Bacterial Microbiota Associated with the Glacier Ice Worm Is Dominated by Both Worm-Specific and Glacier-Derived Facultative Lineages

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The community structure of bacteria associated with the glacier ice worm Mesenchytraeus solifugus was analyzed by amplicon sequencing of 16S rRNA genes and their transcripts. Ice worms were collected from two distinct glaciers in Alaska, Harding Icefield and Byron Glacier, and glacier surfaces were also sampled for comparison. Marked differences were observed in bacterial community structures between the ice worm and glacier surface samples. Several bacterial phylotypes were detected almost exclusively in the ice worms, and these bacteria were phylogenetically affiliated with either animal-associated lineages or, interestingly, clones mostly consisting of glacier-indigenous species. The former included bacteria that belong to Mollicutes, Chlamydiaceae, Rickettsiales, and Lachnospiraceae, while the latter included Arcicella and Herminiimonas phylotypes. Among these bacteria enriched in ice worm samples, Mollicutes, Arcicella, and Herminiimonas phylotypes were abundantly and consistently detected in the ice worm samples; these phylotypes constituted the core microbiota associated with the ice worm. A fluorescence in situ hybridization analysis showed that Arcicella cells specifically colonized the epidermis of the ice worms. Other bacterial phylotypes detected in the ice worm samples were also abundantly recovered from the respective habitat glaciers; these bacteria may be food for ice worms to digest or temporary residents. Nevertheless, some were overrepresented in the ice worm RNA samples; they may also function as facultative gut bacteria. Our results indicate that the community structure of bacteria associated with ice worms is distinct from that in the associated glacier and includes worm-specific and facultative, glacier-indigenous lineages.

Key words: enchytraeid, glacier ecosystem, gut bacteria, symbiosis, psychrophilic

Glaciers, which are harsh environments that are permanently covered with ice and snow, have unique ecosystems composed of psychrophilic/psychrotolerant organisms (3, 33). Recent studies revealed that microorganisms thriving on glacial surfaces have a marked impact on global biogeochemical cycles and surface ice melting (2, 34, 37, 40). Although the importance of glacier ecosystems has been recognized and many glaciers have been examined, most studies have focused on microorganisms on the glacier surface, and knowledge on glacier invertebrates is limited.

The glacier ice worm, Mesenchytraeus solifugus (phylum Annelida; family Enchytraeidae), is the largest metazoan and regarded as the predominant consumer in North American maritime glaciers (17, 35). Ice worms are nocturnal in summer seasons; they reside deep beneath the glacier surface in the daytime to avoid sunlight, and emerge at dusk on the surface to forage food, which mainly consists of unicellular green algae (17, 35). Their behaviors in winter seasons are unknown. Their physiology and phylogeny have also been investigated (9, 14, 18, 38); however, limited information is available on their ecological roles in glacial environments. In our previous study, the bacterial community structures physically associated with the ice worm were analyzed by cloning bacterial 16S rRNA genes (27). The findings obtained revealed that the ice worm harbors glacier-derived and animal gut-specific bacterial lineages, and that the novel Mollicutes bacterium, “Candidatus Vermiplasma glacialis,” colonizes the gut wall of ice worms as a dominant bacterial member. However, the number of analyzed specimens and sequences were limited; therefore, more comprehensive surveys are needed in order to obtain a deeper understanding of the community structure of bacteria associated with ice worms.

In the present study, we aimed to clarify the taxonomic composition of the bacterial microbiota associated with the ice worm in more detail by deep sequencing 16S rRNA gene amplicons. In order to identify the core symbiotic microbiota, if one exists, ice worms were collected from two distinct glaciers in Alaska, Harding Icefield and Byron Glacier, and their associated bacterial communities were compared. The 16S rRNA gene and its transcripts were simultaneously analyzed to assess the activity of each bacterial species. Furthermore, bacterial community structures from the glacier surfaces were examined, and the specificity of the bacterial species associated with ice worms was evaluated. Our results provide an insight into the unique niche occupied by ice worms with their associated bacterial consortium in glacial ecosystems.

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Materials and Methods

Sample collection

Sampling expeditions were carried out in the upper area of Exit Glacier located in Harding Icefield (August 2014) and in Byron Glacier (August 2015). Both glaciers are located in the Kenai Peninsula, Alaska, and are approximately 90 km apart. In Harding Icefield, sampling was conducted at two sites; an ice surface site (60°09.622'N, 149°47.482'W) and snowpack site (60°09.167'N, 149°50.89'W) (Table S1). In Byron Glacier, samples were collected from the ice surfaces of an avalanche cone (60°45.672'N, 148°50.89'W) below the terminus of the glacier. Ice worms and glacier surface samples were collected with sterile stainless steel scoops. All ice worm samples were collected at dusk when ice worms started to appear on the surface of the glaciers. Specimens were preserved on site in RNAlater® solution (Ambion, Austin, TX, USA). Some collected ice worms were kept alive in melted glacier surface ice. All fixed samples were kept frozen during transport to a laboratory in Japan. Samples preserved in RNAlater® were stored at –80°C, and live ice worms were reared at 4°C.

Nucleic acid extraction

All ice worm and glacier ice specimens in RNAlater® solution were slowly thawed on ice. Five ice worm individuals were arbitrarily selected from the specimens and placed into one tube. DNA and RNA were both simultaneously extracted using the PowerViral® Environmental RNA/DNA Isolation Kit (MO BIO Laboratory, Carlsbad, CA, USA), according to the manufacturer’s protocol with modifications. Briefly, ice worms were suspended in 600 μL of solution PV1 (lysis buffer), in which 24 μL of 1 M dithiothreitol (DTT) was added instead of 6 μL β-mercaptoethanol. The suspension was homogenized with a sterile plastic pestle and subjected to a bead-beating procedure. Two-milliliter aliquots from glacier ice samples were centrifuged at 10,000×g at 4°C for 20 min. The pellets were suspended in solution PV1 with DTT, as described above, and processed according to the manufacturer’s protocol. Residual RNA or DNA was digested using RNaseA (Wako, Osaka, Japan) or the TURBO DNA-freeTM Kit (Ambion), respectively. DNA extracts were further purified with the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) to remove RNaseA. The amplification of 16S rRNA genes was not observed from purified RNA samples in 30-cycle PCR with a Bacteria-specific primer set, performed as described previously (27).

PCR, RT-PCR, and amplicon sequencing

RNA samples were reverse-transcribed into cDNA using the SuperScript® III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) with random hexamers. PCR was conducted using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) with the Bacteria-specific 341F and 785R primer set, which targets the V3–V4 region (approx. 400 bp) of 16S rRNA. Primer sequences contained the Illumina overhang adapter sequence (http://support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). The PCR program was as follows: initial denaturation at 98°C for 30 s, 25 cycles of denaturation at 98°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 45 s, and a final extension at 72°C for 7 min. Nextera® XT indices (Illumina, San Diego, CA, USA) were attached to the amplicons by modifications. Briefly, ice worms were suspended in 600 μL of solution PV1 with DTT, as described above, and processed according to the manufacturer’s protocol. Residual RNA or DNA was digested using RNaseA (Wako, Osaka, Japan) or the TURBO DNA-freeTM Kit (Ambion), respectively. DNA extracts were further purified with the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany) to remove RNaseA. The amplification of 16S rRNA genes was not observed from purified RNA samples in 30-cycle PCR with a Bacteria-specific primer set, performed as described previously (27).

The similarity of bacterial community structures was evaluated with QIIME using nonmetric multidimensional scaling (NMDS) based on the Bray–Curtis similarity index. Differences among clusters were statistically tested by an analysis of similarity (ANOSIM) with 999 permutations. The separation of clusters in the NMDS plot was correlated to OTU abundance by calculating Spearman’s correlation coefficient with mothur v1.37.0 (29).

In each of the 16S rRNA OTUs recovered from the ice worm samples, differences in abundance between DNA and RNA samples were statistically tested in R v3.2.2 using the DESeq2 package with the negative binomial Wald test (23). If the adjusted P value was less than 0.05, we considered the OTU abundance to be significantly different between DNA and RNA samples.

Phylogenetic analysis

Nearly full-length 16S rRNA gene sequences obtained in our previous study (27) were aligned using ARB (24) with manual corrections. In total, 1,312 (for Arcicella) and 1,218 (for Herminiimonas) unambiguously aligned nucleotide sites, which corresponded to positions 69–1440 and 94–1390 in Escherichia coli (J01695), respectively, were used for a phylogenetic analysis. Maximum likelihood (ML) and neighbor-joining (NJ) trees were constructed in MEGA7 (22) with 500 bootstrap resamplings. The GTR+Γ+I nucleotide substitution model and maximum composite likelihood model were used for ML and NJ tree reconstructions, respectively.

Fluorescence in situ hybridization (FISH)

In order to detect Arcicella cells associated with ice worms, an Arcicella-specific probe, Arci_1251 (5′-GGTTTACCACATAGCGACTGTC-3′), which corresponds to positions 1251–1272 in J01695, was designed using ARB and labeled with Texas Red at the 5′ end. A non-labeled helper probe (5′-GGTTTGTAGATTACCTGC-3′), which corresponds to positions 1251–1272 in J01695, was used for a phylogenetic analysis. Maximum likelihood (ML) and neighbor-joining (NJ) trees were constructed in MEGA7 (22) with 500 bootstrap resamplings. The GTR+Γ+I nucleotide substitution model and maximum composite likelihood model were used for ML and NJ tree reconstructions, respectively.

Nucleotide sequence accession numbers

Datasets of 16S rRNA reads sequenced in this study have been deposited in DDBJ/EMBL/GenBank under the accession number DRA005157.
Results

Taxonomic composition and species richness of bacteria

Three and six sets of the ice worm samples, each set containing five individual worms, from Harding and Byron (designated as Harding worm/Byron worm), respectively, were subjected to 16S RNA amplicon sequencing. In addition, two and three sets of the glacier surface samples from Harding and Byron (designated as Harding surface/Byron surface), respectively, were similarly analyzed. In total, 4,208 OTUs were identified from 1,965,105 paired-end reads. The mean number (with SD) of the observed OTUs in each sample category was as follows; 79±16 (Harding worm DNA), 546±350 (Harding worm RNA), 101±44 (Byron worm DNA), 1,105±583 (Byron worm RNA), 230±160 (Harding surface DNA), 757±297 (Harding surface RNA), 116±30 (Byron surface DNA), and 810±274 (Byron surface RNA). Thus, the observed number of OTUs as well as the Chao1 species richness estimate and reciprocal Simpson’s diversity index were higher in RNA samples than in DNA samples in any of the worm or glacier surface samples (Table S1). The rank abundance curves of the OTUs showed that RNA samples contained much rare OTUs than DNA samples (Fig. S1).

The taxonomic composition of bacteria was basically consistent among the biological replicates for each sample category (Fig. 1). In contrast, marked differences were observed among the different sample categories. For example, Entomoplasmatales and Mycoplasmataceae sequences were abundantly detected in ice worm samples, whereas only a few were recovered from the glacier surface samples. Most of the Mycoplasmataceae OTUs in the ice worm samples were identical or closely related to the “Ca. Vermiplasma” sequences obtained in our previous study (27), and differed from OTU13756, which accounted for 1.6% of sequences in the “Harding surface DNA 2” sample (Fig. 1). The OTU13756 sequence was identical to the sequences of two Mycoplasmataceae bacteria in the NCBI nr/nt database: the hemotropic cattle pathogen “Candidatus Mycoplasma haemobos” (KJ883514) (19) and uncultured bacterium clone GYS3-84 (JX493242) obtained from soil in China (44). Entomoplasmatales OTUs were also identical or closely related to the phylotypes obtained in our previous study (27). Lachnospiraceae OTUs were exclusively recovered from the Harding worm samples. The closest relative of the dominant Lachnospiraceae OTU742 was a sequence obtained from a termite gut sample (AB089001) with 93.3% sequence similarity. Cytophagaceae, predominated by the genus Arcicella (25 out of 85 Cytophagaceae OTUs and 91.8% of the total Cytophagaceae reads), were frequently detected in both worm and glacier surface samples, but were more abundant in the former, particularly in Byron worms (Fig. 1).

Similarity of bacterial community structures

Bacterial community structure similarities are depicted by an NMDS plot in Fig. 2. Clear separation among sample categories, except for between DNA and RNA samples, was observed (ANOSIM: global $R=0.96$, $P<0.001$). The contribution of the 10 most abundant OTUs to the separation of the plots is also shown in Fig. 2. “Ca. Vermiplasma” OTU11203 greatly contributed to separation between the ice worm and

Fig. 1. Taxonomic composition of bacteria based on 16S rRNA sequences. This classification was mainly performed at the family level.
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In order to identify dominant bacterial OTUs that were consisently associated with ice worms, we first listed OTUs for which the frequency in a sample was >0.1%. These OTUs were then sorted into four groups: (i) OTUs dominant in both Harding and Byron worm samples (designated as “core OTUs”); (ii) OTUs dominant in Harding worm samples only; (iii) OTUs dominant in Byron worm samples only; (iv) other dominant OTUs, which did not fit into groups (i) to (iii), but were included in the 10 most abundant OTUs (see the legend to Fig. 2) (Fig. 3).

Among the core OTUs associated with ice worms, OTU23265 (Entomoplasmatales), OTU11203 (“Ca. Vermiplasma”), OTU19485 (Arcicella), and OTU17386 (Herminiimonas) were only rarely found in the glacier surface samples (Fig. 3). Entomoplasmatales OTU23265 and “Ca. Vermiplasma” OTU11203 phylogenetically belonged to animal-associated bacterial lineages (27), whereas Arcicella OTU19585 and Herminiimonas OTU17386 were closely related to sequences derived from components of the cryosphere or other freshwater environments (Fig. S2). The other core OTUs were frequently and abundantly detected in both ice worm and glacier surface samples.

The majority of OTUs that were only dominant in either the Harding or Byron worm samples were also abundantly recovered from their corresponding habitat glacier surface samples, but were less abundantly detected in the other glacier surface samples (Fig. 3). However, several OTUs, such as OTU742 (Lachnospiraceae), OTU9924 (Parachlamydiaceae), OTU3927 (Entomoplasmatales), and OTU18119 (Rickettsiales), were specifically detected in either the Harding or Byron worm samples, but were almost never found in any of the glacier surface samples. Parachlamydiaceae OTU9924 showed the highest sequence similarity (92.5%) to “Candidatus Metachlamydia lacustris” (GQ221847), which is a parasite of the aquatic amoeba, Saccamoeba lacustris (8). OTU18119 belonged to the uncultured Rickettsiales clade LWSR-14 in the SILVA v111 database, but shared only 84.3% sequence identity with its closest relative in the database, Sphingorhabdus marina (KT899836) isolated from Ny-Alesund, Svalbard. OTU3927 was closest to the Entomoplasmatales core OTU, OTU23265.

**Specificity of bacteria associated with ice worms**

As described above, dominant OTUs in ice worm samples included ice worm-specific and glacier-indigenous lineages. In addition to these dominant OTUs, several less abundant OTUs, such as three Rickettsiales OTUs (OTU21806, OTU13604, and OTU21100), were almost exclusively detected in ice worm samples (Fig. S3). OTU21806 was closely related to the “Candidatus Megaira polyxenophila” clade, which includes endosymbionts of aquatic eukaryotic microbes (31). OTU21806 shared 99.8% sequence identity with endosymbionts of the unicellular green alga, Carteria cerasiformis (AB688628) (21), and of the ciliate, Diophrys oligothrix strain DS12/4 (FR823003) (31). OTU13604 and OTU21100 showed only low sequence similarities to database sequences: 92.6% with a clone obtained from earthworm nephridia (JX644354) and 84.8% with a clone obtained from a termite gut (AB522149), respectively.

OTU23058 (Flavobacterium) was also specifically detected in the ice worm samples, particularly in Byron worms (Fig. S3). This OTU had an identical sequence with clone Ms-09-St2w-2-042 (AB990379) that was previously obtained from ice worms (27). This sequence was the closest (95.7% identity) to the sequence of the isolate, Flavobacterium sp. SIB A4(8) (DQ628949), which was obtained from a Canadian glacier; this Flavobacterium OTU likely originated in the glacier, as we suggested for the Arcicella and Herminiimonas core OTUs. OTU21396 (Brevinema), which was exclusively detected in the Byron worm samples, shared 91.1% sequence identity with clone MsOz-28-Sw-1-012 (AB991225) obtained from M. capillaris (31). Among the core OTUs, Flavobacterium OTU23058 was the second-closest sequence to database sequences: 96.9% with a clone obtained from earthworm nephridia (JX644354) and 84.8% with a clone obtained from a termite gut (AB522149), respectively.

**Differences in taxonomic compositions between DNA and RNA samples**

An analysis using DESeq2 indicated that 62 out of 1,114
OTUs and 217 out of 2,878 OTUs in Harding and Byron worm samples, respectively, showed significant differences in their abundance between DNA and RNA samples (Fig. 3). For example, three Mollicutes OTUs (OTU23265, OTU11203, and OTU18399) were significantly more abundant in DNA samples than in RNA samples. Conversely, several glacier-indigenous OTUs of Alphaproteobacteria, Betaproteobacteria, and Planctomycetacia, including OTU11712 and OTU18399 (Acetobacteraceae), OTU8825 (Comamonadaceae), and OTU25211 (Planctomycetaceae), were more abundantly detected in RNA samples. The dominant Lachnospiraceae OTU742 was also significantly more abundant in RNA samples (Fig. 3).

In situ localization of Arcicella OTUs

We examined the in situ localization of Arcicella OTU cells, which were phylogenetically affiliated with a glacier-indigenous lineage, but greatly enriched in the ice worm samples (Fig. 3). A FISH analysis revealed that Arcicella cells specifically colonized in clusters on the outer surface of the epidermis of Harding and Byron worms (Fig. 4).

Approximately 20–50 cells were observed in each 10-μm-thick section of the ice worms. The Arcicella cells observed were vibroid, 2–5 μm in length, and resembled other Arcicella species described previously (1).

Discussion

The deep sequencing of 16S rRNA genes and their transcripts clearly revealed specific and facultative relationships between bacteria and glacier ice worms. Significant differences were observed in bacterial community structures between ice worms and their associated glacier surface samples. In particular, several OTUs were almost exclusively detected in the ice worm samples. Among them, the “Ca. Vermiplasma” and Lachnospiraceae bacteria reportedly participate in polysaccharide fermentation in the gut of terrestrial earthworms (30, 32). Comparative genomics of Clostridiales families (Lachnospiraceae, Ruminococcaceae, and Clostridiaceae) also indicated that...
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Fig. 4. In situ detection of Arcicella cells in a cross section of an ice worm. An ice worm specimen collected from Byron Glacier is shown as an example. (A) Phase-contrast image. (B) FISH image. Red signals indicate Arcicella cells. (C) DAPI-stained image. Arrowheads indicate Arcicella cells. ep: epidermis. Bar=10 μm.

Lachnospiraceae and Ruminococcaceae bacteria in the mammalian gut are more specialized for the degradation of plant-derived recalcitrant substrates, such as cellulose, than Clostridiaceae bacteria (4). Since algal cells are one of the main food sources of ice worms (17, 27), Lachnospiraceae members may play an important role in the digestion of algal polysaccharides in the ice worm gut.

Several Rickettsiales and Parachlamydiaceae OTUs were also almost exclusively detected in the ice worm samples. These OTUs are phylogenetically related to parasites or endosymbionts of eukaryotes, including algae, ciliates, and amoebas. These OTUs may have been derived from intracellular bacteria that were hosted by ingested eukaryotic organisms, such as green algae and ciliates. However, the frequency of the chloroplast 16S rRNA gene or transcript sequences, which should represent the abundance of food-derived sequences, did not markedly differ between the ice worm and glacier surface samples (Table S2). Therefore, it is more likely that Rickettsiales and Parachlamydiaceae members specifically inhabit the ice worm body, although the localization of these bacteria remains unknown. Alternatively, these bacteria might proliferate in the ice worm body by infection from ingested eukaryotic organisms; it has been reported that certain members of the Rickettsiales or Parachlamydiaceae can be horizontally transferred from their original hosts to phylogenetically distinct host organisms by ingestion or mucosal infection (26, 39, 43).

It is notable that Arcicella OTU19485 and Herminiimonas OTU17386, which belong to glacier-indigenous lineages, were greatly enriched in the ice worm samples. Indeed, the colonization of Arcicella cells was observed on the surface of the ice worm body (Fig. 4). These results indicate that glacier-indigenous bacteria can form tight associations with ice worms, and also suggest that these bacteria have been specialized to associate with ice worms. Arcicella and Herminiimonas are both aerobic and heterotrophic bacteria; the former utilize various carbohydrates (7) and the latter utilize organic acids as carbon sources (15, 20). Arcicella species potentially feed on mucus and excrement secreted by the ice worm, and Herminiimonas species, although their localization remains unknown, may incorporate metabolites produced by the host or other bacterial species.

Marked differences in OTU compositions between the Harding and Byron ice worm samples were largely attributable to differences in bacterial community structures between the Harding and Byron glacier surfaces (Fig. 2 and 3), which may be caused by environmental differences between these two glaciers. For example, the sampling sites in Harding were around the center of the large icefield, in which any influence from the shore should be minimal, whereas those in Byron were located in a small avalanche cone, which was contiguous with soil and covered with plant fragments possibly derived from shore vegetation. These locational differences may lead to different physicochemical and nutritional conditions, which have a marked impact on the bacterial microbiota, as reported in previous studies (25, 36). This is analogous to findings obtained in terrestrial earthworms; the bacterial microbiota in the earthworm gut changes based on the soil used as food (41). In addition, several OTUs specific to the ice worm, such as Mollicutes OTU23265 and OTU11203, Lachnospiraceae OTU742, Parachlamydiaceae OTU9924, and Brevinema OTU21396, also exhibited differential compositional patterns between the Harding and Byron worms (Fig. 1, 3, and Fig. S3). This might reflect the isolation of ice worm populations. Due to the harsh survival conditions of the ice worm, its populations have been geographically isolated among habitat glaciers (9, 10). Thus, bacterial lineages that specifically inhabit the ice worm body might also be isolated and exhibit different taxonomic compositions in the respective glaciers.

An analysis of the 16S rRNA transcript in addition to the gene sequence can provide information on active bacterial species (5, 36). Interestingly, dominant OTUs in the RNA samples of the ice worms were affiliated with both glacier-indigenous and ice-worm-specific bacterial lineages (Fig. 3 and Fig. S2). The glacier-indigenous OTUs of Alphaproteobacteria, Betaproteobacteria, and Planctomycetacia, and animal gut-specific dominant Lachnospiraceae OTUs were significantly (P<0.05) more abundant in RNA samples than in DNA samples (Fig. 3); these lineages are candidates of the major active taxa in the bacterial consortium associated with ice worms. This result indicates that some of the glacier-indigenous bacteria are exploited as facultative gut bacteria, as observed in the close associations between terrestrial earthworms and soil-derived facultative gut bacteria (11). Much OTUs in RNA samples (Table S1) may be attributable to the higher activity of minor OTUs, which were concealed behind several predominant OTUs in DNA samples (Fig. S1) (28), although the
different preparation steps between DNA and RNA templates such as purification and reverse transcription steps may also affect the observed number of OTUs.

Conclusion

The results of the present study strongly suggest that not only animal-associated bacterial lineages, such as Mollicutes, Rickettsiales, Parachlamydiaceae, and Lachnospiraceae, but also glacier-indigenous bacteria, including Arcicella and Herminiimonas, benefit from the presence of the ice worm. The body surface and intestinal tract of the ice worm appear to provide a unique habitat, which is potentially rich with nutrients, to microbes in glacier ecosystems with limited resources, thereby driving the formation of a worm-associated bacterial microbiota that is distinct from that in glaciers. Since ice worms are the predominant metazoa in Alaskan maritime glaciers, the relationships between ice worms and bacteria potentially have an impact on the geochemical cycles in glaciers, as reported previously for terrestrial earthworms (11). Future analyses on the metabolism and genomes of ice worms and their associated microbiota will provide a deeper understanding of their ecological functions in glacier ecosystems.

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