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Genetic analysis of the frozen microbiome at 7900 m a.s.l., on the South Col of Sagarmatha (Mount Everest)

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ABSTRACT

Microbial communities in alpine environments >7,500 m.a.s.l. have not been well studied using modern cultivation-independent sequencing approaches due to the challenges and danger associated with reaching such high elevations. For this reason, we know little about the microorganisms found in sediments on Earth's tallest mountains, how they reach these surfaces, and how they survive and remain active at such extreme elevations. Here, we explore the microbial diversity recovered from three sediment samples collected from the South Col (~7,900 m.a.s.l.) of Sagarmatha (Mount Everest) using both culturing and next generation sequencing approaches (16S rRNA gene, internal transcribed spacer [ITS] region, and 18S rRNA gene sequencing). Both approaches detected very low diversity of bacteria, protists, and fungi that included a combination of cosmopolitan taxa and specialized microorganisms often found at high elevations like those of the genera *Modestobacter* and *Naganishia*. Though we managed to grow viable cultures of many of these taxa, it remains likely that few, if any, can be active in situ at the South Col. Instead, these high-elevation surfaces may act as deep-freeze collection zones of organisms deposited from the atmosphere or left by climbers scaling the Earth's highest mountain.

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Introduction

High-elevation microbial communities must survive a variety of challenging conditions (D. Singh et al. 2012; Merino et al. 2019). With an increase in elevation comes a corresponding shift in altitude-dependent environmental conditions, including colder temperatures, lower atmospheric pressures, lower oxygen availability, and lower water activity (Merino et al. 2019). Sustaining the levels of metabolic activity required to be active becomes more challenging as elevation increases (Margesin et al. 2009; Margesin and Miteva 2011) and, as a result, microbial diversity tends to decrease correspondingly (Bryant et al. 2008; L. Zhang et al. 2009; Margesin and Miteva 2011). For example, in the Transantarctic Mountains, microbial communities in higher elevation soils are significantly less diverse, have lower biomass, and even grow more slowly than those in lower elevation soils (Dragone et al. 2022). The identity and function of the microorganisms can also change as

elevation increases. In the Himalayas, fungi dominate sediment communities at elevations from 3,000 to 3,500 m (Petrovič, Gunde-Cimerman, and Zalar 2000; Margesin and Miteva 2011), archaeal ammonia oxidizers between 4,000 and 5,400 m, and bacterial ammonia oxidizers up to 6,500 m (L. Zhang et al. 2009). In addition, phototrophic microbes are thought to be missing in dry high-elevation soils, but active photosynthetic communities have been detected in periglacial soils above 5,500 m in the Himalayas (S. K. Schmidt et al. 2011) and above 6,000 m in soils receiving water vapor from fumaroles (Solon et al. 2018).

Analysis of samples that have been collected from the highest alpine environments around the world (>7,000 m) suggests that these environments are typically dominated by a small number of polyextremophilic taxa (Lynch et al. 2012; Merino et al. 2019). Psychrophiles, like the fungus *Naganishia friedmannii*, which has been shown to remain active in alpine

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environments above 6,000 m (S. K. Schmidt et al. 2017), or the bacterial genus *Geodermatophilus*, whose type strain was isolated in 1970 from sediment collected at 8,400 m.a.s.l. on Everest (Ishiguro and Fletcher 1975), are common. However, not all of the microorganisms identified above 7,000 m are highly adapted to such an extreme environment. Surface snow samples collected at ~8,000 m on the north side of Everest were found to contain high biomass communities of Proteobacteria and Actinobacteria, including the genera *Actobacterium*, *Acinetobacter*, and *Kocuria* (Liu et al. 2007), which can be found in temperate and tropical soils around the world (Delgado-Baquerizo et al. 2018). Though Liu et al. (2007) suggested that these detected organisms were deposited by snowfall or eolian transport and are probably not active or even viable in situ, their findings highlight that many questions still remain about the microbial communities of the world's highest alpine environments.

Mt. Everest (Sagarmatha, Chomolungma), located on the China–Nepal border within the Mahalangur Himal subrange of the Himalayas, is the world's highest mountain. With an elevation of 8,849 m.a.s.l., Everest's high-elevation environments likely represent some of the most "extreme" terrestrial surfaces on Earth. Though we know that diverse and active microbial communities have been described in many lower elevation environments on the mountain (L. Zhang et al. 2009; Margesin and Miteva 2011) and bacteria have been isolated from samples collected at 8,400 m (Ishiguro 1969; Ishiguro and Fletcher 1975), questions still remain about the habitability of the mountain's highest elevations, in particular of exposed sediment surfaces >7,500 m on the mountain where microbial communities have never been studied using next-generation sequencing approaches. We expect that many microorganisms may never be active in such surface environments, restricted by the extremely challenging conditions experienced at such high elevations.

To better understand the impact that elevation has on microbial habitability and community structure, we analyzed three samples collected from the South Col (~7,900 m.a.s.l.) of Everest. At the South Col, a ridge between Everest's summit and the neighboring Lhotse, temperatures regularly reach -33°C with an air pressure one-third that at sea level (~380 hPa at time of collection; Moore and Semple 2004; Matthews et al. 2020). Additionally, measurements taken by a weather station installed at the South Col in 2019 have recorded a maximum wind speed of 66.5 m/s and a maximum insolation of 1,500 W/m², which is greater than the values expected from the top of the atmosphere due to reflectance (Matthews et al. 2020). Using a combination

of marker gene sequencing and more traditional culture-based methods, we add to previous microbiology research on Mt. Everest by asking two questions: Can we find evidence of microorganisms on terrestrial surfaces >7,000 m and, if so, are these organisms viable and could any be active and able to survive at such extreme elevations?

Materials and methods

Sample collection

Field sampling was performed as part of the National Geographic and Rolex Perpetual Planet Everest Expedition in April to May 2019. A total of three surface sediment samples (0–5 cm depth) were collected in sterile polyethylene bags using aseptic techniques. Sediments were collected about 170 m SE of Camp IV (27.9728°N, 86.9315°E, 7,944 m.a.s.l.; Figure 1).

The three samples were sent to the University of Colorado Boulder, Colorado. Though the samples remained sealed after collection and throughout the entire shipping process, they did not remain frozen throughout the shipment. After arriving at the University of Colorado, Boulder the samples were refrozen and remained in storage at -70°C until they could be processed.

Cultivation-independent analysis

Samples were removed from the freezer and particles <1 mm in size were manually separated from the larger fragments using a sterilized sediment sieve. DNA was then extracted from 0.5 g of these finest particles in a laminar flow hood using the Qiagen DNeasy Powersoil Kit following the manufacturer's recommendations. An extraction blank was included to test for any possible contamination introduced during the DNA extraction.

The DNA aliquots extracted from each of the three sediment samples and the associated extraction blank were used for a variety of polymerase chain reaction (PCR) amplifications to assess the archaeal/bacterial, fungal, and microeukaryotic communities. The primer sets that were used for PCR amplification included a primer pair that targets the hypervariable V4 region of the archaeal and bacterial 16S rRNA gene (515 F: 5'-GTGCCAGCMGCCGCGGTAA-3' and 806-R: 5'-GGACTACHVGGGTWTCTAAT-3'; Walters et al. 2016), a primer pair that targets the internal transcribed spacer of the fungal rRNA operon (ITS1-F: 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS2-R: 5'-GCTGCGTTCTTCATCGATGC-3'; Bellemain et al. 2010), and a primer pair that targets the hypervariable

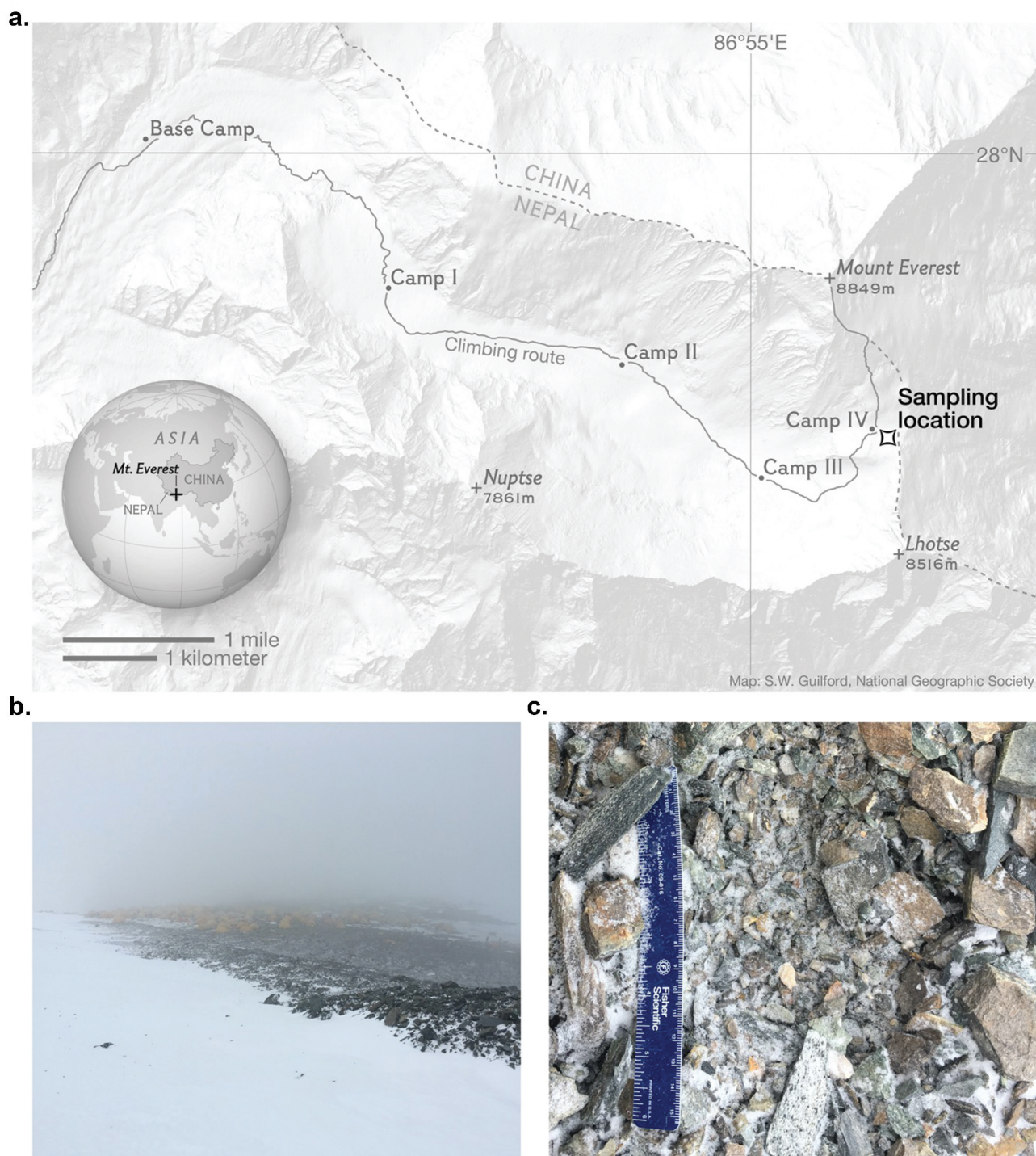


Figure 1. The South Col of Mount Everest (27.9728°N, 86.9315°E). (A) Map displaying the sampling location along the Everest climbing route. Samples were collected ~170 m southeast of Camp IV from an exposed surface environment at 7,944 m a.s.l. (B) View from sampling location back toward Camp IV. (C) The surface at the collection site was made up of fragments of variable grain size, with the smallest fragments <0.5 mm in diameter and the largest fragments ~2-5 cm in diameter. Photo credit: L. Baker Perry/National Geographic.

V9 region of the 18S rRNA gene (1391-F: 5'-GTACACACCGCCCGTC-3' and EukBR: 5'-TGATCCTTCTGCAGGTTACCTAC-3'). One no-template PCR control was run with each set of

amplifications. All three primer sets included the appropriate Illumina adapters and unique 12 bp barcode sequences to permit multiplexed sequencing (Caporaso et al. 2012). PCRs were performed in 25 μ L reaction

volumes. PCR amplification using the ITS1-F/ITS2-R and 1391-F/EukBR primer sets was performed using GoTaq Hot Start PCR Master Mix and PCR amplification using the 515 F/806-R pair used the Platinum II Hot-Start PCR Master Mix (2X). Cycling parameters for the ITS1F/ITS2R primer sets consisted of an initial denaturation step at 94°C for 3 minutes, followed by thirty-five cycles of denaturation at 94°C (45 seconds), annealing at 50°C (60 seconds), extension at 70°C (90 seconds), and a final extension step at 72°C for 10 minutes. Cycling parameters for the 515 F/806 R primer set consisted of an initial denaturation step at 94°C for 2 minutes, followed by thirty-five cycles of denaturation at 94°C (15 seconds), annealing at 60°C (15 seconds), extension at 68°C (60 seconds), and a final extension step at 72°C for 10 minutes. Cycling parameters for the 1391-F/EukBR primer set consisted of an initial denaturation step at 94°C for 3 minutes, followed by thirty-five cycles of denaturation at 94°C (45 seconds), annealing at 57°C (60 seconds), extension at 72°C (90 seconds), and a final extension step at 72°C for 10 minutes. Amplified product was visualized on a gel and then cleaned and normalized to equimolar concentrations using SequalPrep Normalization Plates. Sequencing was performed by the University of Colorado Boulder's next-generation sequencing facility using the Illumina MiSeq platform. The V2 2 × 150 bp Illumina sequencing kit was used for the 16S rRNA gene and the 18S rRNA gene sequencing and the 2 × 250 bp paired-end Illumina sequencing kits were used for the internal transcribed spacer (ITS) region amplicons. Raw sequencing data can be accessed in the National Center for Biotechnology Information Sequence Read Archive, project accession number PRJNA882470.

Bioinformatics

16S rRNA gene sequences, ITS region sequences, and 18S rRNA gene sequences from the soil extractions were processed using DADA2 pipeline v3.8 (Callahan et al. 2016). Sequences were quality filtered and clustered into amplicon sequence variants (ASVs). Taxonomic information was assigned to ASVs using a naive Bayesian classifier method (Wang et al. 2007) that takes the set of ASVs generated and compares them to a training set of reference sequences: the 16S rRNA bacterial and archaeal SILVA database v132 (Quast et al. 2013; Yilmaz et al. 2014), the UNITE database v8.3 for fungi (Nilsson et al. 2019), and SILVA 18S SSU rRNA v132 RefNR 99 database for eukaryotes, which includes fungi (Quast et al. 2013; Yilmaz et al. 2014). A minimum bootstrapping threshold required to return a taxonomic classification of 50 percent similarity was

used for analysis of the 16S rRNA sequences and 18S rRNA sequences and a minimum bootstrapping threshold of 85 percent was used for ITS region sequences.

Downstream analysis of the DADA2 processed data was performed in R v4.0.5 (R Core Team 2017) using the package “mctoolsr” (<https://github.com/leffj/mctoolsr/>) (Leff 2016). For the 16S rRNA sequencing, ASVs associated with chloroplasts, mitochondria, and eukaryotes (sixty-two ASVs) were removed. ASVs that made up at least 1 percent of the extraction blank or no-template PCR control and were at least 1 percent of the samples on average were discarded as likely lab contaminants (ASV-17). After this filtering, a total of fifty-seven bacterial ASVs remained. No archaeal sequences were identified from the next-generation sequencing.

For the fungal ITS region sequencing data, ASVs that could not be classified to the phylum level (only classified as “fungi”) were removed prior to downstream analysis (three ASVs). No reads were identified in the extraction blank. The thirteen reads identified in the no-template PCR control were associated with the second most abundant ASV in the samples (ASV-2, *Naganishia*) that are often dominant members of high-altitude, low-temperature environments (S. K. Schmidt et al. 2017). Therefore, these reads were likely not a result of laboratory contamination but are instead most likely derived from the soil samples and represent “tag switching” events (Schnell, Bohmann, and Gilbert 2015). After this filtering, sixteen fungal ASVs remained.

For downstream analysis of the 18S rRNA gene sequences, we note that two ASVs were associated with human DNA. Only one of these ASVs was identified in the blanks, with 90 percent of the human reads coming from the samples themselves. Based on this, we do not believe that these were contaminated during processing and were likely in the samples at collection. For downstream analysis, these human ASVs were removed. Additionally, two ASVs in the blank samples were not found in any of the three South Col samples and were removed from the data set. After this filtering, fourteen eukaryotic ASVs remained.

Culturing study/culture identification

To determine whether any microorganisms in these samples were viable, the three sediment samples were plated on seven different types of solid culture media in conditions designed to promote growth. Antarctic bacterial medium (P. Singh, Singh, and Roy 2016), modified nutrient broth agar (Pulschen et al. 2017), variable nine-salt solution (Egan, Kjelleberg, and Holmström 2015), and a modified variable nine-salt solution without the nine-salt solution (Egan, Kjelleberg, and Holmström

2015) were chosen because they were designed for the culturing bacteria from oligotrophic environments. Antarctic bacterial medium and modified nutrient broth agar were designed to target organisms found in nutrient-poor, cold, and dry Antarctic sediments (P. Singh, Singh, and Roy 2016; Pulschen et al. 2017) and have been used successfully to grow Proteobacteria, Actinobacteria, Firmicutes, and the fungus *Naganishia* (Pulschen et al. 2015; Dragone et al. 2021), which were the most abundant taxa identified through our 16S rRNA gene and ITS region sequencing effort (Figure 2). Samples were also plated on three nutrient-rich media variants, Luria-Bertani agar (Atlas 2004) and R2A agar at pH 7 and pH 9 (Reasoner and Geldreich 1985), which were chosen to target the copiotroph taxa identified in our next-generation sequencing approach that we felt may be missed if we just used oligotrophic media (Figure 1, Data S1). All media were created following the methods described in the cited literature, but more information about the specific pH and conditions can be found in Dataset S3.

One gram of each sample was vortexed and then shaken for 5 minutes in 1 mL of a 7.2 pH phosphate-buffered solution and 60 μ L of the resulting soil slurry was pipetted onto each plate (surface area of each plate: 58 cm²) and spread across the plates using flame-sterilized cell spreaders. Two blank plates inoculated with 60 μ L PBS were prepared for each media type and handled in an identical manner to the twenty-eight plates inoculated with the soil slurries. Plates of each different media type were incubated at 4°C with regular nocturnal/diurnal light cycles. Plated samples and the uninoculated control plates remained under these conditions for four weeks. All unique colonies that grew were isolated using a streak plate isolation technique. To ensure purity, cultures from the first isolation plate were streaked for isolation two subsequent times.

DNA from each of the isolated colonies was extracted using the Qiagen DNeasy Ultraclean 96 Microbial Kit following the manufacturer's recommendations. A total of six extraction blanks were included to control for contamination introduced during the DNA extraction.

The DNA aliquots extracted from each of the isolated cultures and associated extraction blanks were PCR amplified in duplicate using a primer pair that targets the archaeal and bacterial 16S rRNA gene (S_27 F: 5'-GTGCCAGCMGCCGCGGTAA-3' and S_1429 r: 5'-GGACTACHVGGGTWTCTAAT-3') and using the primer pair (ITS1-F: 5'-CTTGGTCATTTAGAGG AAGTAA-3' and ITS4-R: 5'-GCTGCGTTCTTCATC GATGC-3') that targets the ITS of the fungal rRNA operon. Three no-template PCR controls were run for each set of amplifications. PCRs were performed in

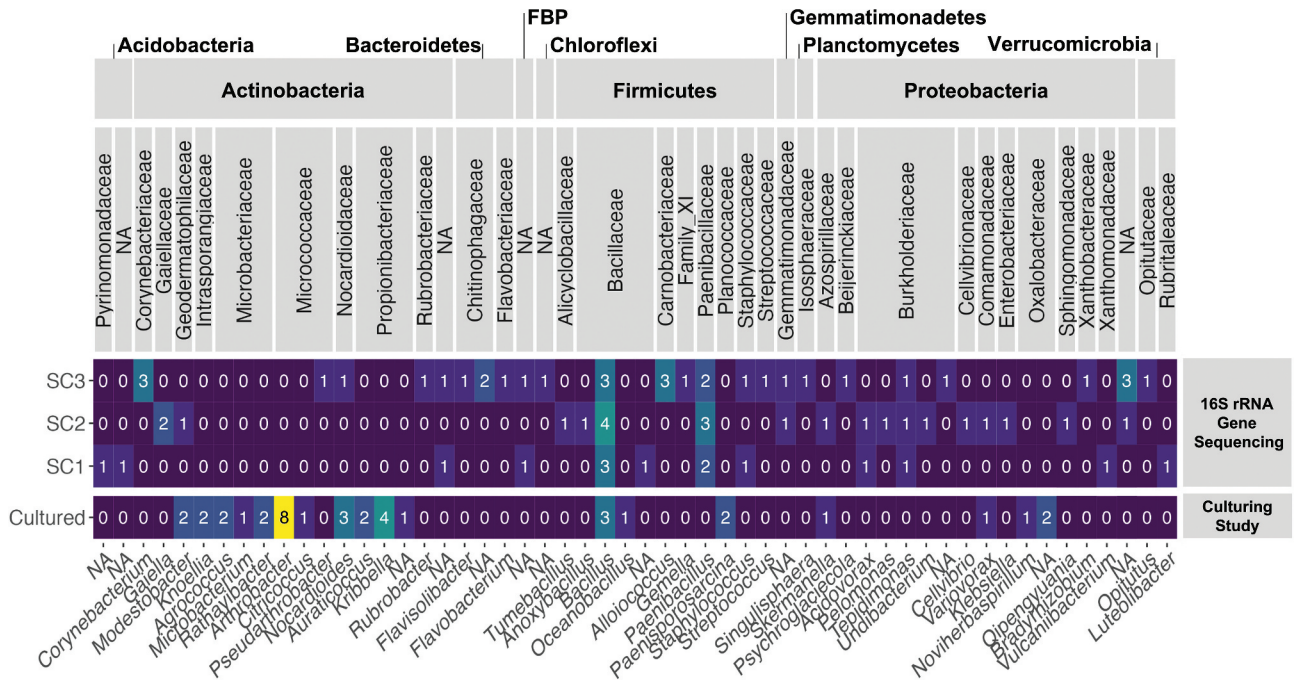
25 μ L reaction volumes. PCR amplification of the ITS region of the rRNA operon was performed using GoTaq Hot Start PCR Master Mix in 25, while PCR amplification of the 16S rRNA gene was performed using Platinum II Hot-Start PCR Master Mix (2X). Cycling parameters for the ITS1F/ITS4R primer sets consisted of an initial denaturation step at 94°C for 3 minutes, followed by thirty-five cycles of denaturation at 94°C (45 seconds), annealing at 50°C (60 seconds), extension at 70°C (90 seconds), and a final extension step at 72°C for 10 minutes. Cycling parameters for the S_27 F/S_1429_R primer set consisted of an initial denaturation step at 94°C for 2 minutes, followed by thirty-five cycles of denaturation at 94°C (15 seconds), annealing at 60°C (15 seconds), extension at 68°C (60 seconds), and a final extension step at 72°C for 10 minutes. Amplified PCR product from all of the cultured isolates, the six extraction blanks, and the three no-template PCR controls were sequenced by GENEWIZ Sanger sequencing service. Fungal DNA was sequenced using the ITS1_F primer and the bacterial/archaeal DNA was sequenced using the S_27 F primer to capture the full-length sequence of the ITS rRNA operon and 16S rRNA gene, respectively.

Sanger sequence data from the isolates were processed following the “swabs to genomes” workflow described in Dunitz et al. (2015). No sequences from any of the six extraction blanks or three no-template controls were recovered. Briefly, sequences were first filtered based on their length, with only sequences >700 bp kept for identifications based on the 16S rRNA gene and only those >200 bp kept for identifications based on the ITS region. These remaining sequences were cleaned with SeqTrace v0.9.1 (Stucky 2012), after which thirty-eight 16S rRNA sequences and six ITS sequences remained. Taxonomy was assigned to all sequences using the RDP classifier v2.10.2 (Wang et al. 2007) and the basic local alignment search tool (Altschul et al. 1990). Recovered sequences for all fungal and bacterial isolates, as well as their likely taxonomic classification, are reported in Dataset S3.

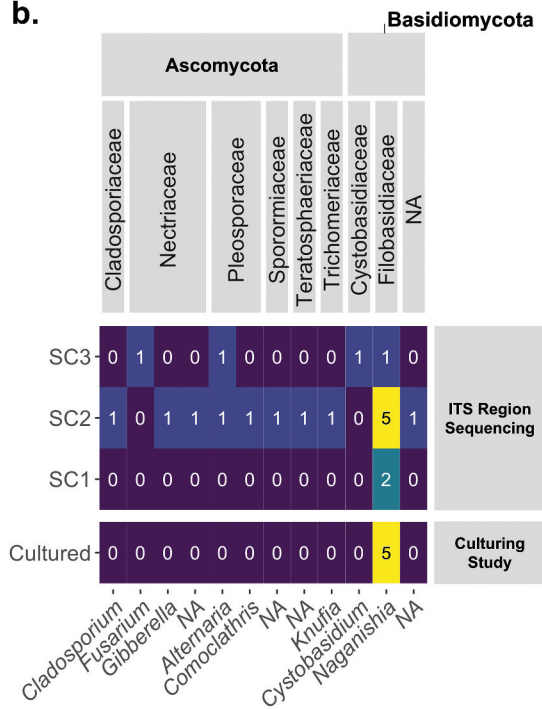
Geodermatophilaceae phylogenetic tree creation

The phylogenetic relationship of our two *Modestobacter* isolates to previously described *Modestobacter* taxa was determined from 850 bp sequences of the 16S rRNA gene via maximum likelihood with RaxML (Stamatakis 2014). Sequences of 850 bp in length were extracted from partial or complete sequences accessed through the National Center for Biotechnology Information Nucleotide Database, including eight species of *Modestobacter* and ten species of *Geodermatophilus*

a.



b.



c.

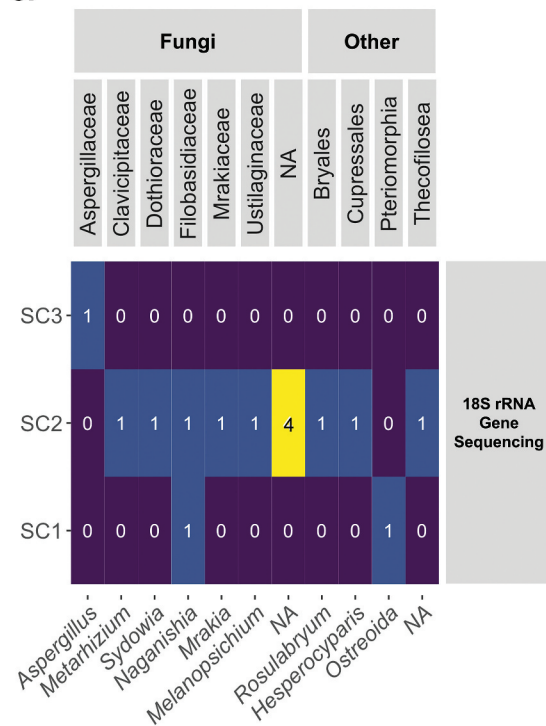


Figure 2. The microorganisms identified in the three South Col samples. (A) The bacterial genera identified through the 16S rRNA gene sequencing and the culturing study. (B) The fungal families identified through the ITS sequencing and the culturing study. (C) The identity of the microeukaryotic ASVs identified through the 18S rRNA gene sequencing. For all heat maps, the number of ASVs (or cultures) assigned with each taxonomic identification is displayed in the corresponding box and colored based on the magnitude. "NA" indicates that a taxonomic classification at that level is not available.

that have been isolated from soils and sediments in cold, dry environments around the world, including the *G. obscurus* that was previously isolated from a high-elevation surfaces on Mount Everest by Ishiguro and Fletcher (1975). Other sequences included *M. lapidis* (Trujillo et al. 2015), *M. multiseptatus* (Mevs et al. 2000), *M. muralis* (Trujillo et al. 2015), *M. marinus* (Xiao et al. 2011), *M. roseus* (Qin et al. 2013), *M. italicus* (Montero-Calasanz et al. 2019), *M. altitudinis* (Golinska et al. 2020), *M. excelsi* (Golinska et al. 2020), *G. poikilotrophi* (Montero-Calasanz et al. 2014), *G. africanus* (Montero-Calasanz, Göker, Pötter et al. 2013a), *G. siccatatus* (Montero-Calasanz, Göker, Pötter et al. 2013b), *G. ruber* (Y. Zhang et al. 2011), *G. pulveris* (Hezbri et al. 2016), *G. arenaris* (Montero-Calasanz et al. 2012), *G. nigrescens* (Nie et al. 2012), and *G. dictyosporus* (Montero-Calasanz et al. 2015), *G. saharensis* (Montero-Calasanz, Göker, Rohde et al. 2013). First, sequences were aligned using MUSCLE v3.8.31 (Edgar 2004). Aligned reads were used to construct a tree with RaxML v8.0.0 (raxmlHPC -f a -m GTRGAMMA -p 12345 -x 12345 -number 100), including *Blastococcus deserti* (NR_169414.1) as an outgroup for tree rooting (Stamatakis 2014). The tree was visualized and annotated using iTOL v6.3.2 (Letunic and Bork 2016).

Geochemical analysis

Sediment pH was determined according to the method described in King et al. (2010). Specifically, 5 g of soil and 5 g of deionized water were placed in a 15-mL conical tube and shaken for 2 hours at 200 rpm. pH was then measured with an Orion Star A211 Benchtop pH meter. Total water content of samples was measured following a method described in Vimercati, Darcy, and Schmidt (2019). In summary, 5 g of sediment of each sample were placed in sterile glass tubes. The tubes were left open to dry at 60°C in an oven for 48 hours. Water content was measured as the percentage difference between the wet and dry sample.

Other geochemical measurements were performed on freeze-dried and crushed aliquots of the three samples. Total nitrogen (TN) content measurements were measured by the Arikaree Laboratory at the University of Colorado Boulder using a Shimadzu TOC-L/TNM-L TOC/TN analyzer. Total organic carbon (TOC) content was measured by Activation Laboratories Ltd.. Litho geochemistry analysis was also performed by Activation Laboratories Ltd. Mineral composition of the samples was performed via lithium borate fusion/inductively couple plasma-optical emission spectroscopy and the composition of trace elements was measured via lithium borate fusion/inductively coupled plasma-mass spectrometry.

Results

Environmental properties of South Col sediment

The properties of these sediments create a unique lithic environment for microbial communities, most notably defined by its extremely high elevation (>7,900 m). Unsurprising, given the high elevation, the sediments were dry (water content <1.0 percent for all samples, mean = 0.31 percent water content) and contained low measured concentrations of organic carbon (average = 0.06 percent g/g) and no detectable nitrogen. Sediment pH was an average of 9.4 across the three samples, suggesting a basic environment. Though DNA extraction and culturing were only performed on the smallest sediment fragments (<1 mm diameter), all three samples were made up of fragments of variable grain size, with the largest sediment fragments ~2-5 cm in diameter (Figure 1) showing the rocky nature of the substrate.

Additional analysis of the three samples that were collected from the South Col show that the mineralogical composition (weight percentage) was on average 59.1 percent SiO₂, 15.7 percent Al₂O₃, 6.4 percent Fe₂O₃, 5.2 percent CaO, 2.6 percent MgO, 3.5 percent K₂O, and 2.1 percent Na₂O, with P₂O₅, TiO₂, and MnO each making up <1 percent (Table 1). These “Everest series greenschists,” which were described by Searle (1999) in more detail, are a type of metamorphic rock formed under the lowest temperatures and pressures experienced by the Himalayan region. These rocks also contained several trace elements, the most abundant of which included barium (average = 596 ppm), strontium (average = 393 ppm), zirconium (average = 221 ppm), rubidium (average = 181 ppm), zinc (average = 100 ppm), cerium (average = 98 ppm), chromium (average = 87 ppm), vanadium (average = 80 ppm), and lanthanum (average = 51 ppm). For more details on mineralogical and elemental composition of samples see Dataset S1.

Cultivation-independent marker gene sequencing

All three samples yielded enough PCR-amplifiable DNA to characterize the bacterial and eukaryotic taxa using marker gene sequencing (see Dataset S2). 16S rRNA

Table 1. Mineralogical composition of the three South Col sediments.

Mineral	Composition (weight percentage ± 1 SD)
SiO ₂	59.15 ± 0.78
Al ₂ O ₃	15.72 ± 0.27
Fe ₂ O ₃	6.35 ± 0.18
CaO	5.17 ± 0.92
K ₂ O	3.48 ± 0.07
MgO	2.56 ± 0.09
Na ₂ O	2.10 ± 0.07
TiO ₂	0.76 ± 0.01
P ₂ O ₅	0.13 ± 0.01
MnO	0.08 ± 0.003

gene reads averaged 4,699 reads/sample (3,101–6,708), ITS region reads averaged 9,556 reads/sample (661–25,284 reads), and 18S rRNA gene reads averaged 14,450 reads/sample (480–28,173 reads). As expected, the number of taxa identified per sample (microbial richness) was low, with average richness of twenty-three 16S ASVs (fifteen to thirty-three), six ITS ASVs (two to fourteen), and five 18S ASVs (one to twelve). No archaeal sequences were identified using these methods.

The thirty-six bacterial ASVs identified by cultivation-independent marker gene sequencing included taxa of the phyla Firmicutes (sixteen ASVs), Proteobacteria (sixteen ASVs), Actinobacteria (ten ASVs), Bacteroidetes (four ASV), Chloroflexi (two ASVs), Acidobacteria (two ASVs), Verrucomicrobia (two ASVs), Planctomycetes (one ASV), Abditibacteriota (one ASV), and Gemmatimonadota (one ASV). In total, thirty-one families were represented, the most common of which were Bacillaceae (six ASVs), Burkholderiaceae (six ASVs), Carnobacteriaceae (three ASVs), Corynebacteriaceae (three ASVs), Chitinophagaceae (one ASV), and Paenibacillaceae (three ASVs). All other families were represented by only one ASV (Figure 2A).

All sixteen of the fungal ASVs identified through the next-generation marker gene sequencing effort were of either the phyla Ascomycota (nine ASVs) or Basidiomycota (seven ASVs). The most common fungal family was Filobasidiaceae (six ASVs), all of which were identified to the genus *Naganishia*. The seven other fungal families included Nectriaceae (three ASVs), Pleosporaceae (two ASVs), Teratosphaeriaceae (one ASV), Sporormiaceae (one ASV), Cladosporiaceae (one ASV), Trichomeriaceae (one ASV), and one Basidiomycota ASV not identified to a fungal family (Figure 2B).

The 18S rRNA gene sequencing effort identified fourteen ASVs. Most of these sequences (ten ASVs) were fungal (Figure 2C) and 77 percent of all reads recovered from the three samples were one ASV that had 100 percent identity over the very short read of 129 bp with members of the genera *Piskurozyma* and *Naganishia*. Nonfungal ASVs were identified as being of the genera *Rosulabryum* (one ASV) and *Crassostrea* (one ASV), the order Ostreoida (one ASV), and an unidentified ASV from the phylum Cercozoa (one ASV; Figure 2C).

Culture dependent methods

Growth of at least one colony occurred on all plates that were inoculated with samples. No growth occurred on any of the fourteen control plates. One hundred eighty-six bacterial colonies and twenty-nine fungal colonies

were streaked for isolation after the four-week incubation. Sanger sequencing revealed that a total of thirty-eight unique bacterial isolates and six fungal isolates were grown from the three sediment samples (Dataset S3).

All bacterial cultures grown were identified as being of the phyla Actinobacteria (twenty-seven isolates), Firmicutes (six isolates), or Proteobacteria (five isolates). Eleven different families were identified, including Micrococcaceae (eight isolates), Propionibacteriaceae (seven), Microbacteriaceae (five), Bacillaceae (four), Oxalobacteraceae (three), Nocardiodaceae (three), Planococcaceae (two), Intrasporangiaceae (two), Geodermatophilaceae (two), Azospirillaceae (one), and Comamonadaceae (one; Figure 2A).

All of the fungal isolates were identified as being of the cold-adapted yeast genus *Naganishia* (Figure 2B), including three isolates most closely related to *N. vishniacii* and two other isolates most closely related to *N. albidosimilis* and *N. adeliensis*.

Discussion

The environmental properties of the South Col sediments we analyzed in this study are not unique to Everest. Many high alpine environments experience limited water and nutrients and high pH, among other elevation-influenced properties (Merino et al. 2019), and still have diverse microbial communities (D. Singh et al. 2012; Yuan et al. 2014; Vimercati, Darcy, and Schmidt 2019). Our three samples, however, are notable for the extremely high elevation from which they were collected (>7,900 m). There are only fifteen mountains in the world, including Mt. Everest, with peaks higher in elevation than the South Col. To our knowledge, these samples represent the highest elevation sediment environment to be explored for microorganisms using cultivation-independent next-generation sequencing methods. Organisms found at such high elevations, if any, must be able to cope with extremely high insolation (Matthews et al. 2020), cold temperatures, low atmospheric pressures, and low oxygen availability, among other corresponding environmental conditions (Merino et al. 2019).

Though the three samples South Col sediment samples were collected using aseptic techniques and sterile sampling material and remained sealed from the moment of collection until they were processed, the samples did not remain frozen through the entire shipping process. For this reason, our analyses focused on the diversity of organisms identified in the samples and not on their abundance.

Taxonomic diversity of bacteria

Both the Illumina marker gene sequencing and culture-based identifications yielded similar microbial diversity in the South Col sediments, though the next-generation sequencing approach identified a greater diversity of microorganisms than the culturing study (ten phyla versus three phyla; Figure 2). Both techniques revealed that the most common bacterial phyla were Firmicutes, Proteobacteria, and Actinobacteria. At finer taxonomic levels, Illumina sequencing identified a greater diversity of Firmicutes, whereas the culturing methods identified a greater diversity of Actinobacteria, though both methods identified many of the same taxa (e.g., Geodermatophilaceae, Nocardioidaceae). These differences are not surprising, because culturing methods are inherently selective. Many of the media we used for our study were designed for culturing of organisms in cold soil environments, where Actinobacteria often are the dominant taxa (Babalola et al. 2009; D. J. Smith et al. 2012; Dragone et al. 2021).

Both methods captured a multitude of taxa with closely related sequences that have been retrieved from other cold, dry, lithic environments. The dominant phyla identified in our South Col sediments were Actinobacteria, Firmicutes, and Proteobacteria, taxonomic groups that often dominate microbial communities in Antarctic soils (Cowan et al. 2014). We see few Acidobacteria and Bacteroidetes, phyla that are common to Arctic tundra (Nemergut et al. 2005). At a finer taxonomic resolution, the families Nocardioidaceae, Planococcaceae, Oxalobacteraceae, Geodermatophilaceae, and Chitinophagaceae have closely related sequences that have previously been retrieved from the Transantarctic Mountains and the McMurdo Dry Valleys (J. J. Smith et al. 2006; Dragone et al. 2021, 2022), as well as from cryoconite holes on Antarctic glaciers (Sommers et al. 2018). We also see taxa that have been identified in other high alpine sediment environments around the world, including near the top of Kilimanjaro (Vimercati, Darcy, and Schmidt 2019) and the Puna de Atacama Volcanic Zone (Solon et al. 2018). Other genera, like *Auraticoccus* and *Kribbella*, have been found on rocks (Cheema et al. 2020), in deserts (Saygin et al. 2019), and on the walls of stone monuments and catacombs (Alonso-Vega et al. 2011).

Of special note is our identification of members of the family Geodermatophilaceae, including *Geodermatophilus obscurus*, which was originally isolated from a sediment sample taken at 8,400 m.a.s.l. by the American Everest expedition of 1963 and later described by Ishiguro and Fletcher (1975). Though *G. obscurus* was not identified in our samples, we did identify taxa of the related genus *Modestobacter* (Figure 3), with one cultured isolate

identified as being most closely related to *M. roseus* (Qin et al. 2013). Organisms of the genus *Modestobacter* are often identified in other cold, dry soils and sediments (Mevs et al. 2000; Busarakam et al. 2016; Golinska et al. 2020) and, like the closely related genus *Geodermatophilus* (Montero-Calasanz et al. 2017), have recently been found to have many psychrophilic and oligotrophic adaptations (Golinska et al. 2020; Tarlachkov et al. 2020). Genomic analysis of *Modestobacter* taxa (see Golinska et al. [2020] for detailed summary) has found genes related to cold-shock regulation (e.g., Csp protein family; Essoussi et al. 2010), pathways related to desiccation tolerance via the uptake of trehalose (Reina-Bueno et al. 2012), and multiple copies of the *uvrD* genes that have previously been linked to increased ultraviolet (UV) tolerance (Normand et al. 2012). Of additional note is the presence of genes that allow for the use of carbon monoxide and other trace gases as a source of carbon and energy (e.g., *coxD*, *coxE*, *coxG*; Lorite et al. 2000). These potential functional attributes suggest adaptations that might allow for survival in environments like the South Col.

Along with Geodermatophilaceae and the other cold-associated organisms described previously, the diversity of bacteria we identified in the South Col samples included more cosmopolitan species that are not characteristic of high alpine or cold soil/sediment environments. Many taxa, including those of the families Paenibacillaceae and Bacillaceae, are endospore-forming species (Montes et al. 2004; Mandic-Mulec, Stefanic, and van Elsas 2015). *Bradyrhizobium* is a nitrogen-fixing genus that is most often found in symbiotic association with legumes, though it is also a common soil taxon (Klepa et al. 2021). *Alloiococcus*, *Staphylococcus*, and *Streptococcus* are genera that include human pathogens (Aguirre and Collins 1992; Becher et al. 2009; Palmieri, Varaldo, and Facinelli 2011), though the latter two often are often found in certain soil environments (Delgado-Baquerizo et al. 2018).

Taxonomic diversity of eukaryotes

Both the Illumina marker gene sequencing of the ITS region and culture-based identifications yielded similar microbial diversity in the South Col and showed that most of the diversity of these samples was made up of the fungal family Filobasidiaceae. More specifically, the majority of the fungal ASVs identified in the marker gene sequencing and all of the fungi that grew in culture were of the genus *Naganishia*. This is not surprising, because *Naganishia* is a genus of cold-adapted polyextremophile yeasts that are commonly found in cold and dry ecosystems, including in Antarctica (Duarte

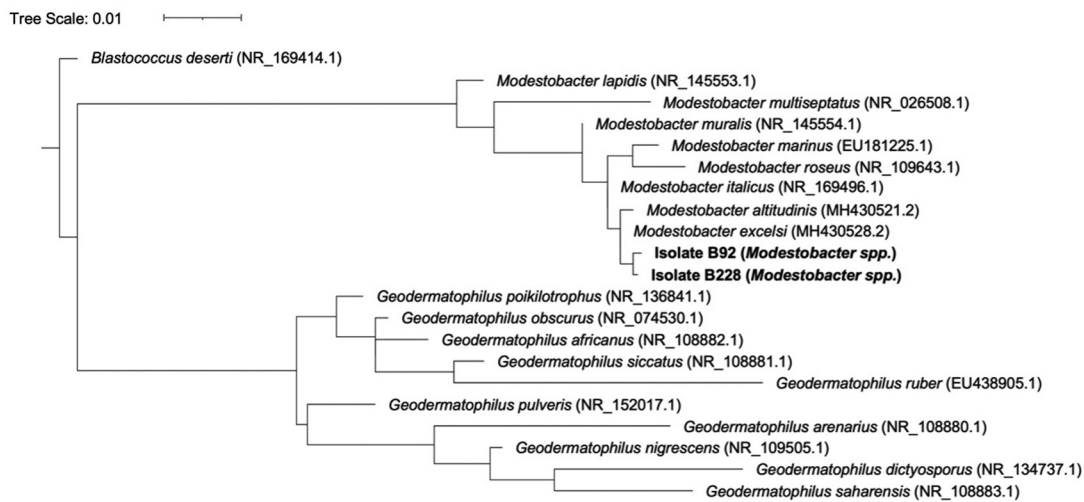


Figure 3. *Modestobacter* and *Geodermatophilus* phylogenetic tree. Phylogenetic relationships displayed are based on partial 16S rRNA gene sequences of at least 800 bp in length. The twenty sequences represented in this tree include two isolates recovered during the culturing study, eight representatives of the genus *Modestobacter*, and ten representatives of the genus *Geodermatophilus*, including *G. obscurus*, the organism isolated from a sediment sample taken at 8,400 m.a.s.l. by the American Everest expedition of 1963. The two cultured Geodermatophilaceae isolates were most closely related to *M. excelsi* and *M. altitudinis*, taxa most often associated with cold, dry, high alpine environments like soils of the Atacama Desert and Antarctica. The tree is rooted by *Blastococcus deserti*, a representative of another Geodermatophilaceae family.

et al. 2018; Nizovoy et al. 2021), on Kilimanjaro (Vimercati, Darcy, and Schmidt 2019), and in high-elevation sediments of the hyperarid Puna de Atacama (Pulschen et al. 2015; S. K. Schmidt et al. 2018). In these other environments, *Naganishia* is thought to survive by periodically coming out of dormancy during brief periods of favorable conditions (S. K. Schmidt et al. 2017). This genus of cold-adapted poly-extremophilic yeasts is commonly found in a variety of extreme cold, dry environments and is adapted to be resistant to high doses of UV radiation, can grow at temperatures below 0°C, and can survive and even grow during extreme daily freeze-thaw cycles (Vimercati et al. 2016; S. K. Schmidt et al. 2017).

The non-*Naganishia* fungal taxa identified in the samples are mostly cosmopolitan, many of which are commonly found in association with plants (*Gibberella*, *Alternaria*, *Cladosporium*; Zanne et al. 2020). One taxon, *Knufia*, belongs to a genus that includes extremotolerant fungi often found on rocks (Breitenbach et al. 2018).

The 18S rRNA gene sequencing results generally supported the patterns described from the ITS data. Though many of the other ASVs were not classified past the phylum level, this sequencing effort revealed a similar mix of cosmopolitan taxa and extremotolerant taxa like that revealed by the ITS region sequencing. The genus *Sporisorium*, for example, includes plant pathogens and mutualists (Ghareeb et al. 2011) and the genus *Mrakia* includes cryotolerant and xerophilic species (Thomas-Hall et al. 2010; Turchetti et al. 2011). Most

notable, 77 percent of all 18S rRNA gene reads were one ASV that was a 100 percent match to several different yeasts in the Filobasidiales, including numerous *Naganishia* like *N. vishniacii* and the genus *Piskurozyma*, a fungus closely related to *Naganishia*. Given the very short reads from the Illumina sequencing run (129 bp), we cannot definitively assign a specific taxonomy to this ASV at this time, but given results from the ITS and long-read 18S data from cultures, it is very likely that this ASV is a member of the genus *Naganishia*.

Implications for microbial survival in extremely high-altitude environments

Our results add to previous work describing the microbial communities on Mount Everest and in the Himalayas (Freeman et al. 2009; L. Zhang et al. 2009; S. K. Schmidt et al. 2011). Previous studies have found that Himalayan microbial communities are dominated by psychrophilic fungi at elevations from 3,000 to 5,300 m (Petrovič, Gunde-Cimerman, and Zalar 2000; Margesin and Miteva 2011). At higher elevations between 4,000 m and 6,500 m, communities of ammonia oxidizers were dominated by archaeal taxa below 5,400 m and bacteria taxa above (L. Zhang et al. 2009), whereas even algae and chytrids were found in soils wet by snowmelt above 5,500 m (Freeman et al. 2009; S. K. Schmidt et al. 2011). Across all studies, microbial diversity decreased with increasing elevation. Our three

sediments from the South Col contained extremely low microbial diversity, much more like the microbial diversity found in the dry tephra “soils” on the volcanic peaks of the inner Atacama Desert (Costello et al. 2009; S. K. Schmidt et al. 2018; Solon et al. 2018) than the diversity found at lower elevations on the mountain. This is not surprising given that microorganisms at such an extreme elevation would have to be able to cope with correspondingly high UV radiation, extreme cold temperatures, low atmospheric pressures, and lower oxygen availability, among other corresponding environmental conditions (Merino et al. 2019).

We expect that most of the organisms we identified may have been seeded by eolian transport from other less extreme terrestrial surfaces. Wind-driven transport of microorganisms and DNA is well described in a variety of environments (Aalismail et al. 2019; Souza et al. 2019) and has been shown to lead to the detection of organisms in environments where they cannot grow (Archer et al. 2019; Maki et al. 2019). This includes on Mount Everest, where Liu et al. (2007) found many cosmopolitan bacterial taxa in snow at 8,000 m. Our identification of cosmopolitan taxa and foreign plant and animal DNA (e.g., *Rosulabryum* and *Crassostrea* DNA; Figure 2C) in these samples supports this theory. In fact, the most dominant prokaryotic phyla (Proteobacteria, Firmicutes, and Actinobacteria) and fungal phyla (Ascomycota) identified in our samples are the same phyla that typically dominate microbial sequences recovered by bioaerosol studies (Aalismail et al. 2019; Archer et al. 2019; Souza et al. 2019; Ruiz-Gil et al. 2020) and include some of the taxa Liu et al. (2007) suggested could not be active at such high elevations on Everest. Even the most highly cold-adapted genera (e.g., *Modestobacter*, *Naganishia*, or Ishiguro’s *Geodermatophilus* as described in Ishiguro and Fletcher [1975] and Ishiguro and Wolfe [1970]) are likely to have been seeded from lower elevation source populations. Representatives of these genera have been detected, and even isolated, from air samples collected around the world (J. J. Smith et al. 2006; Amato et al. 2007; S. K. Schmidt et al. 2017).

The prospect should also be raised that all, or most, organisms found at the South Col in this study rarely, if ever, grow there. The organisms we isolated and grew likely only came out of dormancy due to the less extreme, consistent conditions of the laboratory setting. Though the intense insolation does indicate that melt occurs at the South Col, consistent with reports from climbers, it remains unclear whether this free water would be enough to support brief periods of microbial growth, given that air temperatures rarely rise above -10°C at such high elevations on Mount Everest (Matthews et al. 2020; Potocki et al.

2022). However, previous work at high elevations in the Atacama has shown that even when air temperatures are below -10°C (at 2 m height), surface soil temperatures can reach $+5^{\circ}\text{C}$ due to the intense insolation (D. Schmidt 1999; Schubert 2014). Organisms like *Naganishia* sp. may be able to take advantage of even these short periods of water availability because they can grow during repeated freeze–thaw cycles and at constant temperatures as low as -6°C (Vimercati et al. 2016). Obviously, it would be hard to test this idea in situ, but more work on the unique microbes isolated in the present study could illuminate the potential for organisms to function during brief thaw periods at extreme elevations. It is also very likely that the South Col and other extremely high-elevation environments may just be deep-freeze collection points for deposited organisms (either from the atmosphere or by transiting humans). Given the challenge, and danger, of performing in situ tests at such extreme high-elevation sites, we cannot say whether the brief periods when free water would be available are enough to support microbial growth. Perhaps extended monitoring by the weather station installed at this site where these samples were collected will shed some light on this mystery in the future (Matthews et al. 2020; Potocki et al. 2022).

Though our culturing study could not re-create the extreme conditions at the South Col, our results show that many of the organisms, including the most ubiquitous fungal and bacterial taxa identified, are in fact viable and could be grown at 4°C . Though the average air temperature from 1991 to 2022 at the South Col was -22.6°C , well below our culturing conditions, air temperatures in the Mount Everest region have been increasing at a rate of $\sim 0.33^{\circ}\text{C}$ per decade (Kang et al. 2022), and a record high of -1.4°C was measured at the South Col in July 2022 (National Geographic Society 2022). Organisms that are not active in situ now may become active in the future if the current rate of warming continues (described in Kang et al. 2022).

Evidence of human-associated contamination of Mount Everest

The microorganisms identified in these South Col sediments included taxa that are often associated with human contamination. Many of the sequences recovered could be deposited from mountaineers through coughing, nasal discharge, and associated sputum (Grice and Segre 2011; Wade 2013; Ibrionke et al. 2020). *Staphylococcus*, for example, is one of the most common skin and nose bacteria, and *Streptococcus* is the dominant genus in the human mouth (Otto 2010; Gevers et al. 2012; Ursell et al. 2013; Escapa et al. 2018). Though these genera are widespread in nature and are not always associated with humans (e.g., Ko

et al. 2011; Zhao et al. 2015), the sequences that were identified as being most closely related to our taxa based on analysis using blastn are human associated. For example, the *Staphylococcus* sequence identified in our samples shares a 100 percent identity with the skin colonizers *S. hominis* and *S. epidermis* (Nagase et al. 2002; Otto 2009), and the *Streptococcus* sequence shares a 100 percent identity with oral cavity colonizer *S. oralis* (Kennedy et al. 2000). Because none of these human-associated taxa were identified in our extraction blanks and no-template PCR controls and none grew on our control plates in our culturing study, it remains likely that these organisms were from the collected sediments.

Mount Everest is heavily trafficked by climbers and guides each year, prompting concerns that humans have irrevocably changed the high alpine environment. Our study provides evidence that climbing activity may increase the transport of human-associated microorganisms onto the mountain's highest surfaces. However, we expect that at such high elevations these microorganisms are not active. We predict that if we sampled in the more human-utilized areas on the mountain we may find even more microbial evidence of human impact on the environment, as has been well described at lower elevations in the Himalayas (Rashid and Romshoo 2013; Ahmad et al. 2021; Rather et al. 2022) and around the world (Sinton, Finlay, and Hannah 1998; Joergensen and Emmerling 2006; Harwood et al. 2014).

Conclusions

The South Col of Everest represents a unique surface environment, most notably for being >7,900 m. Despite the extreme elevation and corresponding high UV radiation, cold temperatures, low atmospheric pressures, lower oxygen availability, we have identified bacterial and fungal DNA sequences and have grown bacterial and fungal cultures from sediments collected from the South Col. The mix of cold-adapted and cosmopolitan species identified using our methods suggests that most of these organisms are inactive in situ and may have been seeded by eolian transport from other less extreme terrestrial surfaces. Others may have been transported to the South Col by the activity of climbers. Indeed, our data suggest that the South Col and other extremely high-elevation environments may be deep-freeze collection points for deposited organisms, including human-borne contaminants that may never leave once they arrive.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data and materials availability

All data used in this study are available in the main text or the supplementary material with the raw sequence data available in the National Center for Biotechnology Information (NCBI) Sequence Read Database, project accession number PRJNA882470, BioSample accessions SAMN30937384, SAMN30937385, SAMN30937386.

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