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ORIGINAL ARTICLE

Involvement of laccase-like enzymes in humic substance degradation by diverse polar soil bacteria

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Abstract

Humic substances (HS) in soil are widely distributed in cold environments and account for a significant fraction of soil's organic carbon. Bacterial strains ($n = 281$) were isolated at 15 °C using medium containing humic acids (HA), a principal component of HS, from a variety of polar soil samples: 217 from the Antarctic and 64 from the Arctic. We identified 73 potential HA-degrading bacteria based on 16S rRNA sequence similarity, and these sequences were affiliated with phyla *Proteobacteria* (73.9%), *Actinobacteria* (20.5%), and *Bacteroidetes* (5.5%). HA-degrading strains were further classified into the genera *Pseudomonas* (51 strains), *Rhodococcus* (10 strains), or others (12 strains). Most strains degraded HA between 10 and 25 °C, but not above 30 °C, indicating cold-adapted degradation. Thirty unique laccase-like multicopper oxidase (LMCO) gene fragments were PCR-amplified from 71% of the 73 HA-degrading bacterial strains, all of which included conserved copper-binding regions (CBR) I and II, both essential for laccase activity. Bacterial LMCO sequences differed from known fungal laccases; for example, a cysteine residue between CBR I and CBR II in fungal laccases was not detected in bacterial LMCOs. This suggests a bacterial biomarker role for LMCO to predict changes in HS-degradation rates in tundra regions as global climate changes. Computer-aided molecular modeling showed these LMCOs contain a highly-conserved copper-dependent active site formed by three histidine residues between CBR I and CBR II. Phylogenetic- and modeling-based methods confirmed the wide occurrence of LMCO genes in HA-degrading polar soil bacteria and linked their putative gene functions with initial HS-degradation processes.

Introduction

Arctic and Antarctic tundra is characterized by long periods of low temperatures and short growing seasons. Freezing temperatures result in most tundra-region soils being permafrost that contains a large amount of soil organic matter (SOM) due to a low rate of microbial degradation. Humic substances (HS) account for 60–80% of SOM and are significant components of terrestrial ecosystems, including the Arctic and Antarctic regions. These chemically complex and structurally large organic molecules include various aromatic rings and aliphatic chains with O-, N-, and S-containing functional groups. HS originate through spontaneous condensation reactions among decomposition products of lignocellulosic plant material and surrounding smaller molecules (Gramss et al. 1999; Lipczynska-Kochany 2018). The HS in polar tundra soils are composed of materials with low lignin content, which are mostly derived from grasses, mosses, and lichens (Abakumov and Alekseev 2018). Although HS have been considered as macromolecular assemblies of high-molecular-weight compounds (traditional polymeric model), leading to a rather slow microbial decomposition rate, a new one (supramolecular model) has recently emerged that HS should be regarded as weak supramolecular associations of heterogeneous and relatively small molecules, allowing easier microbial access (Lehmann and Kleber 2015; Lipczynska-Kochany 2018). Large HS and HS-derived small compounds, produced through microbial degradation, regulate the growth of plants and microorganisms through various and continuous interactions within soils.

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Considering their large contribution to SOM, even small increases in the HS-degradation rate due to increases in soil temperature can result in significant changes (Lehmann and Kleber 2015) in the tundra soil ecosystems at both poles.

Although microbial degradation of HS is well-established (Esham et al. 2000; Baldrian 2006; Fodil et al. 2011), reports on polar bacteria with the capacity to degrade HS are rare, and the mechanisms involved in this degradation process are unclear (Park and Kim 2015; Kim et al. 2019). Recently, HS degradation by fungi and bacteria have been ascribed to nonspecific oxidizing enzymes such as manganese peroxidase, lignin peroxidase, versatile peroxidase, and laccase. For example, fungal laccases from *Cladosporium* and *Polyporus* have been characterized for their roles in HS degradation and transformation (Baldrian 2006), and the biodegradation of lignin, an organic compound structurally similar to HS, by *Novosphingobium* spp. (Chen et al. 2012) and by *Pseudomonas* sp. (Granja-Travez and Bugg 2018) has been directly associated with bacterial laccase activity. Considering that HS are derived from lignin-containing plant material and exhibit structural similarities to lignin, bacteria and fungi might share initial HS catabolic pathways similar to those for lignin (Kim et al. 2019).

Laccases are multicopper oxidoreductases with broad substrate specificity, making them potential candidates for scientific and industrial applications, and they can oxidize a variety of polymeric substances, including polyaromatic hydrocarbons (PAH), lignin, and HS. Through substrate oxidation reactions, laccases create reactive radicals, which participate in further polymerizations, degradations, or cleavage of phenolic rings (Ausec et al. 2011). Because the biological functions and mechanisms of laccases are diverse and not yet well defined, some are referred to as laccase-like multicopper oxidases (LMCOs). Soil bacteria may play a critical role in the HS degradation process due to their abundance, diversity, and catabolic versatility (e.g., aromatic ring cleavage) (Van Trump et al. 2006). Evidence supporting a wide occurrence of LMCOs in bacteria is increasing (Claus 2003; Granja-Travez et al. 2018), and bacterial LMCOs may catalyze the initial degradative steps of recalcitrant polymeric HS (Freedman and Zak 2014; Granja-Travez and Bugg 2018). This is in agreement with previous reports that bacterial LMCOs are most diverse in the uppermost humic level of forest soil and high-organic peat soil (Ausec et al. 2011). However, our understanding of bacterial capacity and mechanisms for HS degradation in cold environments is still limited. In this study, we isolated diverse soil bacteria from both polar regions, characterized their cold-adapted ability to degrade HS, and amplified LMCO gene fragments from them. We assume that bacterial LMCOs catalyze the initial oxidative degradation of HS with other nonspecific oxidizing enzymes in tundra soils and expect that they will be applied as biomarkers to predict changes in HS degradation rates due to tundral warming in both polar regions.

Materials and methods

Natural humic acid preparation

Tundra soil was sampled from the top layer (0–20 cm) of a subarctic region (64° 50.68′ N, 163° 42.69′ W, Council, Alaska, USA) rich in organic matter containing HS. The sampling site was composed of moist acidic tussock tundra, with cotton grass, blueberry, and moss beds being dominant vegetation. The soil was characterized as follows: pH 4.6; total organic carbon, 40.5%; total nitrogen, 1.9%; and C/N ratio, 21.3. Soil HS are composed mainly of humic acids (HA) and fulvic acids (FA), the ratio of which differs depending on location and environmental conditions. The detailed structure of HA depends on the plant source and specific formation conditions, yet the average properties are remarkably similar. Natural HA were extracted using a method modified from previously described ones (Dick et al. 1999; Park et al. 2015) as below. The soil sample was completely dried at 45 °C overnight and passed through a 1-mm sieve to remove coarse plant debris and small stones. Twenty grams of soil were treated with 500 mL of 0.5 N NaOH for 3 h with continuous shaking and then incubated for 12 h at 4 °C. The NaOH extract was centrifuged at 5500×*g* for 15 min, and the resultant supernatant was acidified to pH 2.0 with 5.0 N HCl. The insoluble fraction containing HA was separated from soluble fulvic fraction containing FA and more hydrophilic biological molecules by centrifugation at 10,000×*g* for 15 min, redissolved in 0.1 N NaOH, and centrifuged at 4000×*g* for 7 min. The HA pallet was lyophilized, and the resulting solid HA were weighed.

Isolation of HA-degrading bacteria

Two Korean research stations, Dasan Station (78° 55′ N, 11° 56′ E, Ny-Ålesund, Svalbard, Norway) and King Sejong Station (62° 13′ S, 58° 47′ W, King George Island, Antarctica) located in both polar maritime tundra regions, have undergone rapid global climate change over the past several decades. A variety of tundra soil samples containing decaying moss, lichen, or plant debris were collected from surface to 5-cm depth at different locations (23 Arctic sites and 51 Antarctic sites) surrounding the stations in August 2012 and January 2012, respectively. The SOM-rich samples were homogenized and stored at − 20 °C for later analysis. In the laboratory, one gram of each soil sample was mixed with 20% glycerol and the soil-glycerol stocks were homogenized and placed at 4 °C for 1 h. The supernatant

(50 µL) was spread on minimal salt basal (MSB) (Stanier et al. 1966) agar plates containing 0.1% (w/v) HA: 1.0 g of HA powder was dissolved in 0.2 N NaOH and then added to 1.0 L of MSB liquid (MSB + HA; Park and Kim 2015). The plates were incubated at 15 °C for 14 days. To confirm the bacterial cultures' abilities to degrade and utilize HA as a carbon and energy source, colonies were successively inoculated twice on MSB + HA plates. We collected 73 pure cultures from the resulting 281 colonies, identified them as putative cold-adapted HS-degraders, and deposited them into Polar and Alpine Microbial Collection (PAMC) operated by Korea Polar Research Institute under accession numbers PAMC 27290–PAMC 27362.

Taxonomic identification of bacterial isolates

Bacterial isolates were identified by phylogenetic analysis of 16S rRNA gene sequences. The 16S rRNA gene was PCR-amplified from bacterial genomic DNA with the universal primers 27F (5′-AGAGTT TGATCMTGGCTCAG-3′) and 1492R (5′-GGTTAC CTTGTTACGACTT-3′). Amplicons were sequenced with the same primers used for PCR amplification and 926R (5′-CCGTCAATTCCTTTRAGTTT-3′). Each 16S rRNA gene sequence was taxonomically classified using the EzBioCloud database (Yoon et al. 2017). The resultant top hits were further used for phylogenetic analyses to determine evolutionary lineages of the isolates. Phylogenetic trees were reconstructed by the neighbor-joining method based on distance matrices generated by Kimura's two-parameter model using MEGA X program (Kumar et al. 2018). The robustness topologies were assessed by bootstrap analyses based on 1000 replications of the sequences. Species affiliations of the bacterial isolates were predicted when the isolate formed a monophyletic group with a reference species and shared 98.5% or higher similarity with that species. The 16S rRNA gene sequences obtained in this study were submitted to the NCBI GenBank database under the accession numbers MT555326–MT555397.

Temperature-dependent HA-degradation by bacterial isolates

Bacterial HA-degrading rates at various temperatures were measured according to a previous method (Park et al. 2015). Bacterial cells from glycerol stocks in 96-well plates were transferred onto MSB + HA plates and incubated at 4, 10, 15, 20, 25, 30, or 37 °C for 12 days. During incubations, the isolates' growth rates were scored as follows: 4, 3, 2, or 1, based on the formation of dense, ≥ 5.0 mm-diameter colonies within 3, 6, 9, or 12 days, respectively. A score of 0 indicated no cell growth within 12 days.

Cloning, sequencing, and analysis of bacterial LMCO genes

Available bacterial LMCO gene sequences are well-conserved between copper-binding regions (CBR) I and II, which enabled to design degenerate primers (Kellner et al. 2008) for the amplification of LMCO genes from the soil bacterial isolates (Cu1AF for CBR I: 5′-ACMWCBGTY CAYTGGCAYGG-3′ and Cu2R for CBR II: 5′-GRCTGT GGTACCAGAANGTNCC-3′). To PCR-amplify bacterial LMCO gene fragments, 1 µL of bacterial genomic DNA extracted from HA-degrading isolates was added to a 50-µL reaction mixture containing 5 µL 10 × *Taq* buffer, 4 µL dNTPs (2.5 mM each), 2 µL of each primer (10 mM), and 1 U *Taq* DNA polymerase. PCR reactions were carried out using an initial denaturing step (5 min at 95 °C), followed by 35 cycles of amplification (1 min at 95 °C, 45 s at 56 °C, 30 s at 72 °C), and a final elongation step (10 min at 72 °C). Amplicons from each respective source (Arctic and Antarctic bacteria) were pooled by region, and each mixture was ligated into the pTOPO TA V2 vector (Enzynomics, Korea) to generate two bacterial LMCO gene libraries that were introduced into *E. coli* DH5α. Among the white colonies grown on Luria broth plates containing 100 µg/mL ampicillin, we randomly selected 240 clones for insert sequencing with primers M13-Forward and M13-Reverse. After trimming primer sequences and removing identical sequences, the edited sequences (see Supplementary data) were subjected to BLASTP similarity searches against the NCBI non-redundant protein database to determine their putative functions.

Multiple sequence alignment and homology modeling

Nucleotide sequences of laccases or LMCOs from fungi (*Trametes versicolor*, Necochea et al. 2005) or bacteria (*Bacillus* sp. HR03, Mohammadian et al. 2010) were retrieved from the NCBI database. These genes encode enzymes with oxidative activities against laccase-specific substrates such as 2,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid and 2,6-dimethoxyphenol. The gene sequences were translated into amino acid sequences and aligned with those from the soil bacterial LMCOs using ClustalX2 and visualized with the GeneDoc program. To evaluate the alignment predictions, a homology model structure of one LMCO fragment, Antarctic LMCO-08, was generated using the SWISS-MODEL homology modeling web server (http://swissmodel.expasy.org/). The 1.90 Å resolution

crystal structure of the fungal laccase from *T. versicolor* (PDB code 1GYC) was used as a template structure because its amino acid sequence shares high similarity with the Antarctic LMCO-08 sequence, and it is thought to be a sole HA-decolorizing laccase (Fakoussa and Frost 1999). The homology model was validated with PROCHECK and quality-tested using QMEAN, and related structural alignment and figures were generated using the PyMOL molecular graphics system (www.pymol.org).

Results

Isolation and diversity of HA-degrading bacteria

To obtain information on the diversity and spatial distribution of HS-degrading bacteria in polar tundra soils, various soil samples were collected from the Arctic (23 sites) and Antarctic (51 sites) regions. The samples were suspended in 20% glycerol and inoculated on MSB minimal medium containing HA as a carbon source. After incubation for 7 days at 15 °C, many bacterial colonies appeared on the plates (indicative of HA-degrading ability), of which 281 (64 from the Arctic region, and 217 from Antarctica) were randomly selected on the basis of their morphology. After further sub-culturing on MSB + HA plates, 73 isolates (20 from the Arctic region, and 53 from Antarctica) were finally selected for their significant HA-degradative abilities based on colony sizes. Similarities between 16S rRNA gene sequences of type strains from the EzBioCloud database and soil isolates in this study suggested their affiliations with *Gammaproteobacteria* (33 Antarctic isolates and 19 Arctic isolates; a total of 52 isolates, 71.2%), *Actinobacteria* (14 and 1; 15 total isolates, 20.5%), *Bacteroidetes* (4 and 0; 4 total isolates, 5.5%), and *Betaproteobacteria* (2 and 0; 2 total isolates, 2.7%) (Supporting information Tables S1 and S2; Fig. 1). Members of phylum *Proteobacteria* were the most abundant in organic matter-rich soils from both polar regions, with *Gammaproteobacteria* the most prevalent class from that phylum. At the genus level, strains of *Pseudomonas* accounted for 95% and 60% among the Arctic and Antarctic isolates, respectively, followed by strains of genus *Rhodococcus* (phylum *Actinobacteria*) from Antarctic soil.

Temperature-dependent HA-degradation of bacterial isolates

Generally, microorganisms that live in cold environments have optimum growth temperatures of 15 °C or less (Tribelli and Lopez 2018). Therefore, HA-degrading bacteria were initially isolated at 15 °C from the HS-rich soils in Arctic and Antarctic tundra. To characterize HS-degradation rates according to temperature change (4–37 °C), the 73 putative cold-adapted bacterial isolates were scored for their abilities to grow on HA-supplemented MSB agar (Fig. 2). Overall, the number of isolates with higher scores rapidly increased as the temperature rose to 20–25 °C. More specifically, no isolates were observed with a score of 4 at 10 °C, yet 18% of the isolates received a score of 4 at 25 °C. Above 30 °C, no isolates were able to grow at all, indicating that these bacteria have adapted to survive at low temperatures, but increased soil temperatures may enhance their HS-degradation rates up to a certain point.

PCR-based detection of LMCO genes from bacterial isolates

We performed PCR to estimate LMCO gene diversity in cold-adapted HA-degrading bacteria. Laccases and LMCOs usually contain several copper atoms at their catalytic center that are required for enzymatic activity, thus classifying them as multicopper oxidases (Hoegger et al. 2006). Therefore, the nucleotides encoding copper-containing regions are suitable targets for detecting LMCO genes and analyzing their diversity and spatial distributions. When we performed PCR with degenerate primers specific for conserved CBRs I and II, ~ 142 bp amplicons were obtained from 52 of the 73 HA-degrading bacterial isolates. Specifically, putative bacterial LMCO genes were retrieved from 14 Arctic region isolates ($n = 20$ total) and from 38 Antarctic isolates ($n = 53$ total) (Table S2; Fig. 1). This PCR-based approach showed that the majority (71%) of the isolates exhibiting HA-degrading activity possessed at least one LMCO gene. With respect to their microbial sources, the gene fragments were amplified from 38 (75%) and 8 (80%) of 51 *Pseudomonas* spp. and 10 *Rhodococcus* spp. isolates, respectively.

Sequence analysis of bacterial LMCO genes

Two bacterial LMCO libraries (Arc-LMCO and Ant-LMCO) were constructed with LMCO genes amplified from the 14 Arctic and 38 Antarctic isolates. When random clones from each library (80 and 160 clones from Arc- and Ant-LMCO, respectively) were sequenced and analyzed using the NCBI BLASTP algorithm, 75 (94%) of Arc-LMCO and 82 (51%) of Ant-LMCO clones were identified as genes coding for multicopper oxidases such as laccase and LMCO. After removing redundant clone sequences, 14 and 16 different clone sequences were obtained from the Arc- and Ant-LMCO libraries, respectively. When the gene fragments were compared with protein sequences of fungal and bacterial LMCOs, they were analyzed to include the conserved CBR I and II regions that are needed for laccase activity.

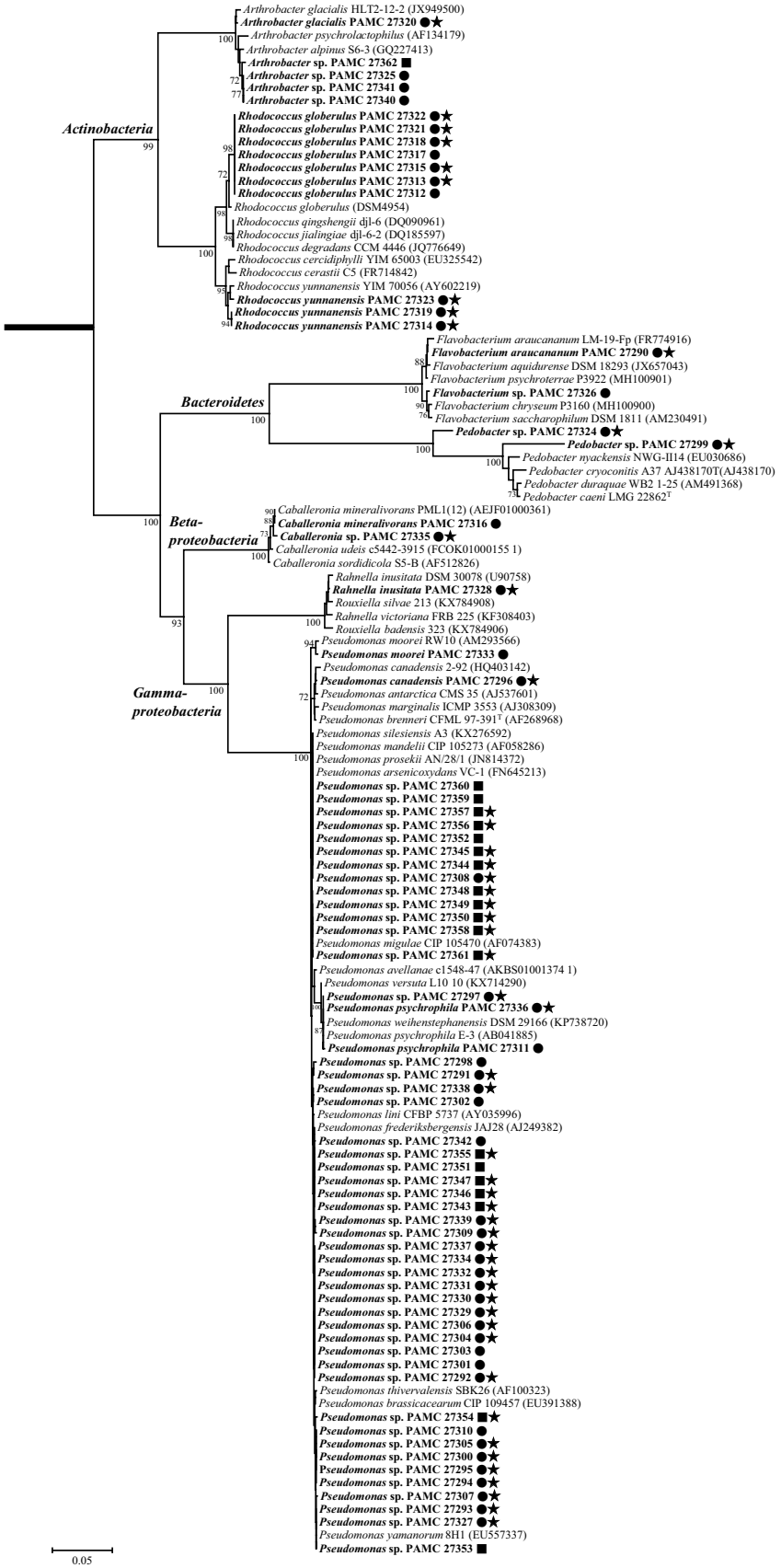


Fig. 1 Neighbor-joining tree of 16S rRNA gene sequences from polar HA-degrading bacterial isolates with closely related reference species. Representative isolates for each phylotype are indicated by bold letters, and branches are supported by high bootstrap values (> 70%) as thick lines. Bar, 10 nucleotide substitutions per 100 nucleotides. Squares and circles indicate the Arctic and Antarctica, respectively. A star indicates the presence of LMCO gene amplicons

Sequence alignment and homology modeling of bacterial LMCOs

Amino acid sequence alignments (Fig. 3a) showed that CBR I and II sequences from HA-degrading bacteria possess highly-conserved regions compared with those from previously characterized laccases or LMCOs from *Bacillus* sp. HR03, *T. versicolor*, and other microorganisms. In addition to CBR 1 and II, bacterial LMCOs shared several conserved residues, including one aspartic acid (D) and one glycine (G) at alignment positions 17 and 18 that were identical in all the isolates. However, the bacterial LMCO sequences showed a significant difference (the absence of cysteine, C) between CBR I and CBR II compared with fungal gene fragments, which has been previously observed in forest and grassland Cambisol soil samples (Kellner et al. 2008). As well as LMCO's activity for lignin (a surrogate substrate for HA), the abundance of LMCOs in bacterial isolates, and their ability to degrade HA, suggests the enzyme may catalyze HS oxidative degradation in natural habitats.

In addition to protein sequence analysis, homology modeling (Fig. 3b) further verified that these LMCOs contain a highly-conserved copper-dependent active site formed by three histidine residues. In a previous study, the copper-binding properties of fungal laccase from *T. versicolor* and its enzymatic mechanism were investigated by X-ray crystallographic structural modeling (Piontek et al. 2002). Taken together, our molecular modeling and protein sequence analysis data suggest LMCOs from polar soil HA-degrading bacteria are functional and may utilize an active site and copper-binding conformation similar to *T. versicolor* laccase to accomplish HS degradation.

Fig. 3 Multiple sequence alignment and homology modeling of bacterial LMCO genes. **a** When laccase or LMCO sequences from various microorganisms and polar bacteria were aligned, highly conserved residues (shaded black) and similar residues (shaded gray) were detected in CBR I and II regions. Three histidine residues present in the copper-binding site of the fungal laccase of *T. versicolor* (corresponding to His84, His86, and His129) were similar to those from *Bacillus* sp. HR03, other microorganisms, and HA-degrading bacteria. Closed circles indicate functionally characterized laccase or LMCO, and open circles denote structurally characterized LMCOs. Histidine residues are indicated by triangles, and the unique cysteine residue for fungal LMCO genes is indicated by a star. **b** Homology modeling structure of Antarctic LMCO-08. The left panel shows a ribbon diagram with superimposed structures of laccase from *T. versicolor* (PDB code 1GYC, green) and a homology model structure of Antarctic LMCO-08 (magenta). The right panel shows a close-up view of the active-site structure of *T. versicolor* laccase. Copper ions are depicted as red spheres, and the copper-binding histidine residues are shown in stick representation. Aligned regions shown in **a** (highlighted in cyan) are similarly highlighted in the structure (corresponding to residues 81–130) of *T. versicolor* laccase. These results revealed that polar bacterial LMCOs have highly conserved histidine residues for copper binding, but they do not have a disulfide bond (Cys104-Cys508) found in *T. versicolor* laccase. The corresponding sequence position of Cys104 of *T. versicolor* laccase is indicated with the star in **a**

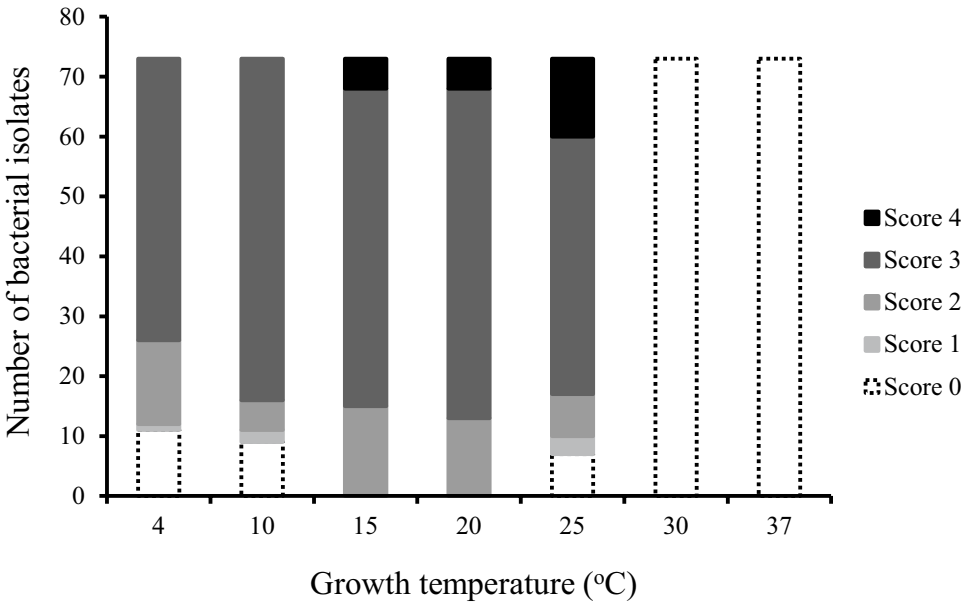
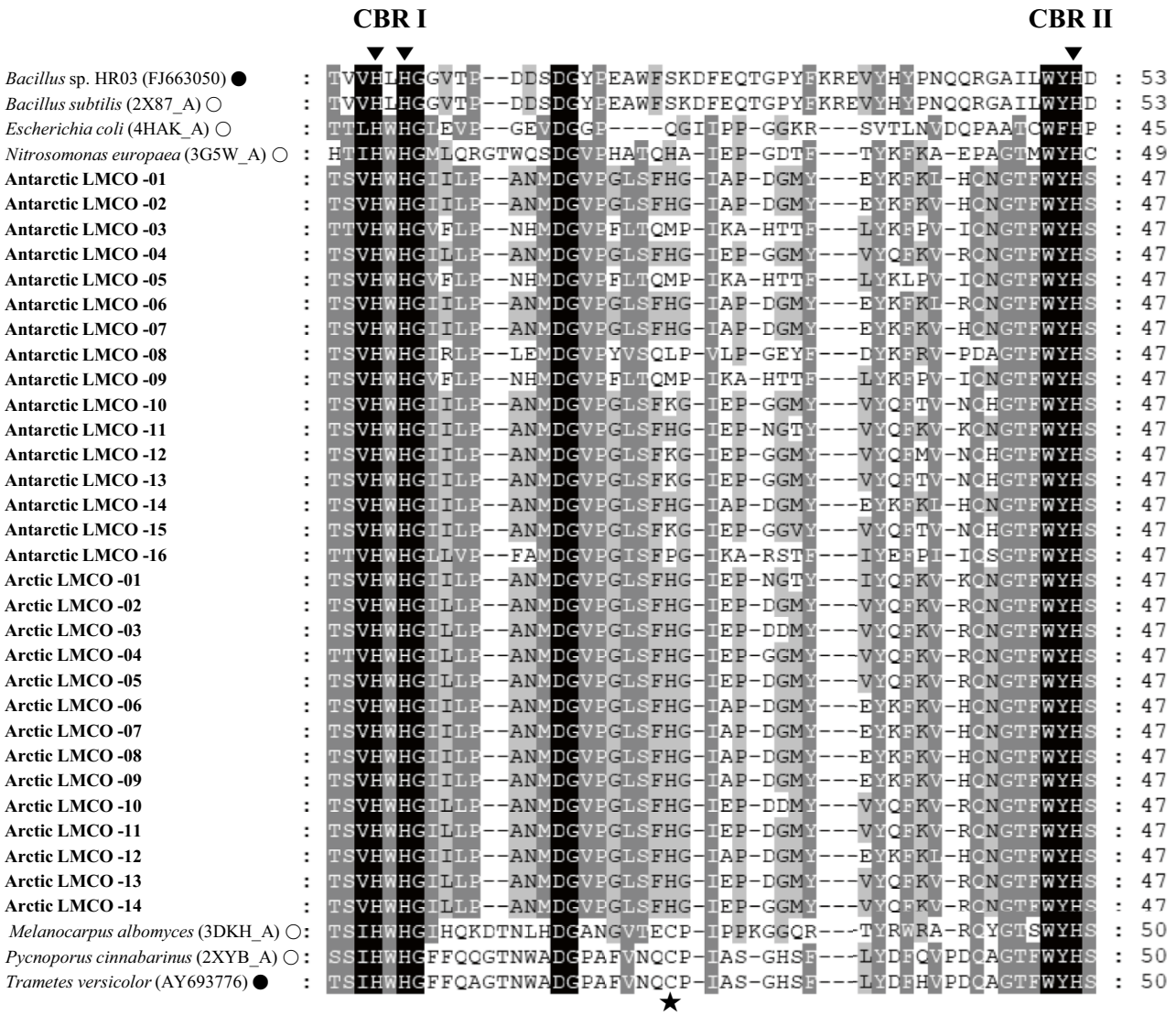
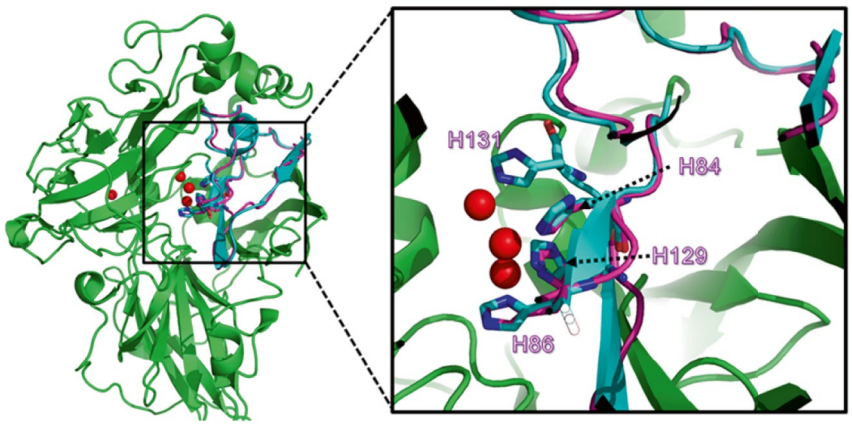


Fig. 2 Temperature effects on HA utilization by polar bacterial isolates. The scores represent the degree of HA utilization from 1 to 4. A higher number indicates better bacterial growth on MSB minimal medium plates supplemented with HA as the carbon source

a


CBR I
CBR II
Bacillus sp. HR03 (FJ663050) ● : TVVHLHGGVTP--DDSDGYEAWFSKDFEQTGPYFKREVYHYPNQQRGAILWYHD : 53
Bacillus subtilis (2X87_A) ○ : TVVHLHGGVTP--DDSDGYEAWFSKDFEQTGPYFKREVYHYPNQQRGAILWYHD : 53
Escherichia coli (4HAK_A) ○ : TTLHWHGIEVP--GEVDGGP-----QGIIPP-GGKR---SVTLNVDQPAATGWFHP : 45
Nitrosomonas europaea (3G5W_A) ○ : HTIHWHGMLQRGTWQSDGVEHATQHA-IEP-GDTF---TYKFKA-EPAGTMWYHC : 49
Antarctic LMCO -01 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKL-HQNGTFWYHS : 47
Antarctic LMCO -02 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKV-HQNGTFWYHS : 47
Antarctic LMCO -03 : TTVHWHGVFLP--NHMDGVPFLTQMP-IKA-HTTF---LYKFPV-IQNGTFWYHS : 47
Antarctic LMCO -04 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-GGMY---VYQFKV-RQNGTFWYHS : 47
Antarctic LMCO -05 : TSVHWHGVFLP--NHMDGVPFLTQMP-IKA-HTTF---LYKLPV-IQNGTFWYHS : 47
Antarctic LMCO -06 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKL-RQNGTFWYHS : 47
Antarctic LMCO -07 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKV-HQNGTFWYHS : 47
Antarctic LMCO -08 : TSVHWHGIRLP--LEMDGVPYVSQLP-VLP-GEYF---DYKFRV-PDAGTFWYHS : 47
Antarctic LMCO -09 : TSVHWHGVFLP--NHMDGVPFLTQMP-IKA-HTTF---LYKFPV-IQNGTFWYHS : 47
Antarctic LMCO -10 : TSVHWHGILLP--ANMDGVPGLSFKG-IEP-GGMY---VYQFTV-NQHGTFWYHS : 47
Antarctic LMCO -11 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-NGTY---VYQFKV-KQNGTFWYHS : 47
Antarctic LMCO -12 : TSVHWHGILLP--ANMDGVPGLSFKG-IEP-GGMY---VYQFMV-NQHGTFWYHS : 47
Antarctic LMCO -13 : TSVHWHGILLP--ANMDGVPGLSFKG-IEP-GGMY---VYQFTV-NQHGTFWYHS : 47
Antarctic LMCO -14 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKL-HQNGTFWYHS : 47
Antarctic LMCO -15 : TSVHWHGILLP--ANMDGVPGLSFKG-IEP-GGVY---VYQFTV-NQHGTFWYHS : 47
Antarctic LMCO -16 : TTVHWHGLLVP--FAMDGVPGISFPG-IKA-RSTF---IYEFPI-IQSGTFWYHS : 47
Arctic LMCO -01 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-NGTY---IYQFKV-KQNGTFWYHS : 47
Arctic LMCO -02 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-DGMY---VYQFKV-RQNGTFWYHS : 47
Arctic LMCO -03 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-DDMY---VYQFKV-RQNGTFWYHS : 47
Arctic LMCO -04 : TTVHWHGILLP--ANMDGVPGLSFHG-IEP-GGMY---VYQFKV-RQNGTFWYHS : 47
Arctic LMCO -05 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-DGMY---VYQFKV-RQNGTFWYHS : 47
Arctic LMCO -06 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKV-HQNGTFWYHS : 47
Arctic LMCO -07 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKV-HQNGTFWYHS : 47
Arctic LMCO -08 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKV-HQNGTFWYHS : 47
Arctic LMCO -09 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKV-HQNGTFWYHS : 47
Arctic LMCO -10 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-DDMY---VYQFKV-RQNGTFWYHS : 47
Arctic LMCO -11 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-DGMY---VYQFKV-RQNGTFWYHS : 47
Arctic LMCO -12 : TSVHWHGILLP--ANMDGVPGLSFHG-IAP-DGMY---EYKFKL-HQNGTFWYHS : 47
Arctic LMCO -13 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-DGMY---VYQFKV-RQNGTFWYHS : 47
Arctic LMCO -14 : TSVHWHGILLP--ANMDGVPGLSFHG-IEP-GGMY---VYQFKV-RQNGTFWYHS : 47
Melanocarpus albomyces (3DKH_A) ○ : TSIHWHGIHQKDTNLHDGANGVTECP-IPPKGGQR---TYRWRA-RQYGTSWYHS : 50
Pycnoporus cinnabarinus (2XYB_A) ○ : SSIHWHGFFQQGTNWADGPAFVNQCP-IAS-GHSF---LYDFQVPDQAGTFWYHS : 50
Trametes versicolor (AY693776) ● : TSIHWHGFFQAGTNWADGPAFVNQCP-IAS-GHSF---LYDFHVPDQAGTFWYHS : 50

b


H131
H84
H129
H86

Discussion

SOM represents the main carbon reservoir in the Arctic and Antarctic tundra, and HS account for the main fraction of SOM. Therefore, elucidating the role of cold-adapted microorganisms in the transformation and/or degradation of HS may be crucial for understanding the effects of rapid warming due to global climate change on terrestrial ecosystems in the polar regions. Soil bacteria dominate cold natural environments and are involved in HS mineralization. To better understand their ecological role at low temperatures, the diversity and spatial distribution of HS-degrading bacteria in polar soils were analyzed. Seventy-three cold-adapted bacterial strains were isolated based on their ability to grow on HA, among which *Pseudomonas* spp. were the most abundant (95% and 60% of Arctic and Antarctic isolates, respectively, Fig. 1). A previous study reported a similar abundance of culturable *Pseudomonas* spp. among 45 HA-degrading bacterial strains isolated from Alaskan subarctic tundra soils (Park and Kim 2015). Members of *Pseudomonas* have predominantly been isolated from sites contaminated with polycyclic aromatic hydrocarbons, and their ability to degrade aromatics and HA in extreme environments, including polar and alpine regions, has been described previously (Grams et al. 1999; Bej et al. 2000; Ma et al. 2006; Tikhonov et al. 2010; Jurelevicius et al. 2012). Taken together, cold-adapted *Pseudomonas* spp. may play a significant role in initial HS degradation and perhaps further mineralization in polar terrestrial environments. As Arctic and Antarctic tundra regions are among the most rapidly warming regions on Earth, increasing temperatures could enhance HS-degradation rates by polar soil bacteria, including *Pseudomonas* spp. Indeed, we demonstrated that HS-utilization efficiency by our isolates gradually increased with incubation temperatures until 25 °C (Fig. 2). Although this growth test was performed on HA-supplemented mineral agar plates with purely isolated bacteria, it is reasonable to assume that temperature increases in tundra soils could enhance bacterial HS-degradation rates, and in turn, increase HS-derived small metabolites that could affect the composition and function of surrounding microbial communities. In the present study, to detect and characterize HS-degrading bacteria, we used a traditional microbial cultivation method that considerably underestimates real microbial diversity. A soil metagenomic approach would have provided greater insight into the extent of bacterial diversity with HS-degrading capacity in the polar tundra regions. However, single-cell culture was a prerequisite to examine changes in HS-degradation rates over a broad range of temperatures, and to detect HS degradation-related LMCOs. With these data obtained using cultured bacteria, metagenomic analysis in future would help to further examine the HS-degrading microbial and LMCO diversity in polar tundra soils.

To date, little information is available regarding bacterial LMCOs in nature, including cold environments. Because bacterial LMCOs may be initially involved in the HS-degradation processes, studying their diversity and distribution within diverse HS-degrading bacteria is important for understanding their actual involvement in HS degradation. In the present study, we determined a majority (71%) of the 73 HA-degrading cultured bacterial strains possessed at least one LMCO gene. This analysis of diverse bacterial strains confirmed the wide occurrence of LMCO genes in polar HS-degrading bacteria and suggested that LMCO function is linked to HS-degradation ability. The failure of LMCO gene detection by PCR could be attributed either to low sequence homology or the presence of different genetic types among them, similar to the laccase enzymes involved in lignin degradation (Granja-Travez et al. 2018). There is a possibility that these strains catalyzed the HA oxidative degradation only with peroxidases, such as bacterial dye-decolorizing peroxidases (DyPs) (Fodil et al. 2011). Therefore, a metagenomic analysis could have detected a larger number of LMCO genes, and better represented the diversity and associations with HS degradation in tundra soils.

From the LMCO clone libraries constructed with pooled PCR amplicons from the 73 cultured bacteria, 30 unique LMCO gene sequences were obtained. All LMCO genes included the conserved CBR I and II sequences essential for laccase activity and one conserved aspartic acid involved in oxygen reactivity of the copper-binding site (Quintanar et al. 2005). When compared with fungal reference sequences from *Melanocarpus albomyces* (3DKH_A) and *T. versicolor* (AY693776), all of the bacterial LMCO protein sequences lacked a cysteine residue between CBR I and II, indicating that bacterial LMCOs could be phylogenetically separate from fungal enzymes (Fig. 3a and b).

In conclusion, we hypothesize that HS-degrading bacteria from Arctic and Antarctic environments play a vital role in soil organic carbon cycling through HS mineralization initiated by bacterial LMCOs. They are cold-adapted psychrotolerant microorganisms that have an optimum temperature between 15 and 25 °C for HS mineralization. In the polar regions where temperatures are continuously increasing, these cold-active enzymes related to HS degradation, including LMCOs, could increase degradation rates by their host bacterial cells. Therefore, characterizing bacterial LMCO expression and their biochemical properties could be a measure by which to gauge bacterial decomposition rates influenced by temperature increases in polar environments. The gene fragments coding for bacterial LMCOs, unique in that they are cold-active and lack a cysteine residue between CBR I and II, could be used as biomarkers to quantify changes in HS decomposition due to temperature increases. In addition, understanding the relationship between bacterial HS-degradation capacity and LMCO function would aid in the elucidation of bacterial HS-degradation pathway(s).

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Compliance with ethical standards

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

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