Stick together: Isolation and characterization of exopolysaccharide producing bacteria from degraded permafrost soils

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ABSTRACT

Permafrost thaw can result in two distinct environmental landscapes (i.e. “wet condition” soils rich in ice wedges and low drainage, and “dry condition” well-drained areas with higher evapotranspiration). These different conditions influence the bacterial communities and may impact their capacity to produce exopolysaccharides (EPSs), which are crucial for bacterial survival and adaptation. This study aimed to isolate and characterize EPSs-producing bacteria from the active layer of two permafrost degrading landscapes and from undisturbed permafrost soil. A total of 54 bacterial isolates were obtained, representing three phyla: Firmicutes, Actinomycetota, and Pseudomonadota. EPSs production was assessed by determining the polysaccharide content measured as glucose equivalent, and 26 isolates were identified as potential EPSs-producers. Among the isolates, *Curtobacterium oceanosedimentum*, *Frigoribacterium faeni*, *Streptomyces* strains, *Neobacillus bataviensis* and *Mesobacillus subterraneus* had the highest polysaccharide yield. They were also found inhabiting in the different horizons of degraded permafrost soil and undisturbed permafrost soil, by determining their relative proportion within the total bacterial community based on 16S rRNA gene sequences similarities.

Key Words

Permafrost; Global change; Exopolysaccharides (EPSs); Bacteria; Soil aggregates; Soil organic matter; Isolation, Sequencing, DNA

INTRODUCTION

Exopolysaccharides (EPSs) are the main part of the complex mixture of organic biopolymers produced and secreted by a wide range of bacteria into their surroundings (Martino 2018). EPSs are high-molecular-weight polysaccharides that can be broadly classified into homopolysaccharides and heteropolysaccharides, depending on their monosaccharide composition (Alvarez-Lorenzo *et al.* 2013; More *et al.* 2014) Homopolysaccharides consist of a single type of monosaccharide and include examples such as dextran, curdlan, and cellulose. Dextran features α-(1,6)-linked glucose chains with occasional branching through α-(1,3)-linkages, while curdlan is composed of β-(1,3)-linked glucose residues, and cellulose consists of β-(1,4)-linked glucose units commonly produced by various bacterial groups (More *et al.* 2014). In contrast, heteropolysaccharides, including alginate, xanthan gum, gellan, and hyaluronic acid, are more structurally complex, incorporating diverse monosaccharides such as glucose, galactose, rhamnose, and glucuronic acids (Wingender *et al.* 2001; More *et al.* 2014; Oleńska *et al.* 2021). Their structural properties are influenced by specific sugar linkages, branching patterns, and the presence of functional substituents like pyruvates, succinates, and acetates, which enhance their versatility and adhesive properties. EPS are categorized into two major types based on the biosynthesis pathway: capsular EPSs, which are tightly associated with the cell surface forming a capsule, and slime EPSs, which are loosely attached to the cell or completely diffuse into the extracellular environment (Ali *et al.* 2020).

The structurally diverse composition of EPSs highlights the ecological significance of EPSs-producing bacteria in soil, where they serve a multitude of purposes primarily associated with the biofilm formation and maintenance (Balducci *et al.* 2023), protecting bacterial cells under various environmental stress, adhesion of the cells to each other and biofilm attachment to the surface (Wingender et al. 1999; Sandhya and Ali 2015) and cryoprotective potentials (Dubey and Jeevaratnam 2015). The chemical compositions of EPSs often contain functional groups such as hydroxyl, carboxyl, sulfate, and phosphate groups, which play a crucial role in cell attachment and bonding with soil particles (Lin *et al.* 2014; Huang *et al.* 2022). These functional groups contribute to the anionic or cationic nature of EPS molecules, enabling electrostatic interactions and hydrogen bonding with various surfaces, including soil minerals and organic matter. Uronic acids, such as glucuronic, galacturonic, and mannuronic acids, commonly found in EPS, introduce negative charges that enhance cation exchange capacity and facilitate the binding of metal ions and clay particles (Mojica, Elsey and Cooney 2007). Additionally, substituents like acetates, pyruvates, and succinates further modify the physicochemical properties of EPSs, improving their adhesive potential and structural stability (Jeong *et al.* 2022). The presence of hydrophilic groups allows EPSs to retain water, creating hydrated microenvironments that promote microbial survival and biofilm formation (Lin *et al.* 2014). This hydrated matrix also acts as a protective barrier against environmental stressors, such as desiccation

and toxins, while providing structural support for biofilm development (Lin *et al.* 2014; Huang *et al.* 2022). Furthermore, the diverse monosaccharide composition of EPS, including glucose, galactose, rhamnose, and mannose, contributes to polymer flexibility, enabling the formation of cross-linked networks that physically entangle soil particles, enhancing soil aggregation and stabilization (Huang *et al.* 2022). These interactions between EPS and soil particles are critical for improving soil structure, nutrient retention, and microbial colonization, underscoring the ecological and environmental significance of EPSs in soil systems.

In harsh environments, such as permafrost soils that remain frozen for two or more consecutive years, the unique properties of EPSs are particularly vital, as they facilitate bacterial survival under extreme cold, nutrient scarcity, and fluctuating water availability. The cryoprotective and stabilizing functions of EPSs are crucial for maintaining bacterial activity, biofilm integrity, and soil aggregation, thereby may enhancing the resilience of soil ecosystems in these extreme conditions. However, the composition and activity of bacterial communities in permafrost soils are significantly influenced by permafrost degradation (Steven *et al.* 2008; Jansson and Taş 2014; Deng *et al.* 2015a; Liu *et al.* 2019). The increase in Arctic temperatures triggers an increase in the depth of active layer, leading to the degradation of permafrost soils and creating conducive environment for bacterial activity (Knoblauch et al. 2013; Koven et al. 2013). When permafrost degrades, it results in several emerging landscape types from which two distinct hydrological landscapes, such as, wet soils, e.g. in areas with high moisture, due lower slope position and poor drainage, and dry soils with better drainage and higher evapotranspiration (Jorgenson and Osterkamp 2005; Jorgenson et al. 2013; Liebmann et al. 2024). These landscapes exert different influences on bacterial community compositions and activities, which have direct impact on soil chemical and physical properties, mainly the degradation/stabilization of exposed soil organic matter previously preserved in the frozen ground, and the soil aggregation (Koven et al. 2013). Therefore, the hydrological regime of soil after the permafrost degradation can be decisive for activity and composition of EPSs-producing bacteria. One way to gain a better understanding of the physiology and ecology of EPSs-producing bacteria is to isolate them from their natural habitats, determine their abundance within the complex soil microbiome, and characterize their EPSs-producing capabilities. In this study we aimed 1) to isolate and identify the EPSs-producing bacteria from the undisturbed permafrost soil and two degraded permafrost landscapes under lower and higher moisture regimes, respectively, 2) to determine the produced polysaccharides from individual bacterial isolates and 3) to determine the proportion of these EPSs-producing isolates in the total bacterial community.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from two different landscapes with degraded permafrost and from an undisturbed intact permafrost soil (hereafter, dry, wet and intact sites) located near Fairbanks, Alaska, USA. The detailed descriptions of the studied sites are reported in Liebmann *et al.* (2024). For each site, a soil profile was prepared with a depth of 100 cm for the dry and wet sites, while for the intact site, the profile was excavated until the permafrost table was reached in 45-55 cm soil depth. Soil samples were collected per horizon including organic layer, topsoil and subsoil by taking the material at various depth within each horizon. All samples were constantly kept at -20°C before processing. For the genetic material extraction, approx. three g of soil sample were immersed with two volumes of LifeGuard Soil Preservation Solution (Qiagen, Hilden, Germany) immediately after sampling. All Lifeguard-treated samples were constantly kept at 4°C until processing.

Isolation of bacteria

For the isolation of bacteria, one g of frozen soil from the topsoil of each site was serially diluted in sterile saline solution (0.9% NaCl) in a total volume of 1 ml. 100 µl of 10^{-2} to 10^{-4} dilutions were plated on three distinct culture media. Standard I (1 g glucose L^{-1}, 15 g peptone L^{-1}, 3 g yeast extract L^{-1}, and 6 g NaCl L^{-1}), Reasoner´s 2A agar (Reasoner and Geldreich 1985) and Nutrient agar (2 g Yeast extract, 5 g Peptone L^{-1}, 1g Lab-Lemco L^{-1} and 5 g NaCl L^{-1}) were utilized to ensure the recovery of a diverse range of bacteria, including general heterotrophic, slow-growing oligotrophic bacteria, and actinomycetes. The plates were incubated at 22°C for 48–72 hours. The 22°C was chosen based in higher CFU. After the incubation, well-grown single colonies were picked from each diluted agar plate and streaked onto corresponding fresh agar plates, followed by incubation. The streaking process was repeated a minimum of three times to ensure the isolation of pure colonies. The pure colonies were subsequently grown in the Nutrient liquid medium for DNA isolation.

Screening soil isolates for EPSs production

For screening of EPSs production, soil isolates were streaked on agar medium (EPS medium) specifically designed exopolysaccharides production described in Rühmann et al. (2015). The plates were incubated at 22°C for 48–72 hours. The isolates that were able to grow on EPS medium and formed a mucoidal/slimy colony were considered as potential EPSs-producers. They were further screened for exopolysaccharides production via automated analytical system involves various polysaccharide detection modules, including determination of total glucose content using phenol-sulfuric-acid transformation, and carbohydrate content by High-throughput-1-phenyl-3-methyl-5-pyrazolone (HT-PMP)-derivatization of the carbohydrates coupled to Electrospray Ionization Mass Spectrometry. as described in Rühmann et al. (2016). The production of polysaccharides measured as glucose equivalent by soil isolates exceeding 100 mg L^{-1} were considered as the greater EPSs-producers. This threshold was set to distinguish between isolates with high and low EPSs production, making it easier to focus on those with significant activity.

DNA extraction and Polymerase Chain Reaction

The extracted DNA from soil samples and isolates were quantified by Quantus fluorometer (Promega BioSystems, Sunnyvale, California, USA). For this, 2 µl of DNA sample was added to to 98 µl 1x TE buffer and 100 µl QuntiFluor dye (QuntiFluor dsDNA dye, Promega Corporation, Madison, USA) in a 1 ml clean Eppendorf tube. A blank and standard were also prepared by mixing 100 ul of 1x TE buffer with 100 µl of QuantiFluor dye or standard DNA provided by manufacturer, respectively. The tubes were incubated in the dark for 10 minutes and the fluorimeter was calibrated by standard and blank samples before quantifying the DNA samples.

Genomic DNA extracted from isolates was utilized to amplify the 16S rRNA gene using master mix composed of DNA, ddH_2O, PCR buffer, BSA and primers: 9bfm (forward primer: 5'-GAGTTTGATYHTGGCTCAG-3') and 1512uR (reverse primer: 5'-ACGHTACCTTGTTACGACTT-3'). For a positive control DNA of *Escherichia coli* (strain ATCC 9637) and for a negative control H_2O were used. The PCR amplification was performed using the PCR thermal cycler (SensoQuest GmbH, Germany) with the following protocol: initial denaturation for three minutes at 95°C, followed by 30 cycles of each denaturation for one minute at 95°C, annealing for one minute at 52°C and elongation for 90 seconds at 72°C. The final elongation was for 10 minutes at 72°C. After the amplification, 4 µl of PCR product was determined by electrophoresis on 1% agarose gel and visualized by trans illuminator (Azure 200, Azure Biosystem, Inc, US). The length of the band was compared to 1 kb DNA ladder and the correct PCR products were then sent to SEQme sequencing company for Sanger sequencing. The obtained sequence data underwent quality trimming and the forward and reverse reads were joined using Geneious Prime bioinformatics software (Java Version 11.0.18+10 (64 bit). The nucleotide sequences were then searched in 16S rRNA gene NCBI database by standard nucleotide BLAST.

Determination of proportion of EPSs isolates in total community

The proportion of the EPSs-producing soil isolates in the total relative proportion of bacterial community from different horizons of each landscape was determined. For this, 16S rRNA gene sequences from the EPSs-producing soil isolates were used as queries to search against a local 16S rRNA gene database constructed from the representative sequences of zero-radius operational taxonomic unit (zOTUs) from the total bacterial community using BLAST (Figure S1, Supporting Information). Afterward, a subset of assigned zOTUs sharing sequences with more than 97% similarity with 16S rRNA sequence of pure isolates was used to calculate their proportion within the total bacterial community across the different horizons within all the three studied sites.

The relative proportion of the bacterial community was calculated by dividing the count of each zOTUs by the sum of counts within each sample and further summarized across the sites and horizons. The data were filtered to include only the phylum level and the mean relative proportions were then

determined. The proportion of EPSs-producing soil isolates among the total bacterial community was calculated by taking the subset of assigned zOTUs with their relative proportions. The data were then filtered at genus level and computed to mean relative proportion. All the figures were generated using the ggplot v 3.4.4 package (H 2016).

RESULTS

Isolation and characterization of potential EPSs-producing bacteria

The bacterial isolation was carried out using three distinct culture media, resulting in the isolation of 17, 20, and 17 bacterial isolates from soil of the dry, wet and intact sites, respectively (Table 1, TableS1, Supporting Information). Isolates were grown on specific polysaccharide production medium for the formation of mucoidal "sticky" colonies as indication for the potential of these isolates to produce EPSs. The majority of soil isolates were able to grow in the form of mucoidal colonies on medium and signifying their potential as EPSs producers (Table S1, Supporting Information). 48 out of 54 isolates were successfully sequenced and categorized into three phyla: Firmicutes, Actinomycetota, and Pseudomonadota. Firmicutes were the most numerous across all three sites, followed by Actinomycetota and Pseudomonadota (Table S1, Supporting Information). Within the Firmicutes, six isolates related to *Bacillus mycoides* were present in all three soils, while isolates of *Peribacillus simplex*, *Neobacillus bataviensis* and *Mesobacillus subterraneus* were found only in wet soil (Table 1). For the Actinomycetota, four isolates closely related to *Frigoribacterium faeni* and *Pseudarthrobacter sp.* were from soil of the intact site and one isolate related to *Microbacterium flavescens* was obtained from dry soil site. *Curtobacterium oceanosedimentum* was found in soil from both intact and wet sites, but could not be found at the dry site. Isolates belonging to *Streptomyces brevispora* and *Microbacterium flavescens* were isolated from dry site (Table 1). Within the Pseudomonadota phylum, *Luteimonas arsenica* was found only in the dry site while *Phyllobacterium sp.* was identintifed in the wet site (Table 1). No potential EPSs-producing isolates of Pseudomonadota were isolated from soil of the intact site.

We found only 5.6% overlapping with one similar isolate such as *Bacillus mycoides* between soil of the dry, wet, and intact sites and another one common isolate such as *Curtobacterium oceanosedimentum* found in soil of both wet and intact sites (Fig. S2, Supporting Information).

Differences in EPSs production by soil isolates

In general, isolates with production of polysaccharide measured as glucose equivalent exceeding 100 mg L^{-1} were considered as potential EPSs-producing isolates. Varying amounts of polysaccharides were reported for different species which was averaged across species level (Fig. 1A, Table 1). Among

the Firmicutes, *Neobacillus bataviensis* exhibited the highest polysaccharide content, reaching up to 1149 mg L^{-1} followed by *Mesobacillus subterraneus* 911 mg L^{-1}. Additionally, the average polysaccharide across six *Bacillus mycoides* was approx. 224 mg L^{-1}, in which one isolate showed the highest at 515 mg L^{-1}. Within Actinomycetota, one isolate (A17) showing similarity to *Curtobacterium oceanosedimentum* yielded the highest polysaccharide about 1309 mg L^{-1} followed by the *Frigoribacterium faeni* isolates yielded average 939 mg L^{-1}. *Streptomyces brevispora* exhibited upto 342 mg L^{-1} and the least polysaccharide content was counted for *Streptomyces luozhongensis* 119 mg L^{-1}. *Pseudarthrobacter sulfonivorans* and *Microbacterium flavescens* was also yielded upto 140 mg L^{-1} (Fig. 1A, Table 1). From the phylum Pseudomonadota, isolate of family Enterobacteriaceae exhibited the polysaccharide up to 667 mg L^{-1}, *Luteimonas arsenica* 227 mg L^{-1} and *Phyllobacterium loti* 177 mg L^{-1}. In total, five isolates from soil of the dry site, 12 isolates from soil of the wet site, and nine isolates from soil of the intact site were identified as potential high EPSs-producers, as their polysaccharide equivalent as glucose surpassed 100 mg L^{-1}. The total amount of polysaccharide produced by these isolates at each site was tended to be different (Fig. 1B). The total polysaccharide content produced by isolates from soil of the wet site was higher approx. 4461 mg L^{-1}, followed by intact site which was approx. 3958 mg L^{-1} (Fig. 1B). The least total polysaccharide production was counted for the isolates of the dry site (1060 mg L^{-1}).

Sugar contents of EPSs produced by soil isolates

The soil isolates identified through 16S rRNA sequencing revealed a diverse array of EPSs enriched with various sugar derivatives determined by High-throughput-1-phenyl-3-methyl-5-pyrazolone (HT-PMP)-derivatization of the carbohydrates coupled to Electrospray Ionization Mass Spectrometry (Table 2). Among the Firmicutes isolates, *Bacillus mycoides* exhibited a substantial presence of hexoses, particularly glucose (Glc) (up to 65 mg L^{-1}) and galactose (Gal) (up to 102 mg L^{-1}), along with notable levels of amino hexoses, such as glucosamine (GlcN) (up to 62 mg L^{-1}) and N-acetylglucosamine (GlcNAc) (up to 12 mg L^{-1}). Similarly**,** *Bacillus proteolyticus* showed elevated concentrations of Gal (13) and GlcN (32 mg L^{-1}), supplemented with rhamnose (Rha) and mannose (Man)**.** The *Neobacillus bataviensis* and *Mesobacillus subterraneus* strains displayed even higher proportions of Glc (103–203 mg L^{-1})**,** Man**,** and uronic acids, particularly glucuronic acid (GlcUA) (up to 21 mg L^{-1}), Among the Actinomycetota, *Frigoribacterium faeni* demonstrated high concentrations of Glc (up to 136) and Man (up to 67 mg L^{-1}), with moderate levels of deoxy hexoses like Rha and GlcN. Similarly, *Curtobacterium oceanosedimentum* produced elevated amounts of Glc (114 mg L^{-1}) and GlcUA (33 mg L^{-1}), along with Rha. *Streptomyces brevispora* and *Streptomyces luozhongensis* were enriched in Glc (101–75 mg L^{-1}) and GlcN (41 mg L^{-1}) and GlcNAc (9 mg L^{-1}), In the Pseudomonadota group, *Luteimonas arsenica* exhibited substantial amounts of Glc (52 mg L^{-1}) and GlcN (48 mg L^{-1}), with levels of GlcNAc (14 mg L^{-1}) and GalN (8 mg L^{-1}).

Table 2: Sugar derivatives of EPSs produced by soil isolates measured in mg L^{-1}

Phylum	ID	Closest Blast Hit 16s rRNA sequencing	Hexoses			Uronic acids		Deoxy hexoses		Amino hexoses		
			Glc	**Man**	**Gal**	**GlcUA**	**GalUA**	**Rha**	**Fuc**	**GlcN**	**GalN**	**GlcNAc**
Firmicutes	S29	*Bacillus mycoides* strain ATCC 6462	45	4	6	0	0	0	0	38	7	11
	S31	*Bacillus mycoides* strain ATCC 6462	0	3	11	0	0	4	0	25	5	0
	S37	*Bacillus mycoides* strain ATCC 6462	53	5	0	0	0	0	0	43	8	11
	A6	*Bacillus proteolyticus* strain MCCC 1A00365	0	6	13	0	0	4	0	32	6	0
	A13	*Bacillus mycoides* strain ATCC 6462	65	5	1	0	0	0	0	62	10	12
	S28	*Bacillus mycoides* strain ATCC 6462	5	5	6	0	0	0	0	42	10	8
	A24	*Bacillus mycoides* strain ATCC 6462	0	3	102	0	0	0	0	0	0	0
	S38	*Bacillus subtilis* strain SBMP4	0	0	11	0	0	0	0	23	5	0
	S39	*Bacillus subtilis* strain SBMP4	0	5	0	0	0	0	0	7	0	0
	S3	*Peribacillus simplex* strain LMG 11160	0	4	0	0	0	0	0	0	0	0
	A25	*Neobacillus bataviensis* strain NBRC 102449	103	0	2	21	0	6	0	4	0	0
	S19	*Neobacillus pocheonensis* strain Gsoil 420	5	3	11	0	0	4	0	30	0	11
	S6	*Mesobacillus subterraneus* strain COOI3B	203	29	0	0	0	12	0	6	0	0
Actinomycetota	S17	*Frigoribacterium faen*i strain 801	68	67	2	0	0	7	0	4	0	0
	S18	*Frigoribacterium faeni* strain 801	136	46	0	0	0	9	0	6	0	0
	A17	*Curtobacterium oceanosedimentum* strain ATCC 31317	114	0	1	33	0	7	0	0	0	0
	S15	*Pseudarthrobacter sulfonivorans* strain ALL	11	7	0	0	0	0	0	7	0	0
	S23	*Pseudarthrobacter sulfonivorans* strain ALL	103	3	0	0	0	0	0	8	0	0
	A11	*Streptomyces brevispora* strain BK160	101	6	0	0	0	0	0	41	7	9
	A2	*Microbacterium flavescens* strain 401	0	12	0	0	0	7	0	0	0	0
	S9	*Streptomyces luozhongensis strain* TRM49605	75	5	0	0	0	0	0	3	0	0

	S7	*Curtobacterium oceanosedimentum* strain ATCC 31317	10	4	14	0	0	5	0	32	5	13
Pseudomonadota	A12	*Luteimonas arsenica strain 26-35*	52	8	0	0	0	0	0	48	8	14
	A22	*Phyllobacterium loti* strain S658	87	3	8	0	0	0	0	30	6	9
	A23	*Phyllobacterium endophyticum* strain PEPV15	39	4	0	0	0	0	0	46	8	15
	S5	*Leclercia adecarboxylata* ATCC 23216 = NBRC 102595 strain LMG 2803	63	4	125	0	0	0	0	0	0	0

Glc: Glucose, Man: Mannose, Gal: Galactose, GlcUA: Glucuronic Acid, GalUA: Galacturonic Acid, Rha: Rhamnose, Fuc: Fucose, GlcN: Glucosamine, GalN: Galactosamine, GlcNAc: N-Acetylglucosamine.

Proportion of EPSs-producing isolates in total bacterial community

The 16S rRNA gene sequence of 24 EPSs-producing isolates showed similarities higher than 97% to the sequence of 13 zOTUs (Table S2). The proportion of assigned zOTUs within the total bacterial community was different among and within soils of the three sites and horizons (Fig. 2). The potential EPSs-producing isolates represented approx. 2% of total bacterial community in soil of the dry site, ~0.5% in soil of the wet site, but below 0.36% in the soil of the intact site. Among the Firmicutes, *Bacillus subtilis* (zOTU3) was predominantly detected in highest proportion in both topsoil (~0.67%) and subsoil (~0.39%) of the dry site. However, it was almost undetected in the horizons of both the other sites (intact and wet sites), with levels below 0.01%. In contrast, *Bacillus mycoides* (zOTU2010) was only found with proportion of 0.03% in the topsoil of the wet site. *Peribacillus simplex* (zOTU2176) was the second after *Bacillus subtilis* found in high proportion of 0.24% in the topsoil of dry site, while it was not detectable in the subsoil. Additionally, their proportion in the topsoil of the wet site was 0.03% and was also detected to have less proportion in the organic layer and subsoil of both intact and wet site. Similarly, *Neobacillus bataviensis* and *Neobacillus pocheonensis* assigned to zOTU702 and *Mesobacillus subterraneus* (zOTU3623) were observed in proportions of 0.14% and 0.02% respectively, in the topsoil of the wet site, contrasting with their proportions of less than 0.001% in other horizons across all three sites (Fig. 2).From the Actinomycetota, two assigned zOUTUs, *Streptomyces luozhongensis* (zOTU1852) and *Streptomyces brevispora* (zOTU1848) were found in high proportions in the organic layer of the dry site of 0.36% and 0.1%, respectively, while the proportion of *Curtobacterium oceanosedimentum* (zOTU2238) was high in the organic layer of the intact site with 0.14%. Moreover, *Frigoribacterium faeni* (zOTU1136), *Pseudarthrobacter sp.* (zOTU1072) and *Microbacterium flavescens* (zOTU1055) shared almost similar proportion across the topsoil and subsoil in all three sites, with the exception of *Pseudarthrobacter sp.*, which was also found in the organic layer of the dry site (Fig. 2).The *Phyllobacterium endophyticum* of Pseudomonadota assigned to zOTU3875 exhibited a range of high proportion of 0.03% in the topsoil to a low proportion of 0.001% in subsoil of the wet site, while it was not fund in any horizons of the dry and intact sites. Lastly, *Luteimonas arsenica* (zOTU2611) was also found in all horizons except organic layer of dry site and subsoil of intact site.

DISCUSSION

Bacterial community and EPSs producers

Climate change globally induces elevated temperatures in both, atmosphere (Crowley 2000) and soil (Davidson and Janssens 2006). In permafrost regions, this temperature rise causes permafrost degradation which is one of the most challenging physical transitions for biological systems, involving alteration in soil moisture and temperature conditions (Gilichinsky et al. 1993). Our previous study

(Liebmann *et al.* 2024a) documented distinct differences in environmental conditions across the study sites. In the undisturbed permafrost soil, moisture content ranged from 43% to 57%, with temperatures between -0.3°C and 0.6°C. In contrast, the degraded permafrost sites showed a range of 44% moisture and temperatures between 3.4°C and 4.2°C for the wet site, and 27% to 36% moisture with temperatures ranging from 8.6°C to 10.5°C for the dry site (Liebmann et al. 2024). These changes collectively shift the bacterial community in both abundance and composition (Deslippe *et al.* 2012; Ganzert, Bajerski and Wagner 2014; Luláková *et al.* 2019; Zaitseva *et al.* 2022). To date, one of the dominant focuses on permafrost soil is to reveal the microbial distribution patterns and to identify the factors influencing the microbial diversity and abundance (Wilhelm et al. 2011; Deng et al. 2015b; Aksenov et al. 2021; Varsadiya et al. 2021a, 2021b, 2022; Petters et al. 2024). From our cultivations of EPSs-producing strains, we observed that *Bacillus mycoides* was the only isolate common to all sites with additional common *Curtobacterium oceanosedimentum* between wet and intact site, highlighting the significant variation in the EPSs-producing strains among the three sites (Table 1, Figure S2, Supporting Information). This variation can be attributed to the distinct environmental conditions among the sites, particularly in terms of moisture content and temperature fluctuations.

Effect of environmental conditions on EPSs-producing soil isolates

Bacteria employ a range of strategies to cope with cold temperatures, nutrient limitation, and fluctuating moisture levels, with the production of cryoprotectants like EPSs (Krembs *et al.* 2002). EPSs act as cryoprotective agent that stabilizes bacterial aggregates within the frozen soil or during the thaw when the moisture level is high and temperature fluctuations, providing physical protection by biofilm formation (Krembs *et al.* 2002; Casillo *et al.* 2017; Ali *et al.* 2020). From screening analysis of our isolates for their EPSs production capabilities, we found higher number of EPSs-producing isolates including *Neobacillus bataviensis*, *Mesobacillus subterraneus* and *Phyllobacterium* strains from soil of the wet sites, followed by intact site isolates including *Bacillus mycoides*, *Curtobacterium oceanosedimentum,* and *Frigoribacterium* strains (Table 1 and Fig. 1B). Similarly, the total polysaccharide content produced by isolates of each site was higher for the wet sites compared to that of the intact and dry site (Fig. 1B). Due to their high polysaccharides producing ability, these bacteria may utilize this strategy in their natural habitat to endure the extreme conditions, characterized by lower temperatures and higher moisture levels, as the conditions mentioned in Liebmann et al. (2024). However, bacterial species are modified according to their surrounding conditions (Lowe, Jain and Zeikus 1993) and produce EPSs accordingly (Xia et al. 2014; Jyoti et al. 2024). We found same strain *Curtobacterium oceanosedimentum* isolated from both intact and wet site, but the polysaccharide production was much higher for the one isolated form the intact site (Table 1). This difference may be due to subspecies-level genetic variation (Tu and Lin 2016) or an enhanced ability for EPSs

production in response to the higher SOC content characteristic of the intact site. Furthermore, previously identified as EPSs producers such as *Bacillus mycoides* (Farag *et al.* 2020), *Bacillus subtilis* (Voběrková *et al.* 2016; Mohite *et al.* 2019) and *Streptomyces* species (Homero et al. 2021) were also found in dry site, and produced different concentration of the polysaccharide. The presence of these isolate in dry conditions and their EPSs production may also represent an adaptive strategy to mitigate the challenges posed by low moisture availability by forming protective biofilms, retaining moisture, ensuring soil structural integrity and facilitating nutrient cycling (Deng et al. 2015a).

EPSs-producing isolates proportion in total community

We employed a bioinformatics approach to assess the proportion of EPSs-producing soil isolates within the proportion of total bacterial community present in the different horizons of intact and degraded permafrost soil. The utilization of high-throughput sequencing and bioinformatic tools allowed us to discern the taxonomic affiliations and relative abundance of EPSs-producing isolates within the overall microbial community structure (Table S2, Supporting Information). The proportion of EPSs-producing isolates within total community varied among the three sites, with the community structure exhibited greater diversity in soil of the wet and intact sites compared to the one on the dry site (Fig. 3). However, some of the assigned genera such as *Streptomyces* (zOTU1852 and zOTU1848), *Bacillus* (zOTU3) and *Peribacillus* (zOTU2176) to EPSs producing soil isolates found only or with higher proportion in the different horizon of the dry site (Fig 3). They are known for their ability to produce EPSs as potential response to low soil moisture level (Wang et al. 2003; Marvasi et al. 2010; Homero et al. 2021) and were also investigated for their role in soil aggregation (Aspiras *et al.* 1971; Raliya *et al.* 2014). On the other hand, the assigned genera *Mesobacillus*, *Neobacillus*, *Microbacterium and Pseudarthrobacter* were higher in topsoil and subsoil of the intact and wet sites compared to the dry site, whereas some of them were only found more prominent in the subsoil (Fig. 3). Similarly, *Curtobacterium oceanosedimentum* (zOTU2238) was found in wet subsoil and intact throughout all the horizon. The presence of these genera in the wet and intact sites suggests their acclimation to high moisture content and low temperatures through the EPSs production in their surroundings. Previous studies also reported the EPSs production by the strains of *Microbacterium*, *Curtobacterium* and *Pseudarthrobacter*, and contribute to soil aggregate formation (Godinho and Bhosle 2009; Bourles *et al.* 2019; Liu *et al.* 2022). The varying proportions of these EPSs-producing isolates across the three sites (Fig. 2), alongside their EPSs production capabilities (Fig. 1A), highlight their adaptation to the environments after permafrost degradation, that may have role in soil aggregate stability (Kaci et al. 2005; Vardharajula and Sk 2014; Deka et al. 2019).

CONCLUSION

Bacteria have adopted unique mechanisms to cope with and survive in cold habitats experiencing environmental fluctuations. One of these adaptations includes the production of cryoprotective agents in the form of EPSs (Krembs *et al.* 2002). These agents also act as a gluing substance, physically promoting soil aggregation (Costa et al. 2018). The current study provides information about the bacterial strains isolated from two degrading permafrost soils and an undisturbed intact permafrost soil. These isolates are particularly important for EPSs production in order to survive in extreme conditions characterized by low temperatures, varying moisture levels and limited nutrients. We observed a notable tendency for high polysaccharide production with different sugar derivatives by the strains, indicating that these are potentially high EPSs producers. They were also observed in varying proportion within the total bacterial community in both degraded permafrost soils and undisturbed intact permafrost soil. The results of this study suggest that the presence of these EPSs-producing strains may influence the soil aggregate formation in different degradation landscapes of permafrost soils.

FUNDING

The work was financially supported by the joint German-Czech Project "CRYOVULCAN – Vulnerability of carbon in Cryosols", with the individual grants GU 406/35-1, UR 198/4-1, VO 2111/6-1, GACR project n. 20-21259J

DATA AVAILABILITY

Raw fastq 16S rRNA gene sequences of the whole microbiome were uploaded to European nucleotide archive (ENA) under the study n. PRJEB79237. Sequences of the pure strains were uploaded also to ENA under the project n PRJEB79239.

AUTHOR CONTRIBUTIONS

CV, JB, PL, TU and GG completed fieldwork and collected samples from Fairbanks, Alaska, USA. MW and SZ screened soil samples for bacterial isolation. MW, SZ performed molecular analysis and MW JB and MV analyzed the sequencing data. BR screened the isolates for EPSs production, MW and JB wrote the manuscript, and CV, GG, HW and OS contributed to and have approved the final manuscript.

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Table 1. Taxonomic classification of soil isolates identified as potential EPSs producers, that produced more than 100 mg L^{-1}. Isolates sharing more than 97% sequence similarity are presented at the species level and lower than 94% are presented at family level. Polysaccharide contents are listed for isolates exceeding 100 mg L^{-1} production

Phylum	**ID**	**Closest Blast Hit 16s rRNA sequencing**	**% Identity**	**Locality**	**Polysaccharide content** (mg L^{-1})
Firmicutes					
	S29	*Bacillus mycoides* strain ATCC 6462	99.9	Intact	145.34
	S31	*Bacillus mycoides* strain ATCC 6462	100	Intact	180.69
	S37	*Bacillus mycoides* strain ATCC 6462	97.6	Intact	150.05
	A6	*Bacillus proteolyticus* strain MCCC 1A00365	98.0	Intact	170.17
	A13	*Bacillus mycoides* strain ATCC 6462	100	Dry	212.37
	S28	*Bacillus mycoides* strain ATCC 6462	100	Dry	140.79
	A24	*Bacillus mycoides* strain ATCC 6462	100	Wet	515.24
	S38	*Bacillus subtilis* strain SBMP4	99.2	Wet	171.48
	S39	*Bacillus subtilis* strain SBMP4	98.0	Wet	164.54
	S3	*Peribacillus simplex* strain LMG 11160	96.5	Wet	100.0
	A25	*Neobacillus bataviensis* strain NBRC 102449	99.0	Wet	1148.6
	S19	*Neobacillus pocheonensis strain Gsoil 420*	99.1	Wet	146.0
	S6	*Mesobacillus subterraneus* strain COOI3B	98.2	Wet	911.44
Actinomycetota					
	S17	*Frigoribacterium faeni* strain 801	99.4	Intact	1309.14
	S18	*Frigoribacterium faeni* strain 801	99.5	Intact	735.79
	A17	*Curtobacterium oceanosedimentum* strain ATCC 31317	99.3	Intact	1309.14
	S15	*Pseudarthrobacter sulfonivorans* strain ALL	98.6	Intact	115.63
	S23	Micrococcaceae	91.7	Intact	208.21
	A11	*Streptomyces brevispora* strain BK160	99.5	Dry	342.92
	A2	*Microbacterium flavescens* strain 401	98.3	Dry	136.85
	S9	*Streptomyces luozhongensis* strain TRM49605	99.5	Wet	118.63
	S7	*Curtobacterium oceanosedimentum* strain ATCC 31317	98.8	Wet	216.69
Pseudomonadota					
	A12	*Luteimonas arsenica* strain 26-35	99.8	Dry	227.26
	A22	*Phyllobacterium loti* strain S658	99.0	Wet	177.28
	A23	*Phyllobacterium endophyticum* strain PEPV15	99.5	Wet	124.15
	S5	Enterobacteriaceae	91.3	Wet	667.23

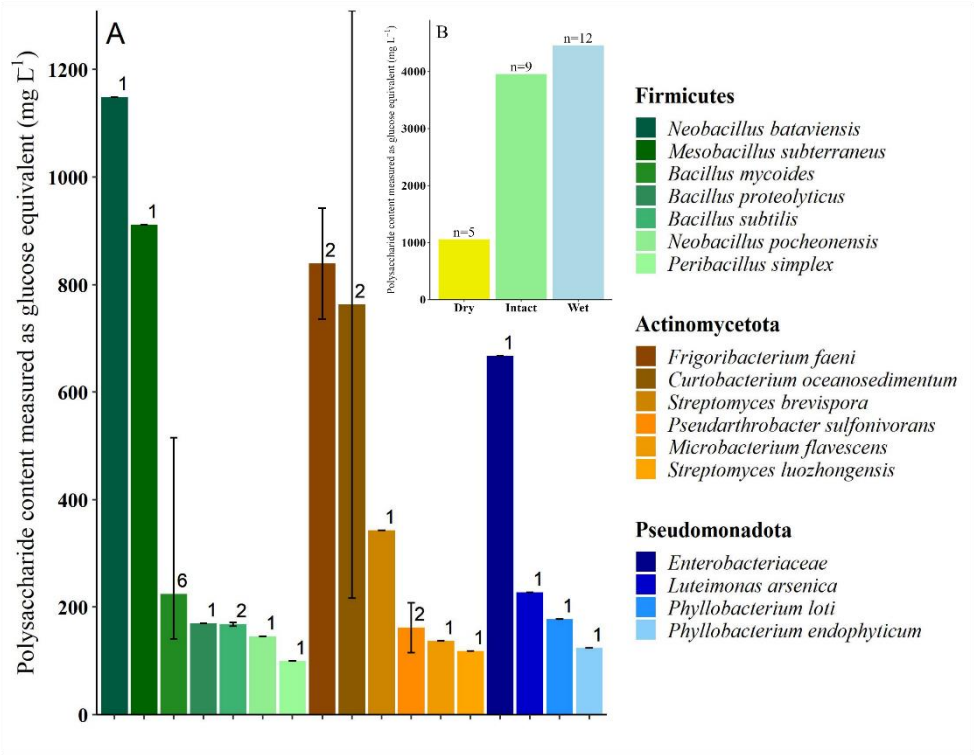

Figure 1. A: Polysaccharide production measured as glucose equivalent in mg L^{-1}. Each bar represents the average polysaccharide content for a family/species, with the numbers on top indicating the number of isolates. Whiskers represent the higher and lower amount of polysaccharide contents. B: Total polysaccharide content produced by isolates of each site, including only those with production exceeding 100 mg L^{-1}. The variable "n" represents the number of isolates.

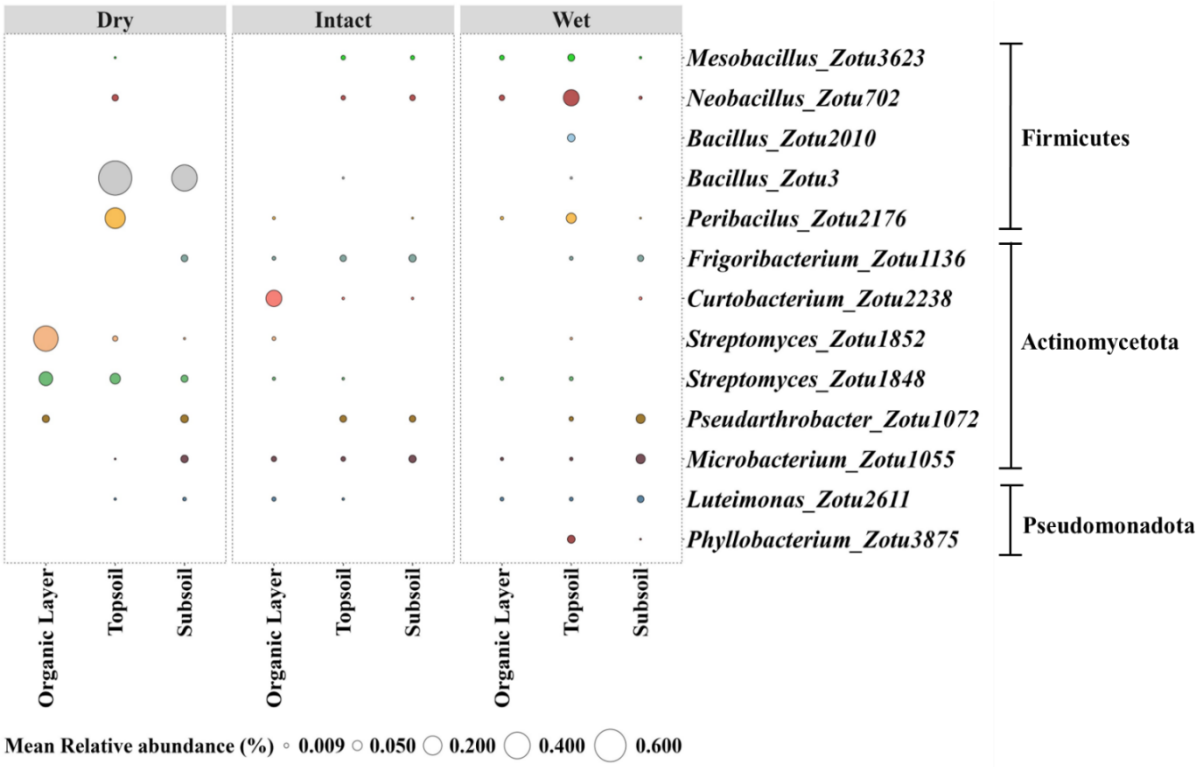

Figure 2. Relative proportion of assigned zOTUs for EPSs-producing soil isolates at genera level in total bacterial community of two sites with degraded permafrost soil (dry and wet site) and sire with undisturbed permafrost soil (Intact site).