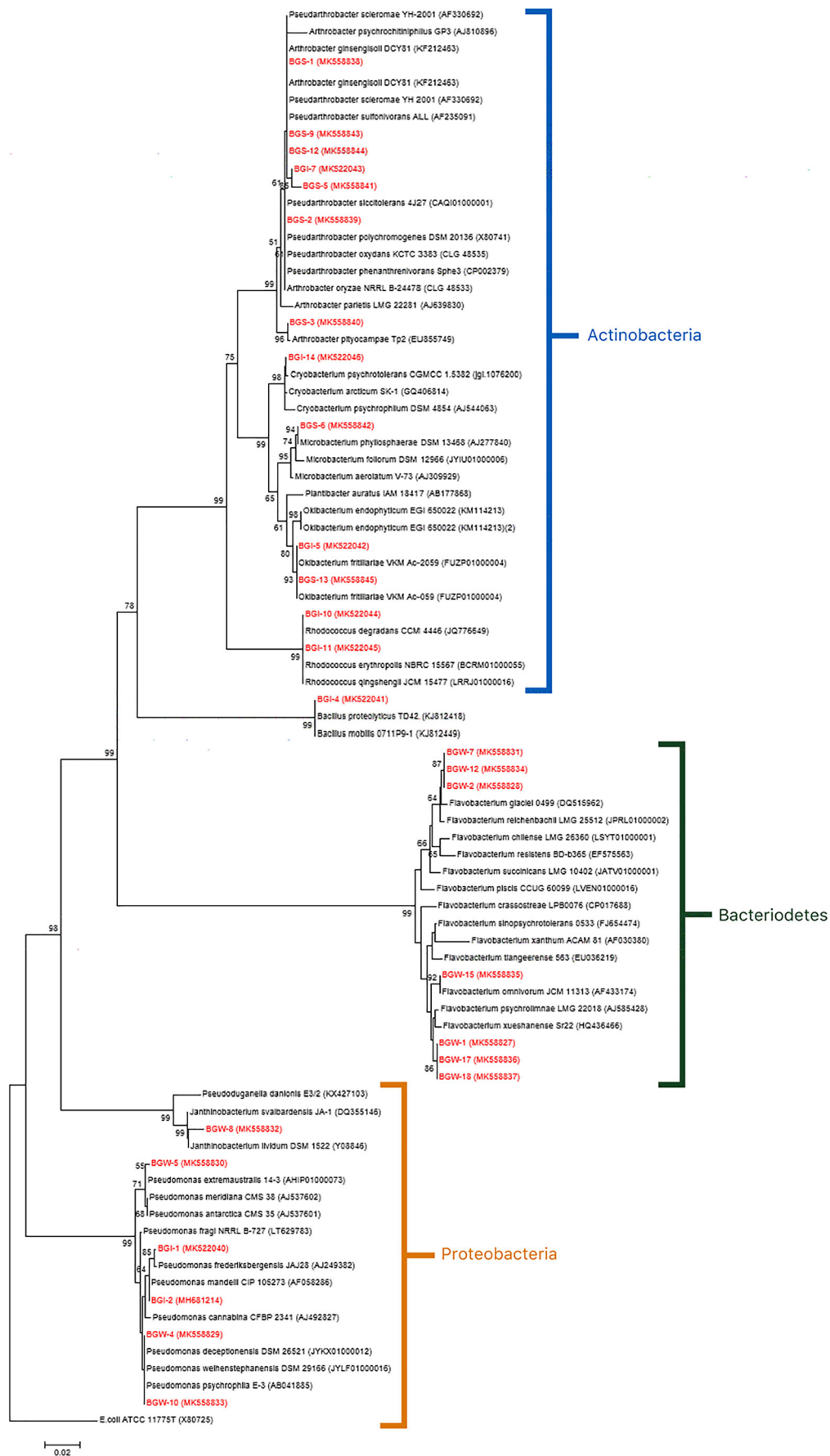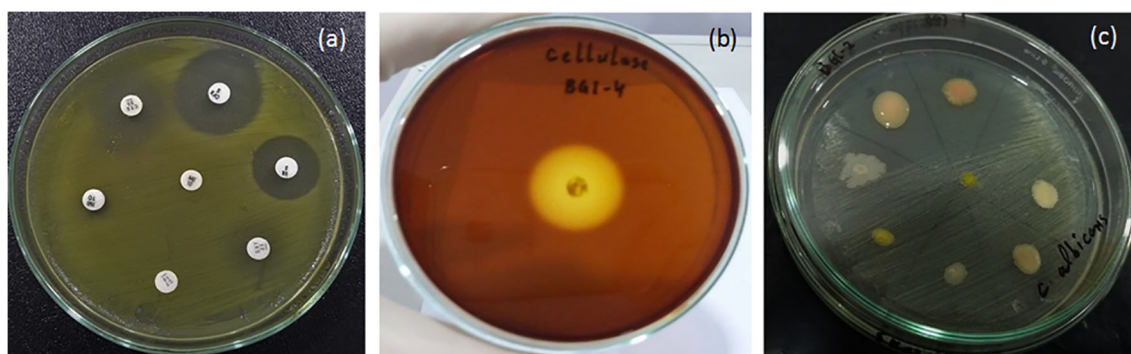
Fig. 3 **a** Antibiotic susceptibility test for *Okibacterium* sp. BGS-13 shows resistance to methicillin, imipenem, and trimethoprim sulfamethoxazole. **b** Cellulolytic enzyme activity by the *Bacillus* sp. BGI-4 on carboxymethylcellulose (CMC) plate. **c** Antimicrobial activities by *Pseudomonas* sp. BGI-1 and *Pseudomonas* sp. BGI-2 against *Candida albicans* by spot on lawn assay using Mueller Hinton agar plate

Hilden, Germany) and DNeasy® PowerSoil® Kit (Qiagen GmbH, Hilden, Germany). One hundred milliliters each of water and ice samples and 50 g sediment sample were used for community DNA extraction. Quality and quantity of the extracted DNA were determined using NanoDrop 2000 spectrophotometer (Thermo Scientific). 16S rRNA genes for the three samples were amplified using the forward primer (5′ GTGCCAGC MGCCGCGGTAA) and reverse primer (5′ GGACTACHVGGGTWTCTAAT) designed for the V3 and V4 regions (Klindworth et al. 2013). Size of the PCR amplicons was checked through 1% agarose gel electrophoresis. Sequencing was done using MiSeq (Illumina, San Diego, CA) at the BioAnalytical Laboratory, Institute of Marine and Environmental Technology (IMET), University of Maryland Center for Environmental Sciences (UMCES). The libraries were prepared using the Nextera XT kit (Illumina) and sequencing was done using 2×300 cycle run using the MiSeq reagent kit v.3. The 16S rRNA gene dataset consisted of 189,890 raw sequence reads for the ice sample, 451,614 for water, and 225,910 reads for the sediment sample.

16S rRNA gene data and operational taxonomic unit classification

16S rRNA gene MiSeq sequencing reads for the three glacier samples were assembled and classified using QIIME software v 1.9.1 (Caporaso et al. 2010). Alignment of the unique sequences was performed with the 'align.seqs' command against a Greengenes database. Quality filtered sequences were clustered into operational taxonomic units (OTUs) according to sequence similarity using a 97% similarity threshold against the respective reference databases (Marzorati et al. 2008). The remaining sequences were clustered into de novo OTUs with UCLUST within QIIME.

RDP classifier in QIIME was used for the assignment of taxonomy to these sequences.

Nucleotide sequence accession numbers

The 16S rRNA gene accession numbers for isolates from different samples were designated as follows: bacteria from ice (MK522040-MK522046), from water (MK558827-MK558837), and from sediment (MK558838-MK558845). The bacterial 16S rRNA gene raw data from MiSeq sequencing for the ice, water, and sediment samples was deposited into the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the BioProject accession number PRJNA533649.

Results

Bacterial count, isolation, and identification

Microscopic and viable count

Direct bacterial count using epifluorescence microscopy revealed the presence of high number of bacterial cells in all three samples. The highest bacterial count was observed in sediment (3.3×10^6 cell g^{-1}), followed by water (2.8×10^6 cells mL^{-1}) and ice (6.4×10^5 cells mL^{-1}) (Table 1).

For viable count in the ice sample, no bacterial colony was observed on any of the three media used (R2A, TSA, LBA) even after 9 months of incubation at 4 °C and 6 months of incubation at 15 °C. Viable bacterial count from the water and sediment samples varied depending on the incubation temperature and the type of medium used. The maximum viable count for water (2.0×10^3 CFU mL^{-1}) and sediment (1.8×10^4 CFU g^{-1}) samples was recorded when R2A medium was used at 15 °C (Table 1).

Table 1 Bacterial counts from the three glacier samples (ice, water, sediment). Table includes viable counts and direct bacterial cell counts using CYBR Gold with epifluorescence microscopy

Sample type	Viable count at 4 °C (CFU/ml or g) (SD±)			Viable count at 15 °C (CFU/ml or g) (SD±)			Epifluorescence microscopy (cells/ml or g) (SD±)
	R2A	TSA	LBA	R2A	TSA	LBA	
Ice	–	–	–	–	–	–	6.40×10^5 (1.10)
Water	1.45×10^3 (0.89)	1.07×10^3 (1.07)	1.14×10^3 (1.01)	1.98×10^3 (0.66)	1.55×10^3 (1.2)	1.58×10^3 (1.04)	2.84×10^6 (1.0)
Sediment	1.13×10^4 (0.78)	1.05×10^4 (0.99)	8.9×10^3 (1.05)	1.83×10^4 (0.85)	1.41×10^4 (0.55)	1.07×10^4 (0.98)	3.30×10^6 (0.91)

Isolation of bacteria

A total of 27 bacterial isolates were recovered from three glacier samples, including 11 from water and 8 from each ice and sediment samples. Most of the isolates were recovered on the oligotrophic medium R2A, whereas the nutrient-rich media (TSA and LBA) yielded less colonies. Most of the colonies appeared after 2 to 3 weeks of incubation (4 °C and 15 °C) and only few appeared in the first week. However, the same isolates formed visible colonies within 2 to 5 days on subsequent re-streaking. Initial identification was done based on the colony and cell morphologies, pigmentation, and growth characteristics.

Direct plating of the melted ice sample on agar media (R2A, TSA, and LBA) did not yield colonies on any of the plates even after 9 and 6 months of incubation at 4 °C and 15 °C, respectively. On the other hand, in enriched with liquid media (R2B, TSB, and LB), growth was observed in the second week at 15 °C as indicated by turbidity in the media. When inoculated on the respective solid medium, colonies appeared within the first to the 3rd week of incubation at 4 °C and 15 °C. A total of 8 different bacterial isolates were obtained after plating the liquid enrichment culture on agar media; six colonies from R2B and one each from TSB and LB enrichments were recovered.

Gram staining revealed Batura Glacier is inhabited by representatives from both Gram-positive (14 isolates) and Gram-negative (13 isolates), with majority of the isolates exhibited coccobacillary cell morphologies (Table 2). Almost half of the isolates showed pigmented colonies. Ten of the isolates exhibited yellowish pigmentation while one each orange and pink (Table 2). Strains isolated from the glacier ice, water, and sediment were named with the prefix BGI (Batura Glacier Ice), BGW (Batura Glacier Water), and BGS (Batura Glacier Sediment).

Taxonomic classification of culturable isolates based on 16S rDNA gene sequences

Molecular identification based on 16S rDNA gene sequences revealed Batura Glacier is inhabited by a diverse group of bacteria. The isolates belong to 3 major phylogenetic groups: Actinobacteria (48%), Bacteroidetes (26%), and Proteobacteria (22%) (Fig. 2). The dominant genera were *Flavobacterium* (26%), *Arthrobacter* (22%), and *Pseudomonas* (19%). A phylogenetic tree was constructed to determine the affiliation of 27 isolates with other bacteria in the NCBI GenBank database (Fig. 2). The majority of 16S rRNA sequences was similar to previously determined sequences with 98% or above identical values (Table 3). Some of the isolates had 98 to 99% 16S rRNA gene sequence similarity with psychrophilic bacteria isolated from various cold environments. BGI-14 was closely related to *Cryobacterium psychrotolerans* (> 99%), BGW-1/BGW-17/BGW-18 to *Flavobacterium psychrolimnae* (> 99%), BGW-2/BGW-7/BGW-12 to *Flavobacterium glaciei* (> 98%), and BGW-5 to *Pseudomonas antarctica* (> 99%) (Table 3).

Characterization of bacterial isolates

Growth characteristics of the isolated bacteria

Phenotypic characteristics including temperature, pH, and salinity ranges for growth were determined. Results revealed that most of the isolates could grow at a wide range of temperature (4–35 °C) and pH (5–12) (Table 2). A few strains, including BGI-10 and BGI-11, demonstrated the ability to grow at the extremes of pH range (3 and 13). The majority of the isolates were either slight halophiles or halotolerant. BGI-10 and BGI-11 were able to grow at salt concentrations of up to 8% (w/v); however, none of the isolates demonstrated growth at 10% NaCl. Most of the isolates preferred the oligotrophic medium R2A and also demonstrated growth at nutrient-rich media (Table S1). BGI-10 and BGI-11 were able to grow in all the 8 media used. Five *Pseudomonas* species BGI-1, BGI-2, BGW-4, BGW-5, and BGW-10 demonstrated growth on MacConkey's agar and could not ferment lactose (Table S1). BGS-6, BGI-10, and BGI-11 demonstrated good growth on mannitol salt agar with high salt concentration (7.5% w/v) and deemed halotolerant.

Extracellular enzymes and antimicrobial activities

Isolates were screened for enzymatic and antimicrobial activities. Most of the isolates demonstrated the ability to produce one or more extracellular enzymes

Table 2 Physiological characteristics of the glacial isolates

Isolates	Temperature range (°C)	pH range	NaCl range (%)	Biochemical properties				Pigmentation	Cell morphology (Gram staining)
				Citrate utilization	Glucose/ sucrose/ lactose	Catalase test	Nitrate reduction		
BGI-1	4–30	5–11	0–4	+	±/–	+	–	–	−ve (short rods)
BGI-2	4–35	5–11	0–5	+	±/–	+	–	–	−ve (short rods)
BGI-4	4–40	4–12	0–2	+	–/–/–	+	+	Orange	+ve (thin long rods)
BGI-5	4–40	5–12	0–4	–	–/–/–	+	–	Pale yellow	+ve (cocci)
BGI-7	4–25	4–12	0–4	–	–/–/–	+	+	–	+ve (coccobacilli)
BGI-10	4–35	3–13	0–8	+	–/–/–	+	–	–	+ve (coccobacilli)
BGI-11	4–35	3–13	0–8	+	–/–/–	+	–	–	+ve (coccobacilli)
BGI-14	4–25	3–12	0–2	–	+/+/+	+	+	–	+ve (cocci)
BGS-1	4–30	5–12	0–6	+	+/–/–	+	–	–	+ve (coccobacilli)
BGS-2	4–30	5–12	0–4	+	±/–	+	+	–	+ve (short rods)
BGS-3	4–35	6–11	0–2	–	±/–	+	–	Pink	+ve (cocci in clusters)
BGS-5	4–30	5–12	0–4	–	–/–/–	+	+	–	+ve (short rods)
BGS-6	4–35	4–13	0–8	–	–/–/–	+	–	Pale yellow	+ve (coccobacilli)
BGS-9	4–30	4–13	0–6	–	–/–/–	+	+		+ve (cocci in clusters)
BGS-12	4–30	5–13	0–4	+	–/–/–	+	–		+ve (coccobacilli)
BGS-13	4–35	4–12	0–6	–	+/+/+	+	–	Pale yellow	+ve (bacilli)
BGW-1	4–25	5–11	0	–	+/–/–	+	+	Yellow	−ve (coccobacilli)
BGW-2	4–25	5–11	0	–	–/–/–	+	–	Yellow	−ve (short rods)
BGW-4	4–35	5–13	0–6	+	–/–/–	+	–	–	−ve (coccobacilli)
BGW-5	4–35	5–13	0–6	+	–/–/–	+	–	–	−ve (short rods)
BGW-7	4–20	5–12	0	–	–/–/–	+	–	Yellow	−ve (coccobacilli)
BGW-8	4–35	4–13	0–4	+	–/–/–	+	+	–	−ve (short rods)
BGW-10	4–35	4–13	0–4	+	–/–/–	+	–	–	−ve (bacilli)
BGW-12	4–25	5–10	0	–	+/–/–	+	–	Yellow	−ve (thick rods)
BGW-15	4–20	6–9	0	–	+/–/–	+	–	Yellow	−ve (small cocci)
BGW-17	4–20	5–11	0–2	–	+/+/+	+	+	Yellow	−ve (short rods)
BGW-18	4–20	5–10	0	–	+/+/+	+	+	Yellow	−ve (short rods)

(Table S2). Enzymatic assays revealed that 70% of the strains produced cellulases, 48% lipases, 41% proteases, 41% DNases, and only 7% amylases. Some isolates demonstrated remarkable ability to produce 3 or more different enzymes including strains BGS-9, BGI-1, BGI-2, BGS-2, BGS-12, BGW-4, BGW-5, and BGW-15. *Flavobacterium* sp. BGW-15 and *Flavobacterium* sp. BGW-17 were the only strains able to produce amylases (Table S2).

The majority of the glacier isolates demonstrated antimicrobial activity against one or more test microorganisms: 33% against *C. albicans*, 30% against *Bacillus* sp., 26% against *P. aeruginosa*, 26% against *S. aureus*, 22% against *Acinetobacter* sp., and only 4% against *K. pneumoniae*. Some strains exhibited broad-spectrum activity including BGI-2, BGW-4 and BGW-5, BGI-1, BGW-8, and BGW-10 (Table S2).

Prevalence of antibiotic resistance in the glacier isolates

Antibiotic resistance to commercial antibiotics was found among the glacial isolates (Table S3). As many as 67% of the Gram-negative and 46% of the Gram-positive bacteria demonstrated resistance to trimethoprim/sulfamethoxazole (30 μg) (Table 4). Among the Gram-negative bacteria, 11% were resistant to colistin sulfate (10 μg) and nalidixic acid (30 μg) each. Resistance against methicillin (10 μg) and vancomycin (5 μg) among the Gram-positive isolates was 23% and 15%, respectively. All the Gram-negative bacteria were sensitive to the broad-spectrum antibiotics imipenem (10 μg) and ofloxacin (5 μg). Only one Gram-positive isolate exhibited resistance to imipenem (10 μg) and all 27 isolates were sensitive to ofloxacin (5 μg) (Table S3).

Table 3 Taxonomic classification of 16S rRNA gene sequences of culturable bacterial isolates with accession number and closely related species in the GenBank database with similarity index

Isolate	Sample type	Accession no.	Phylogenetic group	Nearest phylogenetic neighbor with accession number	Similarity (%)
BGI-1	Ice	MK522040	Proteobacteria	*Pseudomonas mandelii* (BDAF01000092)	99.72
BGI-2	Ice	MH681214	Proteobacteria	*Pseudomonas frederiksbergensis* (AJ249382)	99.59
BGI-4	Ice	MK522041	Firmicutes	*Bacillus proteolyticus* (MACH01000033)	99.81
BGI-5	Ice	MK522042	Actinobacteria	*Okibacterium fritillariae* (FUZP01000004)	99.81
BGI-7	Ice	MK522043	Actinobacteria	*Arthrobacter ginsengisoli* (KF212463)	98.84
BGI-10	Ice	MK522044	Actinobacteria	*Rhodococcus erythropolis* (BCRM01000055)	99.81
BGI-11	Ice	MK522045	Actinobacteria	*Rhodococcus erythropolis* (BCRM01000055)	99.76
BGI-14	Ice	MK522046	Actinobacteria	*Cryobacterium psychrotolerans* (jgi.1076200)	99.12
BGS-1	Sediment	MK558838	Actinobacteria	*Arthrobacter ginsengisoli* (KF212463)	99.44
BGS-2	Sediment	MK558839	Actinobacteria	*Arthrobacter ginsengisoli* (KF212463)	99.62
BGS-3	Sediment	MK558840	Actinobacteria	*Arthrobacter agilis* (X80748)	97.68
BGS-5	Sediment	MK558841	Actinobacteria	*Pseudarthrobacter sulfonivorans* (AF235091)	95.90
BGS-6	Sediment	MK558842	Actinobacteria	*Microbacterium phyllosphaerae* (AJ277840)	99.68
BGS-9	Sediment	MK558843	Actinobacteria	*Arthrobacter ginsengisoli* (KF212463)	99.38
BGS-12	Sediment	MK558844	Actinobacteria	*Arthrobacter ginsengisoli* (KF212463)	99.42
BGS-13	Sediment	MK558845	Actinobacteria	*Okibacterium fritillariae* (FUZP01000004)	99.91
BGW-1	Water	MK558827	Bacteroidetes	*Flavobacterium psychrolimnae* (AJ585428)	99.03
BGW-2	Water	MK558828	Bacteroidetes	*Flavobacterium glaciei* (DQ515962)	98.72
BGW-4	Water	MK558829	Proteobacteria	*Pseudomonas deceptionensis* (JYKX01000012)	99.48
BGW-5	Water	MK558830	Proteobacteria	*Pseudomonas antarctica* (AJ537601)	99.82
BGW-7	Water	MK558831	Bacteroidetes	*Flavobacterium glaciei* (DQ515962)	98.74
BGW-8	Water	MK558832	Proteobacteria	*Janthinobacterium svalbardensis* (DQ355146)	99.43
BGW-10	Water	MK558833	Proteobacteria	*Pseudomonas deceptionensis* (JYKX01000012)	99.64
BGW-12	Water	MK558834	Bacteroidetes	*Flavobacterium glaciei* (DQ515962)	98.94
BGW-15	Water	MK558835	Bacteroidetes	*Flavobacterium omnivorum* (AF433174)	99.82
BGW-17	Water	MK558836	Bacteroidetes	*Flavobacterium psychrolimnae* (AJ585428)	99.35
BGW-18	Water	MK558837	Bacteroidetes	*Flavobacterium psychrolimnae* (AJ585428)	99.43

Culture-independent characterization of bacterial community

The results from Illumina amplicon sequencing of 16S rRNA genes revealed Proteobacteria, Actinobacteria, and Bacteroidetes as major phyla from the three glacier samples (Fig. 4a). Proteobacteria dominated in all the three samples, followed by Actinobacteria and Bacteroidetes. A small fraction of the bacterial communities was also represented by the Saccharibacteria and Firmicutes and a considerably large fraction of the sequences (20%) in the sediment sample was unassigned.

The sequences in glacier samples were assigned to 24 genera (> 0.5%) and the majority of them could not be refined to the genus level and were then grouped under the category "Bacteria (others)." The genera included the following: *Arthrobacter*, *Flavobacterium*, *Pseudomonas*, *Kaistobacter*, *Mycoplana*, *Ochrobactrum*, *Devosia*, *Rhodococcus*, *Rhodobacter*, *Janthinobacterium*, *Acinetobacter*, *Segetibacter*, *Flavisolibacter*, *Methylotenera*, *Polaromonas*, *Sphingomonas*, *Novosphingobium*, *Agrobacterium*, *Methylobacterium*, *Lysinibacillus*, *Chryseobacterium*, *Salinibacterium*, *Mycetocola*, and *Microbacterium* (Fig. 4b).

Mycoplana (16%) and *Arthrobacter* (12%) represented the major genera in the ice sample. The genera *Kaistobacter* (20%) and *Arthrobacter* (16%) dominated in sediment sample; *Ochrobactrum* (30%), *Janthinobacterium* (24%), and *Flavobacterium* (11%) dominated in ice sample. *Arthrobacter* was found predominantly in all the three samples. Graphs (Fig. 4a and b) depict the phyla and genera with operational taxonomic units (OTUs) that have abundance greater than 0.5%.

Discussion

In the present study, we examined the possibility of using a glacier in the Karakoram Range of Pakistan as source for isolation of novel cold-adapted bacteria, and identification

Table 4 Antibiotic susceptibility test of the glacier isolates using broad-spectrum and narrow spectrum antibiotics.
Table represents the number of isolates (in percentage) sensitive and resistance in each group (Gram-positive and Gram-negative bacteria)

Antibiotics	Gram-negative bacteria		Gram-positive bacteria	
	Sensitive (%)	Resistance (%)	Sensitive (%)	Resistance (%)
Imipenem	100	0	96.30	3.70
Ofloxacin	100	0	100	0
Trimethoprim/sulfamethoxazole	33.33	66.67	53.85	46.15
Vancomycin	NA	NA	84.62	15.38
Methicillin	NA	NA	76.92	23.08
Colistin sulfate	88.89	11.11	NA	NA
Nalidixic acid	88.89	11.11	NA	NA

and exploration of some of their metabolic potential to be used for biotechnological or industrial applications. The glaciers of the Karakoram, particularly in the Pakistani geographical region, are not commonly explored in terms of their bacterial community characterization and metabolite production.

Viable count was the highest on R2A among the three media and the majority of the isolates were recovered using this medium. R2A is a low nutrient medium designed for the recovery of bacteria from oligotrophic samples (Reasoner and Geldreich 1985). Many studies have previously been reported where R2A was considered the medium of choice for isolation of psychrophilic or psychrotrophic bacteria from cold environments (Christner et al. 2001; Segawa et al. 2011; Tam et al. 2015). The maximum viable counts for water and sediment samples were measured at 15 °C on R2A agar. A similar

range of viable counts has been reported previously for samples from the cold habitats (Zhang et al. 2013; Segawa et al. 2011). In our study, isolates from the ice were recovered through culture enrichment in liquid media, which is in accordance with a previous study (Antony et al. 2012). Only a small fraction of microorganisms in any environment is culturable and a large fraction remains uncultivable and extreme environments make it even harder for the isolation of bacteria; their nutritional requirements are limited and somewhat unknown. Other studies have also reported extremely low viable bacterial counts from the glacier ice samples (Zhang et al. 2008; Christner et al. 2001).

Another feature, which was predominant in the glacial isolates, was the formation of pigmented colonies. More than half of the isolates formed pigmented colonies that appeared
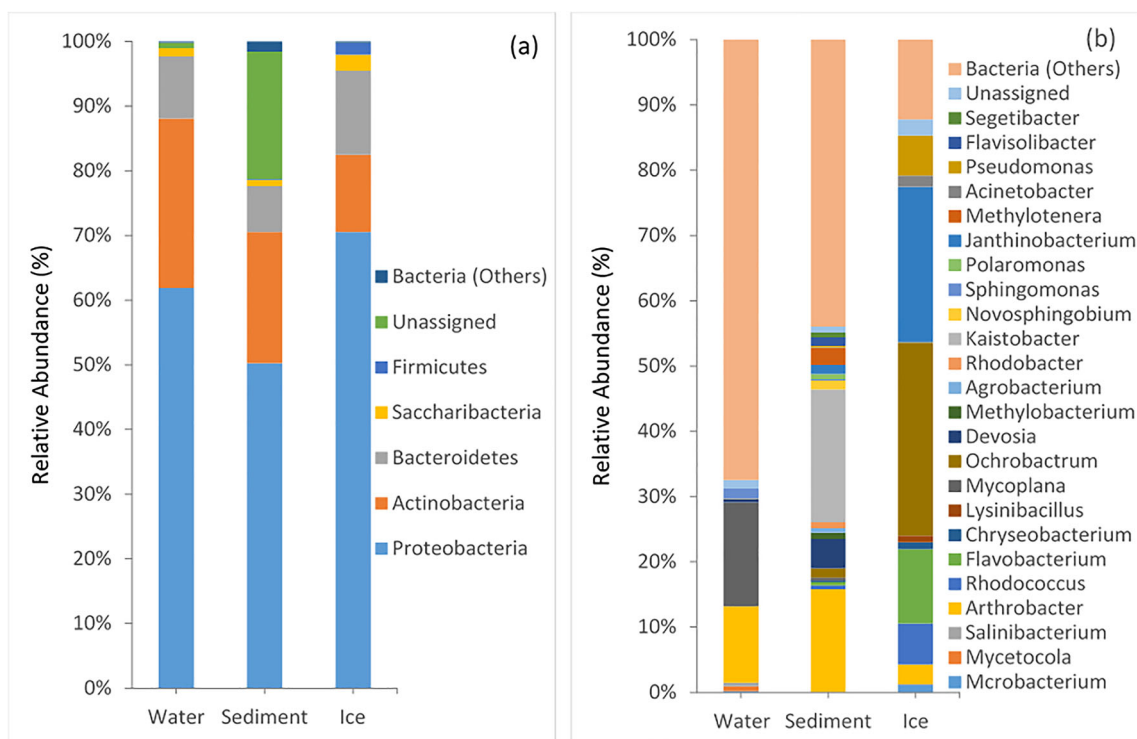

Fig. 4 Culture-independent bacterial community characterization of the glacier samples using Illumina amplicon sequencing of 16S rRNA genes. Distribution of bacterial community at the **a** phylum and **b** genus level

yellowish and orange. Studies showing pigmented bacteria dominating bacterial communities have been reported from glaciers and other cold habitats (Shen et al. 2018; Christner et al. 2000; Antony et al. 2012). Carotenoid pigmentation is considered a strategy to withstand environmental stresses including low temperatures (Chattopadhyay and Jagannadham 2001; Dieser et al. 2010). Fong et al. (2001) found correlation between temperature and carotenoid production, and a decrease in cultivation temperature resulted in a concomitant increase in carotenoid production, which is speculated to contribute to membrane stabilization at low temperatures.

All the isolated bacteria belonged to 3 major phyla including Actinobacteria, Proteobacteria, and Bacteroidetes. The predominance of these groups in glaciers and other cold environments has been previously reported (Silva et al. 2018; Shen et al. 2018; Liu et al. 2018; Cheng and Foght 2007). *Flavobacterium*, *Arthrobacter*, and *Pseudomonas* were the dominant genera in overall the three glacier samples which conforms to the previous reports from cold habitats (Thakur et al. 2018; Reddy et al. 2009; Cheng and Foght 2007). All the isolated bacteria from sediment samples were members of Actinobacteria with 63% representing the genus *Arthrobacter*. Zhao et al. (2018) studied the culturable bacteria in soil along an altitudinal gradient in the Tianshan Mountains and found that almost 50% represented the genus *Arthrobacter*.

16S rRNA gene meta-barcoding also revealed Proteobacteria, Actinobacteria, and Bacteroidetes as the dominant taxa in the glacier samples. Proteobacteria accounted for more than 50% of relative abundance in all the three glacier samples. Proteobacteria, Actinobacteria, and Bacteroidetes have been previously reported as the dominant phyla from glacier environments using culture-independent methods (Lopez et al. 2018; Venkatachalam et al. 2015). *Mycoplana*, *Arthrobacter*, *Kaistobacter*, *Ochrobactrum*, *Janthinobacterium*, and *Flavobacterium* represented the major genera in the glacier samples. *Arthrobacter* was consistently found as one of the dominant genera in terms of the relative abundance in all the three glacier samples. *Arthrobacter* has been previously reported as the dominant genus from cold environments (Thakur et al. 2018; Zhao et al. 2018). Similarly, *Mycoplana* (Yang et al. 2016), *Kaistobacter* (Yang et al. 2016), *Janthinobacterium* (Rassner et al. 2016), *Ochrobactrum* (Liu et al. 2009), and *Flavobacterium* (Segawa et al. 2011) have also been reported previously from glacial environments. Interestingly, *Ochrobactrum* was the dominant genus among all in the ice sample and accounted for 30% of the relative abundance. Members of the genus *Ochrobactrum* have rarely been reported from glaciers and other cold environments. *Ochrobactrum* species are metabolically versatile and produce a variety of enzymes to degrade recalcitrant compounds including pesticides, phenols, and petroleum hydrocarbons, making them potential candidates for bioremediation

technologies (Ermakova et al. 2017; Arulazhagan and Vasudevan 2011).

According to Moyer and Morita (2007) definitions of cold-adapted bacteria, Batura Glacier is mainly dominated by psychrotrophic bacteria as the majority of the isolates exhibited a wide growth temperature range (4–30 °C or 4–35 °C). *Flavobacterium* isolates BGW-15, BGW-17, and BGW-18 could not grow above 20 °C and were determined to be representatives of psychrophilic bacteria. According to Russell (1990), psychrophiles are found in permanently cold environments and psychrotrophs dominate habitats which experience more thermal fluctuations. This is the case for non-polar glaciers like Batura Glacier in the Karakoram, as it experiences wide seasonal temperature fluctuations. Therefore, psychrotrophic bacteria are expected to dominate over their psychrophilic counterparts. The dominance of psychrotrophic bacteria compared to true psychrophilic bacteria has been previously reported in similar cold environments (Liu et al. 2018; Thakur et al. 2018; Reddy et al. 2009; Steven et al. 2007).

Antibiotic resistance patterns in the glacier isolates revealed resistance against some commercial antibiotics. High resistance was documented against trimethoprim/ sulfamethoxazole among the Gram-negative and Gram-positive bacteria. However, resistance against the broad-spectrum antibiotics (ofloxacin and imipenem) was the least observed. Imipenem is a β-lactam antibiotic and resistant bacteria produce β-lactamases to inactivate the drug. In a recent study, Van Goethem et al. (2018) found a very low abundance of β-lactamase genes in the metagenome of a pristine cold habitat of Antarctica. Nevertheless, antibiotic resistance genes and organisms have been reported from non-clinical and natural environments including the ocean and polar region (Hatosy and Martiny 2015; Van Goethem et al. 2018; Segawa et al. 2013). The resistance found in the remote pristine regions with no or less human impact suggests a natural process that is acquired by these microorganisms over time through the process of evolution and the passing off through generations. Nevertheless, Batura Glacier is located close to living populations and anthropogenic transfer through migratory birds and airborne bacteria could be a possibility (Segawa et al. 2013).

Most of the isolates in our study exhibited antimicrobial activities against one or more of the test organisms. All cultured *Pseudomonas* species demonstrated broad-spectrum antimicrobial activities. Cold-adapted *Pseudomonas* species with wide-spectrum antimicrobial activities have been reported previously from Antarctica (Silva et al. 2018; Tedesco et al. 2016; O'Brien et al. 2004). Isolated strains from Batura Glacier also exhibited abilities to produce a variety of extracellular enzymes including cellulases, proteases, lipases, DNases, and amylases. Cold-adapted bacteria with enzyme activities have been reported previously from the cold

environments (Reddy et al. 2009; Margesin et al. 2003). The majority of the isolates demonstrated cellulolytic activity (70%), similar to a recent study by Thakur et al. (2018); their result revealed 71% of cellulolytic bacteria in their strains using carboxymethyl cellulose (CMC) plates. Overall, *Pseudomonas* species dominated among the glacier isolates in terms of enzymatic activities. *Pseudomonads* have been reported previously as producers of lipases (Ganasen et al. 2016), proteases (Zeng et al. 2003), and cellulases (Menéndez et al. 2015).

Conclusions

Results of the culture-dependent and culture-independent study revealed bacterial community from the glacier samples belonged to 3 major phylogenetic groups including Actinobacteria, Bacteroidetes, and Proteobacteria. The range of temperature for growth of the isolates indicated that Batura Glacier is mainly inhabited by psychrotrophic bacteria rather than the true psychrophiles. The majority of the isolates were either slight halophiles or halotolerant. Our results also indicated that cold environments in the Karakoram Range are promising sources of cold-adapted bacteria with enormous potential for biotechnological exploitation. Most of the isolates produced one or more extracellular enzymes and exhibited antimicrobial activity against a number of microorganisms including multidrug-resistant pathogenic strains. Almost all the isolates were sensitive to the broad-spectrum antibiotics (ofloxacin and imipenem), while varying degree of resistance was found against the narrow spectrum antibiotics.

Compliance with ethical standards

Conflict of interest   The authors declare that they have no conflict of interest.

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