RESEARCH ARTICLE

Census of bacterial microbiota associated with the glacier ice worm *Mesenchytraeus solifugus*

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One sentence summary: We analyzed the taxonomic composition of bacteria physically associated with the glacier-adapted worm *Mesenchytraeus solifugus*, and provided the first report of symbiosis between bacteria and the glacier invertebrates.

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ABSTRACT

The glacier ice worm, *Mesenchytraeus solifugus*, is a unique annelid, inhabiting only snow and ice in North American glaciers. Here, we analyzed the taxonomic composition of bacteria associated with *M. solifugus* based on the 16S rRNA gene. We analyzed four fixed-on-site and 10 starved ice worm individuals, along with glacier surface samples. In total, 1341 clones of 16S rRNA genes were analyzed for the ice worm samples, from which 65 bacterial phylotypes (99.0% cut-off) were identified. Of these, 35 phylotypes were closely related to sequences obtained from their habitat glacier and/or other components of cryosphere; whereas three dominant phylotypes were affiliated with animal-associated lineages of the class *Mollicutes*. Among the three, phylotype Ms-13 shared less than 89% similarity with database sequences and was closest to a gut symbiont of a terrestrial earthworm. Using fluorescence in situ hybridization, Ms-13 was located on the gut wall surface of the ice worms. We propose a novel genus and species, *Candidatus Vermiplasma glacialis*, for this bacterium. Our results raise the possibility that the ice worm has exploited indigenous glacier bacteria, while several symbiotic bacterial lineages have maintained their association with the ice worm during the course of adaptive evolution to the permanently cold environment.

Keywords: glacier ecology; enchytraeid; symbiosis; 16S rRNA; gut bacteria

INTRODUCTION

On glaciers, which are extreme environments characterized by low temperature and poor nutrients, a variety of psychrophilic or psychrotolerant organisms form unique ecosystems (Anesio and Laybourn-Parry 2012). Many studies on their diversity, physiology and the impact of their activity on the surrounding environment have been reported (Takeuchi, Kohshima and Seko 2001; Napolitano and Shain 2004; Anesio et al., 2009; Cameron, Hodson and Osborn 2012; Lutz et al., 2014); however, most of these investigations have focused on microbial communities on...
glacier surfaces, while information on glacier invertebrates remains limited.

The glacier ice worm, *Mesenchytraeus solifugus* (family Enchytraeidae, phylum Annelida), is the most dominant and largest metazoan in North American maritime glaciers from Alaska to Oregon (Fig. S1, Supporting Information) (Shain et al., 2001; Hartzell et al., 2005). *Mesenchytraeus solifugus* is the only known annelid that lives their entire life in glacier ice/snow, i.e. an environment permanently at the freezing point. Physiological and protein structure analyses have revealed that their metabolism, e.g. ATP synthesis capabilities, is adapted to cold environments (Farrell, Hohenstein and Shain 2004; Napolitano, Nagae and Shain 2004; Tartaglia and Shain 2008; Marotta, Parry and Shain 2009). A recent phylogeographic study has suggested that their distribution reflects past glacial dynamics (Dial et al., 2012). However, little is known about their life cycle, nutrition and ecological niches in the glacier ecosystem.

Terrestrial earthworms play crucial roles in soil organic matter decomposition and biogeochemical cycles in temperate regions (Curry and Schmidt 2007). It has been postulated that the gut microbiota of earthworms contributes significantly to these activities (Edwards and Fletcher 1995; Drake and Horn 2007). The majority of gut microorganisms harbored by earthworms are derived from the soil ingested by the host; anaerobes stimulated by the gut environment participate in various bioactivities such as fermentation of recalcitrant materials and denitrification (Karsten and Drake 1995; Thakuria et al., 2010; Wüst, Horn and Drake 2011; Depkat-Jakob et al., 2013). Aquatic and semi-aquatic oligochaetes also have an impact on aquatic ecosystems via food webs and the formation of sediment structure (Martin et al., 2008). Given that it is a dominant fauna in the habitat, it is conceivable that the ice worm occupies a similar niche in the glacier ecosystem and has a significant impact on it.

In the present study, we aimed to clarify the microbial community structure physically associated with the ice worm to elucidate the ice worm’s ecological niche and its nutrient sources. We performed both culture-dependent and -independent censuses of the bacterial microbiota based on 16S rRNA genes, which we compared with those of the glacier surface habitat and those of two other enchytraeid worms. We also performed fluorescence in situ hybridization (FISH) analysis for a dominant gut bacterium and transmission electron microscopy (TEM) of the intestinal tract. Our results provide an insight into the relationship between invertebrates and bacteria on a glacier and should contribute to further understanding of the glacier ecosystem.

**MATERIALS AND METHODS**

**Sample collection**

The field research was carried out at Byron Glacier and Harding Icefield in the Kenai Peninsula, Alaska in August 2010, 2011 and 2014 (Fig. S1, Supporting Information). Ice worms and glacier surface ice/snow were collected with sterilized stainless steel scoops. A portion of samples was suspended in RNAlater solution (Ambion, Austin, TX, USA) on site and stored at −30°C until use. The remaining live ice worms were kept in melted glacier surface ice at 4°C. Collected samples were kept cold with the glacier ice during transportation.

Two other Enchytraeidae species were used for comparison with the ice worm. A snow worm, *Mesenchytraeus sp.*, was collected from snow at Ozegahara, Japan in April 2012 and provided by Takaaki Torii (IDEA Consultants Inc.) (Fig. S2, Supporting Information). The collected worms were kept in melted snow at 4°C in a laboratory. This species is active in snow during spring, and in summer, inhabits waterside soil (Torii 2012). A tiny earthworm, *Enchytraeus japonensis*, reared in a laboratory, was provided by Arata Tochinai (Hokkaido University).

To wash out ingested food materials, individual worms were starved in Petri dishes filled with double-distilled water (DDW) at 4°C for a period (ice worms: 1 week, 2 weeks, 1 month and 2 months; snow worms: 3 days). DDW was sterilized by ultraviolet (254 nm) irradiation for 30 min before use and changed every 3 days for the first week, and weekly after that. *Enchytraeus japonensis* were kept in agar plates at room temperature for 3 weeks without food.

**DNA extraction**

DNA was extracted from the entire body of individual worms using the Ultra Clean® Soil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA, USA), according to the manufacturer’s protocol. Prior to DNA extraction, the body surface of the worms was washed twice by submerging them in DDW for 10 min. The glacier surface samples were melted and 2 mL aliquots were centrifuged at 4°C and 17 000 × g for 15 min. The pellets were suspended in 2 mL of phosphate-buffered saline (PBS) and centrifuged under the same conditions. The pellets were used for DNA extraction as above.

**PCR, cloning and sequencing**

PCR was performed with Ex-Taq polymerase (Takara, Shiga, Japan) and a Bacteria-specific primer pair, 27Fmix (5′-AGAGTTTGATYMTGGCTCAG-3′) and 1492R (5′-GGHTACCTTGTAGACTT-3′) (Hornog et al., 2007), to amplify near-full-length 16S rRNA genes. DNA extracted from the samples was diluted 10 times with TE buffer, and 10 μL of each was used as a template for PCR in a 100 μL reaction. To minimize PCR drift, the reaction mixture was divided into four tubes and combined after amplification (Polz and Cavanaugh 1998). The reaction was performed in a C-1000 Thermal Cycler (Bio-Rad) and the following program was used: 1-min initial denaturation at 95°C, 25 or 30 cycles of denaturation (15 s at 95°C), annealing (30 s at 50°C) extension (90 s at 72°C) and a final 4-min extension at 72°C.

PCR products were purified using the MonoFas™ DNA Purification Kit I (GL Sciences, Tokyo, Japan) according to the manufacturer’s protocol. Clone libraries were prepared with the TOPO® TA cloning® Kit for Sequencing (Invitrogen, Carlsbad, CA, USA) and Competent Quick DH5α (Toyobo, Osaka, Japan). The clones were chosen at random and the inserts were amplified with an M13 primer pair with the following program: 3-min initial denaturation at 95°C, 30 cycles of denaturation (15 s at 95°C), annealing (30 s at 50°C), extension (2 min at 72°C) and a final 5-min extension at 72°C. The amplicons were purified with the Multiscreen™ PCR μ96 Filter Plate (Millipore, Billerica, MA, USA) and sequenced with T3 and T7 primers, using the Big Dye® Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Austin, TX, USA) in an ABI 3130xl or ABI 3730 Genetic Analyzer (Applied Biosystems).

**Phylogenetic analysis**

All sequences were subjected to identification of chimeras in USEARCH 6.0 (Edgar et al., 2011) on the Ribosomal Database Project website (http://fungene.cme.msu.edu/FunGenePipeline/chimera_check/form.spr). After elimination of putative
chimeras, low-quality sequences and plastid 16S rRNA gene sequences, the remaining ones were aligned using the SILVA SSURef nr release111 database (Pruesse et al., 2007) using the SILVA Incremental Aligner in ARB software (Ludwig et al., 2004; Pruesse, Peplies and Glöckner 2012). The alignment was manually corrected and sequences were assigned to phylotypes defined by 99.0% sequence similarity criterion in the mothur program package ver. 1.33 with the furthest neighbor algorithm (Schloss et al., 2009). Sequences closely related to each phylotype were retrieved from the GenBank nr database by BLAST searches. The Chao1 richness, Simpson’s diversity index, Good’s coverage and rarefaction curve were calculated in mothur.

Unambiguously aligned 1049 nucleotide sites, corresponding to positions 110–1277 in Escherichia coli (J01695), were used for phylogenetic analysis of Mollicutes phylotypes. A maximum-likelihood (ML) tree was constructed in MEGA6 using the general time reversible (GTR) model and a gamma distribution (G) with 500 bootstrap resamplings (Tamura et al., 2013). A Bayesian inference of phylogeny was performed with the MrBayes software package ver. 3.2 using a GTR + G nucleotide substitution model (Huelsenbeck and Ronquist 2001). The analysis was run for 1 000 000 generations with a sample frequency of 100. The initial 250 000 generations were discarded as burn-in.

Unweighted UniFrac analysis implemented in the QIIME program package ver. 1.7.0 was conducted to measure bacterial community structure similarities (Lozupone and Knight 2005; Caporaso et al., 2010). The robustness of sample clustering was evaluated by jackknife sampling in QIIME.

Transmission electron microscopy

Sample preparation for TEM was conducted according to Shain et al. (2000). Briefly, an ice worm individual was dissected into three parts with a sterile razor, pre-fixed with 2% paraformaldehyde and 2.5% glutaraldehyde, and post-fixed with OsO₄. Fixed specimens were rinsed five times in 0.1 M cacodylate buffer and then dehydrated and embedded in EPON™ resin (Quetol 651; Nisshin EM, Tokyo, Japan). Thin sections were observed using an H-7500 transmission electron microscope (Hitachi, Tokyo, Japan).

FISH and cell enumeration

To detect Mollicutes phylotypes, the probe Tcutes1-825 (5′-CGCTGCTTATGCAAACATAAT-3′, positions 825–855 in E. coli), which targets the Entomoplasmatales phylotypes Ms-05 and Ms-08, and the probe Tcutes2s-823 (5′-CGCTAGCCGACCTCAATAC-3′, positions 823–844 in E. coli), which targets the Mycoplasmataceae phylotype Ms-13, were designed using the probe-designing function implemented in ARB and labeled with Texas Red at their 5′ extremity. Specificity of the probe was checked using the probeCheck web server (Loy et al., 2008) and it was found that each probe had four or more mismatches against any other sequences in the SILVA database. The optimal temperature for hybridization was estimated in silico using the DNAMElt web server (Markham and Zuker 2005). A non-labeled helper probe (5′-AGGGCCGAGCCGACTAT-3′) was also designed and used with the probe Tcutes2s-823 (Fuchs et al., 2000). To evaluate the degree of non-specific binding of oligonucleotide probes to specimens, probe EUB338-III (5′-GCTGCCAGCGTACT-3′) (Daims et al., 1999) labeled with 6-carboxyfluorescein (6-FAM), mainly targeting verrucomicrobia, which we did not detect in the clone analysis, was used. We employed the EUB338 probe (5′-GCTGCTCCGAGGTAGT-3′) labeled with 6-FAM to detect most bacterial taxa (Amann et al., 1990).

Ice worm specimens preserved in RNA later solution were dissected into three parts with a sterile razor and fixed in 4% paraformaldehyde at 4 °C overnight. After three washes in DDW, the specimens were sequentially treated with 10, 15 and 20% sucrose, embedded in Tissue-Tek® OCT tissue-freezing medium (Sakura Finetechanical, Tokyo, Japan), and cut into 10-μm-thick cross-sections on a Leica CM 1850 cryostat (Leica Biosystems, Nüföch, Germany). The sections were placed on a MAS-coated glass slide (Matsunami, Osaka, Japan) and treated with 0.25 N HCl at room temperature for 10 min. The sections were then washed with DDW and dehydrated in a series of 50, 80, 90 and 100% ethanol. Alternatively, ice worm specimens were homogenized in 300 μL DDW with a sterile plastic pestle, fixed and dehydrated as above. Hybridization was conducted according to Noda et al. (2003) at 50 °C for 90 min. Specimens were enclosed using Slowfaze Gold with 4,6-diamidino-2-phenylindole (DAPI) (Life Technologies, Carlsbad, CA, USA) and observed under an Olympus BX51 epifluorescence microscope (Olympus, Tokyo, Japan).

To count the total number of prokaryotic cells associated with an ice worm individual, the entire body was homogenized with a sterile plastic pestle in 250 μL PBS after washing the worm surface as above. The homogenate was fixed with 4% paraformaldehyde overnight and centrifuged at 10 000 × g for 10 min. The pellet was washed twice in DDW and then immobilized onto a polycarbonate hydrophilic membrane filter (Miplore; pore size, 0.2 μm). The cells were stained with DAPI and counted microscopically.

Bacterial isolation and identification

Individual ice worms were homogenized in 500 μL LB medium (1% Bacto Tryptone, 0.5% Bacto Yeast Extract, 1% NaCl and 0.1 mM NaOH in 1 L of DDW) or its 100-fold dilution. The homogenate was spread on either LB or 20-fold diluted LB agar plates and incubated at either 4 °C or −1 °C. Representative colonies that exhibited unique morphologies and colors were picked up and suspended in lysis buffer (20 mM Tris-HCl (pH 8.0), 200 μg mL⁻¹ proteinase K (Takara), 5 mM EDTA, 400 mM NaCl, and 0.3% SDS) at 55 °C for 1 h. The lysate was treated with AmpDirect® Plus reagent (Shimadzu, Kyoto, Japan) and directly used as a template for PCR with the primer set 27F-mix and 1492R. The PCR conditions were as follows: an initial denaturation for 5 min at 94 °C, 30 cycles of denaturation (30 s at 94 °C), annealing (30 s at 52 °C), extension (150 s at 72 °C) and a final 7-min extension at 72 °C. Sequencing was performed as described above. The isolates were identified on the basis of their 16S rRNA gene sequences and stored in both glycerol solution and DMSO solution at −80 °C.

Nucleotide sequence accession numbers

The nucleotide sequences reported in this study have been deposited in DDBJ/EMBL/GenBank under the accession numbers AB989660-AB991625 (clones), AB991626-AB991700 (isolates), AB991701-AB992193 (plastids) and LC012708 (18S rRNA gene of the snow worm Mesenchytraeus sp.).

RESULTS

Taxonomic composition of ice worm-associated bacteria

General features

The samples analyzed for the census of bacteria in this study are given in Table 1. Two worm individuals were examined for
The bacterial taxonomic compositions were similar between individual worms in the same category, although the relative abundance of bacterial taxa differed (Fig. 1). The dominant phyla, which accounted for 67% of the clones analyzed, were Proteobacteria (Alphaproteobacteria: 10 phylotypes, Betaproteobacteria: 13, Gammaproteobacteria: 7 and Deltaproteobacteria: 1) and Bacteroidetes (Sphingobacteria: 7 phylotypes, Flavobacteria: 2 and Bacteroidia: 1) (Fig S6, Supporting Information).

Ice worm samples fixed on site
The bacterial taxonomic composition of the ice worm samples fixed on site in 2010 (Fx10) and 2011 (Fx11) was considerably different; only six phylotypes were shared among the 21 (2010) and 31 (2011) identified. For example, Flavobacterium was only detected in 2010 while Singulisphaera (Planctomycetes) was only detected in 2011 (Fig. 1). Similarly, Entomoplasmatales was predominant in 2010, while Singulisphaera was so in 2011. The diversity was also different; the reciprocal Simpson index was 2.30 and 5.20 in 2010, while it was 10.94 and 12.08 in 2011 (Table 1).

Table S2 (Supporting Information) shows the closest database sequences for each phylotype obtained in this study. Phylotypes belonging to the genus Aricella (Bacteroidetes) were closest to database sequences derived from ice/snow environments. A phylotype belonging to Albidiferax (Betaproteobacteria) shared more than 97% similarity with database sequences obtained from an ice/snow environment. Several other phylotypes belonging to the genera Streptococcus (Bacillii), Rhodovulum (Alphaproteobacteria) and Delfia (Betaproteobacteria) shared more than 97% similarity with database sequences recovered from various environments, including human specimens, soil, freshwater and glaciers. Dominant phylotypes, Ms-05 (Entomoplasmatales) and Ms-13 (Mycoplasmataceae), only shared less than 90% similarity with database sequences, and each formed a monophyletic cluster with uncultured gut symbionts of invertebrates such as isopods and earthworms (Fig 2).

**Starved ice worm samples**
The taxonomic composition of bacteria shifted with starvation period. Ferruginibacter (Bacteroidetes) were predominant in
the ice worms starved for 1 week (St1w), while Arcicella was predominant in the 2-week-starved samples (St2w) (Fig. 1). A Phycisphaera-like phylotype (Planctomycetes) dominated in St1m and Variovorax (Betaproteobacteria) predominated in St2m. Ferruginibacter, Arcicella, Albidiferax and Variovorax were frequently detected through the starvation period (Figs S7, Supporting Information) and all of these phylotypes shared >97% sequence identity with described species and/or uncultured clones obtained from ice/snow environments. The Phycisphaera-like phylotype Ms-04 shared only 84% sequence identity with described species and showed 95% similarity to uncultured clones obtained from soil or aquatic environments (Table S2, Supporting Information).

Comparisons of bacterial communities between fixed-on-site and starved ice worms
Clear-cut differences in taxonomic composition of bacteria were observed between fixed-on-site and starved ice worm specimens. Mollicutes (Mycoplasmataceae and Entomoplasmatales) were abundant in the fixed-on-site samples but decreased or disappeared in the starved samples. Entomoplasmatales were not detected from samples starved for longer than 1 month, except for sample St1m-1, and Mycoplasmataceae was never detected in the starved samples. Rhodovulum was also never detected in the starved samples (Figs 1 and S7, Supporting Information). Chao 1 richness and the reciprocal Simpson diversity index indicated that bacteria from the fixed-on-site ice worms were generally more diverse than those from the starved samples (Table 1).

Of the 65 phylotypes that were obtained from the ice worm samples, 35 phylotypes in total, accounting for 68% of the clones analyzed, were also detected from the habitat glacier and/or from other components of cryosphere. These putatively psychrophilic or psychrotolerant phylotypes were detected in both the fixed-on-site worm specimens collected in 2010 (9 and 10% of analyzed clones for each of the two individuals), 2011 (84 and 96%) and the starved individuals (22–100%).

Comparisons of bacterial communities with reference samples
The taxonomic composition of bacteria detected in the glacier surface samples exhibited striking differences between years (Byron10 and 11) (Fig. 1). After elimination of plastid sequences, 24 and 46 phylotypes were found from Byron10 and 11 samples, respectively, only 5 phylotypes of which were shared between them. Three of these 5 phylotypes were affiliated with Ferruginibacter and one of them was also found in the ice worm samples. In total, 65 phylotypes were identified from the glacier surface samples. Among them, 24 phylotypes were shared by ice worm samples, accounting for 45% of the ice worm clones analyzed. Mollicutes phylotypes, abundant in the ice worms, were not detected in the glacier samples (Figs 1 and S7, Supporting Information).

From the snow worm Mesenchytraeus sp., 28 phylotypes were identified in total and these were not detected in the ice worm samples. Of these, 10 phylotypes were closely related to bacteria previously detected from the snow surface in their habitat area (Kojima, Fukuhara and Fukui 2009) and/or other components of cryosphere. The bacterial microbiota of the earthworm *E. japonensis* was predominated by a single Spirochaeta phylotype, Ej-01 (Figs 1, S3 and S4, Supporting Information).

UniFrac analysis showed the dissimilarity of bacterial community structure among the samples (Fig. 3). As implied in Fig. 1, the fixed-on-site ice worm samples were closely related to the glacier surface samples, while the starved ice worm samples clustered together. The bacterial communities associated with the *Mesenchytraeus* snow worm and *E. japonensis* were distinct from each other and from the ice worms.
Figure 2. Phylogenetic positions of Mollicutes phylotypes detected from *M. solifugus* based on 16S rRNA gene sequences. The phylogenetic tree was constructed using a ML method. Numbers indicate bootstrap support values of the ML tree and Bayesian posterior probabilities, respectively (left/right).

Figure 3. Unweighted UniFrac analysis showing bacterial community similarities. Jackknife support values >50% are shown.
Analysis of plastid sequences

We observed numerous cells of red-pigmented algae in the gut of the ice worms (Fig. S8, Supporting Information). Concordantly, we obtained abundant plastid sequences. Plastid sequences were more abundant in samples collected in 2010 than those in 2011, for both ice worms and glacier surface ice (Table S1, Supporting Information). When incorporated into the SILVA database, the majority were affiliated with the green algal family Trebouxiophyceae and the most dominant phylotype showed 93.9% sequence similarity to Pseudochlorella pringsheimii (Fig. S9, Supporting Information).

Microscopic observation of ice worm-associated bacteria

Dense prokaryotic cells were observed in the lumen of ice worm digestive tracts by TEM (Fig. 4a–c). Prokaryotic cells also abundantly existed within or proximal to the mucus layer that covered the cilia of the gut epithelium (Fig. 4a and b). Several prokaryotic cells were found in division form (Fig. 4d). No microbial cells were found either on the epidermis or in the other ice worm tissues surveyed. The ice worms fixed on site at Byron Glacier in 2011 were used for FISH analysis. The cells of a dominant phylotype, Ms-05 (Mycoplasmataceae), were specifically detected on the surface of the gut epithelium in a cross-section (Fig. 5). They were also detected in a homogenized ice worm sample and cohybridized with the EUB338 probe but not with EUB338-III (Fig. S10, Supporting Information). We failed to detect the Entomoplasmatales phylotypes Ms-05 and Ms-08 signals in any specimens.

The ice worm individuals collected on Byron Glacier and Harding Icefield in 2014 were kept alive at 4°C for 1 month and then used for enumeration of associated prokaryotic cells. The number of prokaryotic cells per ice worm individual was estimated to be $1.7 \times 10^5 \pm 3.7 \times 10^4$ (Byron, $n = 3$) and $9.4 \times 10^5 \pm 2.1 \times 10^5$ (Harding, $n = 3$) (mean $\pm$ SD).

Bacterial isolation

A total of 14 phylotypes were isolated by culture on LB plates at either 4°C or −1°C (Table S3, Supporting Information). All of the phylotypes were affiliated with either Betaproteobacteria (Janthinobacterium: 2 phylotypes; Albidiferax, Actinobacterium, Glaciegerax, Massilia, Polaromonas, Undibacterium and Variovorax: 1) or Gammaproteobacteria (Pseudomonas: 4 phylotypes and Rhodanobacter: 1). Of these, 6 phylotypes were also found in the culture-independent analysis of the ice worm samples, and 11 phylotypes exhibited >97% sequence similarity with described species and/or uncultured clones obtained from other components of cryosphere (Table S4, Supporting Information).

DISCUSSION

This is the first report specifically focusing on the bacterial microbiota associated with invertebrates that inhabit a glacier environment. We revealed that the majority of bacteria that ice worms harbor are closely related to those generally detected in the components of cryosphere. This raises the possibility that the ice worm has exploited glacial bacteria as transient gut symbionts. This is analogous to the earthworms, where bacteria ingested with soil act as transient gut symbionts and contribute to the host’s nutrition (Wüst, Horn and Drake 2011).

Discrimination of transient and more tightly associated gut bacteria was attempted in the present study by starving the host ice worms. For example, phylotypes assigned to Rhodovastum were frequently detected in both fixed-on-site ice worms and glacier surface samples, but not in starved worm samples. These bacteria might be digested or pass through the digestive tract without intimate interaction with ice worms. In contrast, Albidiferax, Variovorax and Arcicella phylotypes were detected in both fixed-on-site and starved ice worms. The loss of diversity and predominance of Variovorax in the samples starved for 2 months (St2m) might be attributable to the long starvation period that would have led to unhealthy gut microbiota. The difference in taxonomic compositions of bacteria between sampling years or individuals may reflect the conditions, such as the abundance of algae (Table S1, Supporting Information), of the collection sites.

In earthworms, the taxonomic composition of gut bacteria differs in relative abundance from that of the surrounding soil because of differences in redox potential and nutrient concentration (Drake and Horn 2007). Similarly, the relative abundance of putatively transient ice worm gut bacteria was distinct from the surrounding glacier surface ice/snow. While the ice worms might receive nutritional benefits from the transient gut bacteria, for glacier bacteria, the ice worm gut might be a hotspot for proliferation, given that organic matter is much concentrated in the gut, compared to the oligotrophic glacier environment. According to Segawa et al. (2010), the prokaryotic cell density on the surface of a glacier in the Alaska Range is $10^6$ cells mL$^{-1}$. Thus,
the density of prokaryotic cells associated with ice worms was far higher than that on the glacier surface.

In the culture-independent analysis of 16S rRNA genes, diverse bacteria that were not obtained by the culture-dependent approach were recovered, as in other environments (Singleton et al., 2003). Among them, phylotypes belonging to the class Mollicutes were dominant in several ice worm individuals. These Mollicutes phylotypes were related to lineages that have tight and species-specific associations with the gut epithelium or midgut gland of terrestrial earthworms and isopods (Singleton et al., 2003; Wang et al., 2004, 2007; Kostanšek, Štrus and Avguštin 2007; Fraune and Zimmer 2008; Nechitaylo, Timmis and Golyshin 2009). In the present study, we found that the Mollicutes phylotype Ms-13 was also localized on the gut wall surface of the ice worms. Thus, it seems likely that these Mollicutes lineages have maintained association with the host worm during the course of their evolution. Although the Mollicutes phylotypes obtained from the ice worms were not detected in the glacier surface samples in our Sanger sequencing analysis, we identified a few V4 region sequences of 16S rRNA gene identical to those of Ms-13 and nearly identical to Ms-05 and Ms-08 among the sequence reads generated by Choudhari et al. (2013) using an Illumina HiSeq from a Byron Glacier sample. This implies that the gut-associated Mollicutes phylotypes are deposited on the glacier surface with ice worm feces and re-infected to other individuals.

Previous observations have revealed that ice worms ingest algae blooming on the surface of glaciers (Goodman 1971). Concordantly, we observed abundant algal cells in the ice worm guts. Red-pigmented algae were dominant, and they morphologically resembled Chlamydomonas cf. nivalis that were found on the surface of glacier snow in Alaska (Takeuchi et al., 2006). We also identified numerous plastid 16S rRNA genes of algae in the ice worm samples fixed on site. It is conceivable that ice worm gut microbiota, including those transiently acquired with ingested food, play a role in the digestion of recalcitrant matter such as algal cell wall polysaccharides. For example, the phylotype Ms-01, one of the most frequently detected phylotypes, exhibited 98.0% sequence similarity with Variovorax paradoxus strains, which participate in a wide variety of catabolic pathways, including...
cellulolysis (Satola, Wübbeler and Steinbüchel 2013). Because
the ice worm permanently resides in glacier ice/snow, exploita-
tion of psychrophilic or psychrotolerant bacteria as gut sym-
bionts might be important.

Our results provide basic information to elucidate the eco-
logy and evolution of the ice worm and its associated bacteria.
However, it remains unclear whether the detected bacterial se-
quences represent truly active gut symbionts and what their
functional roles are. Future metagenomic and metatranscrip-
tomic analyses of the ice worm gut microbiota should address
these issues. We propose a novel genus and species for the Mol-
lictures phylotype Ms-13.

Description of ‘Candidatus Vermiplasma glacialis’

Vermiplasma glacialis (ver. mi. plas’ma. L. merc. n. vermis worm;
Gr. neut. n. plasma something formed or molded, N.L. neut. n.
vermiplasma something from worm; gla. cia. lis. L. fem. adj.
glacialis icy or glacial).

Cells are curved rods, 1.0–1.5 μm in length and specifically
localized on the gut wall surface of the glacier ice worm M.
solifugus. The assignment of ‘Candidatus Vermiplasma glacialis’
is based on the 16S rRNA sequence (AB989845) and hybridiza-
tion with the 16S rRNA-targeted oligonucleotide probe (5’-
CGCTAGCGACCTCATAATAC-3’).

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

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