Korean Journal of Microbiology (2019) Vol. 55, No. 4, pp. 367-376
DOI https://doi.org/10.7845/kjm.2019.9141

pISSN 0440-2413
eISSN 2383-9902

Humic substances degradation by a microbial consortium enriched from subarctic tundra soil

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아북극 툰드라 토양 내 농화배양된 미생물 컨소시엄에 의한 부식질 분해대사경로 연구

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(Received November 18, 2019; Revised November 28, 2019; Accepted November 28, 2019)

The largest constituent of soil organic matter in polar cold regions, humic substances (HS), are natural aromatic heteropolymers, with a composition similar to lignin. The microbes in subarctic tundra soil from Alaska, USA, were able to degrade humic acids (HA, a major component of HS) during microcosm experiments at a low temperature of 5°C, which is similar to natural soil temperature during the thawing period (an average temperature of 5.6°C in 2011~2012). The relative abundance of HA decreased to approximately 71% compared with the non-incubated soil control (100%). The microbes, however, were unable to degrade HA at 25°C, which is in the ideal soil temperature range for planting most plants. When enriched at 15°C in liquid mineral medium provided with HA as a sole carbon source, the HA-enriched microbial consortium was metabolically activated to degrade abundant soil carbons (e.g., 4-hydroxybenzoic acid and D-cellobiose) and completely degraded 2-methoxy phenols (ferulic and vanillic acids), which are lignin-derived mono-aromatics. Our data indicate that the microbial community of Alaska tundra soil is cold-adapted and symbiotically degrades HS, possibly via a bacterial lignin-catabolic pathway in which vanillic acid is a primary metabolite. To our knowledge, this is the first report describing a HS-degradative pathway at the microbial consortium level.

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Keywords: bacterial community, catalytic gene, humic acids, low temperature

Humic substances (HS) are highly complex aromatic heteropolymers found throughout the environment, including the Arctic and Antarctic tundra, and they are the largest constituent (up to 70%) of total soil organic matter (SOM). HS are formed by spontaneous condensation of biomolecules, mainly lignin and small organic compounds, originating from the decay and transformation of plants and other surrounding organic materials. Therefore, HS are considered modified lignin and thus more resistant to microbial degradation. Recent studies suggest that HS are supramolecular aggregates of heterogeneous molecules with relatively low molecular weight, resulting in considerably smaller organic complexes than previously assumed. Based on their solubility in acids, HS are able to be divided into two main fractions, insoluble humic acids (HA) and soluble fulvic acids. HS and HS-derived low molecular weight compounds regulate the growth of plants and microorganisms through various and continuous interactions within the soil (Granja-Travez *et al.*, 2018; Lipczynska-Kochany, 2018).

An enormous amount of organic carbon is stored in the Arctic

tundra soil due to long-term, low-level microbial degradative activity, which results from low atmospheric and soil temperatures. Soil freezing-derived environmental constraints, such as physical distance between substrates and extracellular enzymes, dryness, and anaerobic conditions, can affect microbial decomposition —either temporarily or indefinitely (Davidson and Janssens, 2006). Because temperature is one of the primary factors determining microbial decomposition of SOM, increasing temperatures increase microbial degradation of various organic compounds. Large and complex organic compounds are generally characterized by lower decomposition rates and inherently higher temperature sensitivity, as their activation energy for decomposition is higher (Fierer *et al.*, 2005; Davidson and Janssens, 2006; Lehmann and Kleber, 2015). Due to their large contribution to SOM and the putatively higher temperature sensitivity of microbial decomposition, even small changes in the rate of HS degradation can result in a significant change in the soil ecosystem of the Arctic tundra (Lehmann and Kleber, 2015).

It is likely that soil bacteria are generally directly and/or indirectly involved in *in situ* HS biodegradation even in cold environments. This biodegradation includes both the depolymerization of the HS polymer and the catabolism of HS-derived small compounds. The degradative activities and community composition of soil bacteria have been reported (Park *et al.*, 2015; Kim *et al.*, 2018a), with several bacterial strains known to degrade HS under adequate physiological conditions (Esham *et al.*, 2000; Badis *et al.*, 2009; Rocker *et al.*, 2012; Park and Kim, 2015; Ueno *et al.*, 2016; Lipczynska-Kochany, 2018). However, information on these degradative pathways for HS remains limited and unclear. Using HA as a surrogate for HS, previous work detected several HA-degradative genes through transcriptome analysis of *Pseudomonas* sp. PAMC 26793, a subarctic tundra soil isolate, proposing partial HS catabolic pathways for the isolate (Kim *et al.*, 2018b).

Lignin is a heterogeneous phenolic polymer typically composed of guaiacyl and syringyl units, or guaiacyl units alone. These units are connected through various type of C-O-C and C-C bonds. Recent studies on microbial lignin degradation have focused only on the catabolism of lignin-derived monoaromatics with a guaiacyl moiety, such as guaiacol (2-methoxy phenol), ferulic acid, and vanillic acid. Thus, bacterial catabolic genes and pathways for these aromatics are well characterized in *Sphingobium* sp. SYK-6 (Masai *et al.*, 2007) and *Rhodococcus jostii* RHA1 (Bugg *et al.*, 2011). Lignin is assumed to be depolymerized by bacterial extracellular oxidoreductases, such as dye-decolorizing peroxidases and laccase-like multicopper oxidases, and β-etherases. The various resulting bi-aromatics (e.g., β-aryl ether and biphenyl) and ferulic acid are funneled into a main metabolite, vanillic acid, which is *O*-demethylated to produce protocatechuic acid. Protocatechuic acid is further degraded through a *meta*- or *ortho*-cleavage pathway (Kamimura *et al.*, 2017). Considering that HS are derived from decaying plant material containing lignin and exhibit partial structural similarity to lignin, bacteria might degrade HS via catabolic processes similar to the lignin degradation pathways.

Materials and Methods

Microcosm design, incubation, and HA component extraction

Subarctic tundra soils rich in HS were collected on June 29, 2012 from the active layer (0~20 cm) of a grassland site [designated AK1-75; temperature at 20 cm depth, approximately 5.6°C in mid-June to early September] in Council, Alaska, USA (Park *et al.*, 2015). The AK 1-75 soils were homogenized and stored at -20°C until used. For testing, the frozen samples were slowly thawed in a refrigerator, and 200 g of soil was incubated in a 500 ml beaker at 5 or 25°C for 33 days. As a control, one sample was autoclaved at 121°C for 15 min to exclude the possibility of non-biological degradation of HS. The beakers were wrapped in plastic to maintain the initial water content of the soil, but homogenized with a spatula for aeration every 2 weeks. After 33 days of incubation, the soils were subjected to HA extraction with 0.5 N NaOH according to previously described methods (Park *et al.*, 2015).

Pyrolysis-gas chromatography-mass spectrometry

HA extracted from AK 1-75 soil (hereby referred to as $HA_{AK\ 1\text{-}75}$), or commercial lignin (Cat. No. 471003, Aldrich), was wrapped with pyrofoil (Japan Analytical Industry) and pyrolyzed for 5 sec in a quartz tube with a Curie temperature of 590°C using a Curie Point Injector (Japan Analytical Industry). The products

of pyrolysis were transferred immediately to a 7890A-5975C GC-quadrupole-MSD instrument (Agilent Technologies Inc.). The GC column used was 30 m × 0.25 mm × 0.25 μm DB-5ms (Agilent Technologies Inc.), using a helium carrier gas with a flow rate of 1.0 ml/min. Each sample was injected with a split ratio of 1:10, and an injection temperature set to 250°C. The oven temperature was programmed to increase from 40°C (held for 5 min) to 300°C at 7°C/min. The final temperature was maintained for 10 min. The ionization mode was electron ionization at 70 eV. Pyrolysis products were identified by comparing the resulting spectra with spectra from the NIST 08 mass spectral library and from published literature.

Enrichment and metabolic activity assessment of soil microbes

A small fraction (5 g) of AK 1-75 soil, which was thawed in a refrigerator, was added to 10 ml mineral salts basal medium (MSB; Stanier *et al.*, 1966), strongly homogenized by vortexing, and placed at 5°C overnight. After centrifugation twice at low-speed (123 × *g*, 2 min, 5°C), the MSB supernatant was obtained for use as a soil microbe suspension for enrichment culturing with $HA_{AK\ 1\text{-}75}$. Enrichment culture was performed by inoculating the cell suspension (3 ml) into a 250-ml Erlenmeyer flask containing 50 ml MSB and 6 ml $HA_{AK\ 1\text{-}75}$ solution (0.83% $HA_{AK\ 1\text{-}75}$ dissolved in 0.1 N NaOH) and incubating this at 15°C with shaking. After 27 days of enrichment culture, 2 ml of the cell culture was added to new 50 ml MSB medium, which was used as an inoculum for following experiments.

For community level physiological profiling, 0.1 ml of the above dilution was transferred into each well of a microtiter EcoPlate (Biolog) containing 31 of the most useful carbon sources for soil community analysis. The plates were placed in a covered plastic container containing moist paper towels and incubated at 15°C for 7 days. Utilization of the carbon sources was indicated by reduction of tetrazolium violet redox dye, which changed from colorless to purple when microbes utilized the substrate. The development of purple color was measured at 595 nm every 24 h. The average metabolic response (AMR, n = 3) was calculated as follows: AMR = Σ(OD well − OD neg)/95, where OD well is the optical density of each carbon source-containing well and OD neg is the optical density of the negative control well. As a control, an MSB soil suspension of AK 1-75, which was not enriched, was directly transferred to the EcoPlate.

To test for the ability to degrade various mono-aromatics, 2.0 ml of the above dilution was inoculated into each of several 250 ml-Erlenmeyer flask containing 50 ml MSB, to which a substrate (5 mM of benzoic acid, vanillic acid, ferulic acid, phenol, or coniferyl alcohol) was added. The flasks were incubated at 15°C for 9 days with shaking. Every 24 h, half a milliliter of each culture was harvested, and the concentration of substrate remaining was determined using high-pressure liquid chromatography (HPLC).

Results

HA degradation by cold-adapted soil microbes

A microcosm experiment with HS-rich AK 1-75 soil was performed for 33 days at 5°C, the approximate thawing-period temperature at the sampling site. HA with high molecular weight was extracted from 2 g of uncultured dried soil to produce approximately 0.95 g of solid HA ($HA_{AK\ 1\text{-}75}$), which comprised 48% of the soil weight. The initial HA level (100%) from a -20°C soil control decreased to approximately 71%, indicating that some HA was removed through a biologically-mediated degradation process, possibly resulting in an accu-

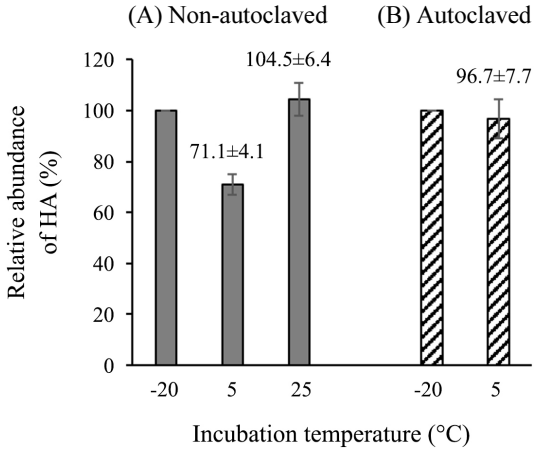

Fig. 1. Temperature-dependent changes in HA content during microcosm experiments. The microcosm beakers with HS-rich AK 1-75 tundra soil were incubated at different temperatures for 33 days. The weight of extracted HA was measured (n = 3) for (A) non-autoclaved intact AK 1-75 soil and (B) autoclaved inert AK 1-75 soil both incubated under the same conditions.

mulation of small HS-derived compounds (Fig. 1A). To show that the HA degradation was biologically mediated, we measured HA levels in an autoclaved sample of AK 1-75 under the same conditions. As shown in Fig. 1B, after 33 days, there was almost no change in HA content of autoclaved AK 1-75, in contrast to that in active AK 1-75. However, we found no decrease in HA content in samples incubated at 25°C for 33 days (Fig. 1A), indicating that HA degradation was processed by cold-adapted indigenous microbes.

Comparative compositional analysis of HA and lignin

$HA_{AK\ 1\text{-}75}$ was analyzed for the presence and relative abundance of organic constituents in its chemical structure using pyrolysis-gas chromatography-mass spectrometry (Py-GC/MS, 590°C). On the pyrolysis GC chromatogram, a wide range of different decomposition products were detected including: substituted phenols (phenol category, five compounds); 2-methoxy phenols (guaiacol category, seven compounds); substituted benzenes (benzene category, 11 compounds); bicyclic compounds containing one benzene ring (bicyclic category, seven compounds); heterocyclic compounds (heterocyclic category, two compounds); and both substituted alkanes and alkenes (aliphatic category, two compounds). This data is presented in Table 1. Among these compounds, five were observed in higher abundance [toluene (**1**), phenol (**2**), 4-methyl phenol (**3**), 2-methoxy phenol (guaiacol, **4**), and 2-methoxy-4-vinyl phenol (**5**)] (Fig. 2A). The compounds in the phenol (total area of 24.9%) and guaiacol (total area of 14.3%) categories were detected as the main thermal decomposition products produced by cleavage at the weakest bonds of large heteropolymer $HA_{AK\ 1\text{-}75}$. Although the structure and formation of HS are still disputed, HS should be regarded as supramolecular associations of rather small molecules, with phenolic and carboxylic functional groups conceivably the most important groups for surface charge and reactivity of HS (Lipczynska-Kochany, 2018). Thus, various mono-aromatics with carboxylic and/or phenolic substitutions are assumed to be derived from $HA_{AK\ 1\text{-}75}$ pyrolysis at 590°C. However, these polar aromatics with a carboxylic group, such as benzoic acid, vanillic acid, and ferulic acid, were not detected on the Py-GC chromatogram, because compounds with very low volatilities did not readily vaporize.

As a reference, commercial lignin was also analyzed to confirm that it consisted of the same two main mono-aromatics as $HA_{AK\ 1\text{-}75}$.

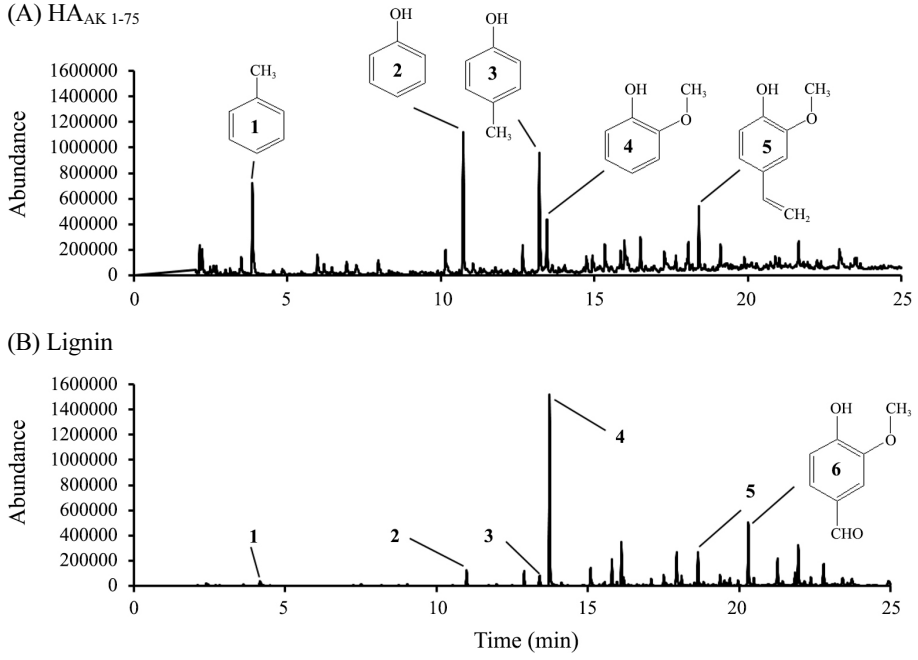

Fig. 2. Pyrograms of (A) natural HA extracted from AK 1-75 ($HA_{AK\ 1\text{-}75}$) and (B) commercial lignin. The abundance of compounds **1**, toluene; **2**, phenol; **3**, 4-methyl phenol; **4**, 2-methoxy phenol (guaiacol); **5**, 2-methoxy-4-vinyl phenol; **6**, vanillin (4-formylguaiacol) are noted in each pyrogram.

Table 1. Comparative analysis of natural HA_{AK1-75} and commercial lignin pyrolysis products

Pyrolysis product[a]	Category	HA_{AK1-75}		Lignin	
		Time (min)	Area (%)	Time (min)	Area (%)
Benzene	Benzene	2.2	0.5	2.4	0.4
Butanal, 2-methyl-	Aliphatic	2.2	1.0		
Toluene	***Benzene***	***3.9***	***6.8***	4.2	1.0
Furfural	Heterocyclic	6.0	1.8		
Ethylbenzene	Benzene	6.9	1.0		
p-Xylene	Benzene	7.2	0.9	7.5	0.4
Styrene	Benzene	8.0	1.2		
2-Furancarboxaldehyde, 5-methyl-	Heterocyclic	10.1	1.8		
Phenol	***Phenol***	***10.7***	***10.3***	11.0	2.3
Benzene, 1,3,5-trimethyl-	Benzene	11.0	1.1		
Phenol, 2-methyl-	Phenol	12.7	2.1	12.9	2.0
Phenol, 4-methyl-	***Phenol***	***13.2***	***8.9***	13.4	2.0
Phenol, 2-methoxy- [guaiacol]	***Guaiacol***	***13.5***	***3.8***	**13.7**	**27.5**
1-Undecene	Aliphatic	13.6	1.0		
Benzyl nitrile	Benzene	14.7	1.5		
Phenol, 2,4-dimethyl-	Phenol	14.9	1.1		
Benzene, 1,2-dimethoxy-	Benzene			15.1	3.1
Phenol, 4-ethyl-	Phenol	15.3	2.5	15.5	0.2
Phenol, 2-methoxy-3-methyl- [3-methylguaiacol]	Guaiacol			15.8	3.8
Phenol, 2-methoxy-4-methyl- [4-methylguaiacol]	Guaiacol	15.9	1.5	16.1	6.0
1,2-Benzenediol	Benzene	16.0	2.7	16.2	1.3
Benzofuran, 2,3-dihydro-	Bicyclic	16.5	2.4		
3,4-Dimethoxytoluene	Benzene			17.1	1.1
1,2-Benzenediol, 3-methoxy-	Benzene	17.3	2.1		
1,2-Benzenediol, 3-methyl-	Benzene			17.5	1.7
Phenol, 2-methoxy-4-ethyl- [4-ethylguaiacol]	Guaiacol	17.7	1.0	17.9	5.7
1,2-Benzenediol, 4-methyl-	Benzene	18.0	0.4	18.1	1.4
Indole	Bicyclic	18.1	2.3		
Phenol, 2-methoxy-4-vinyl- [4-vinylguaiacol]	***Guaiacol***	***18.4***	***3.7***	18.6	4.9
Phenol, 2,6-dimethoxy- [syringol]	Guaiacol	19.1	1.6	19.4	1.9
Phenol, 2-methoxy-4-allyl- [eugenol]	Guaiacol			19.5	0.8
Phenol, 2-methoxy-4-propyl- [dihydroeugenol]	Guaiacol			19.7	1.2
1H-Indole, 3-methyl-	Bicyclic	19.9	1.0		
Vanillin [4-formylguaiacol]	***Guaiacol***			***20.3***	***10.0***
Phenol, 2-methoxy-4-(1-propenyl)- [isoeugenol]	Guaiacol			20.5	1.3
Naphthalene, 1,3-dimethyl-	Bicyclic	20.6	0.4		
1,2,4-Trimethoxybenzene	Benzene	20.9	0.8		
Phenol, 2-methoxy-6-(2-propenyl)- [6-allylguaiacol]	Guaiacol	21.0	0.7	21.3	4.6
Benzaldehyde, 3,4-dimethoxy-	Benzene			21.8	2.1
1-(4-Hydroxy-3-methoxyphenyl)ethanone [acetovanillone]	Guaiacol	21.7	2.0	22.0	6.9
Benzimidazole, 5-tert-butyl-2-methyl-	Bicyclic	22.3	1.0		
Naphthalene, 1,6,7-trimethyl-	Bicyclic	23.0	0.9		
Naphthalene, 2,3,6-trimethyl-	Bicyclic	23.5	0.9		
Ethanone, 1-(3,4-dimethoxyphenyl)-	Benzene			23.4	1.7
Others			27.3		4.7
Total			100		100

[a] For a clear comparison, HA_{AK1-75}- and lignin-derived products with areas >3% and >10%, respectively, are highlighted in bold italics.

On the GC chromatogram, four compounds in the phenol category (total area of 6.5%) and 12 compounds in the guaiacol category (total area of 74.6%) were detected. In the guaiacol category, 2-methoxy phenol (**4**) and vanillin (4-formylguaiacol, **6**) were detected with the highest abundances (27.5 and 10.0%, respectively) showing that lignin is a heterogeneous phenolic polymer typically composed of guaiacyl units (Fig. 2B and Table 1). Comparative Py-GC/MS analysis of $HA_{AK\ 1\text{-}75}$ and lignin showed that these two aromatic heteropolymers are structurally similar, because phenols (aromatic nuclei with 1-hydroxyl group) and 2-methoxy phenols (aromatic nuclei with 1-hydroxyl and 2-methoxyl groups) are their main compositional moieties.

Lignin-derived compound degradation by HA-enriched microbes

EcoPlates were used to comparatively evaluate the changes in the community-level physiological activity of AK 1-75 soil microbes, with or without HA enrichment. As shown in Fig. 3A, obtained with EcoPlates containing 31 substrates of the most useful carbon sources for soil community analysis (Biolog brochure), the AMR values by HA-enriched microbes were significantly greater than non-enriched ones (for example, an AMR value of 0.6 vs. 0.1 after a 7-day incubation). Especially with the enriched microbes, significantly higher AMR values compared with water were found for the following substrates: β-methyl-D-glucoside (4.5 times), 4-hydroxy benzoic acid (6.9 times), D-glucosaminic acid (31.7 times), and D-cellobiose (17.0 times) (Fig. 3B). This analysis showed that the substrate utilization rate and functional diversity of AK 1-75 microbes significantly increased through HA-enrichment culture, indicating that HA-degradation related genes were induced.

The degradative ability of HA-enriched microbes was examined against various lignin-derived mono-aromatics in MSB medium using HPLC chromatography. As shown in Fig. 3C, the enriched microbes were able to degrade a simple aromatic

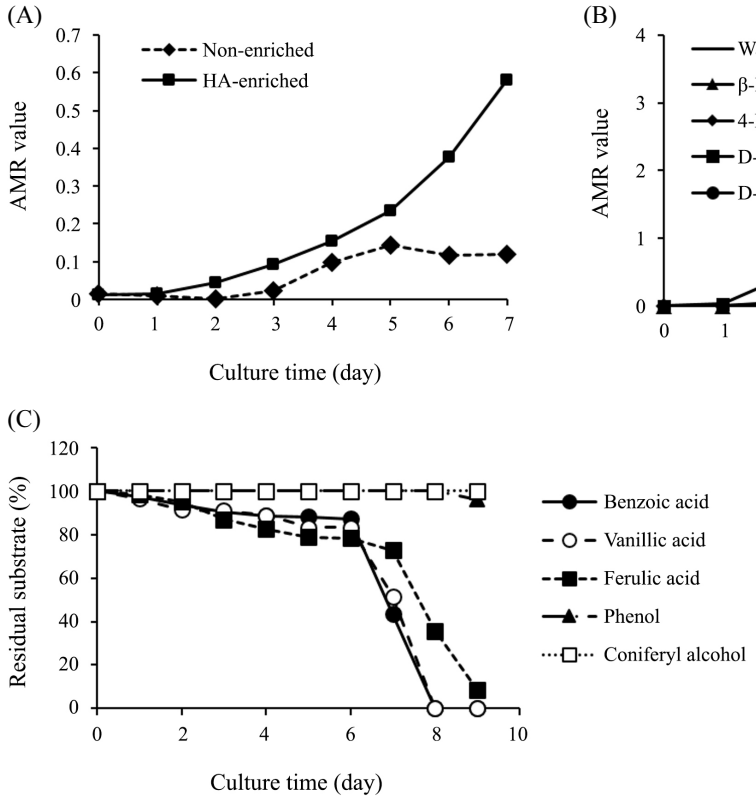

Fig. 3. Assessment of function and catalytic activity of the AK 1-75 microbial consortium enriched by HA. The experiments were performed at 15°C with a cell suspension from AK 1-75 which had previously been enriched in HA-containing MSB media at 15°C. Average metabolic response (AMR, $n = 3$) values were calculated from EcoPlates wells containing (A) all the thirty-one substrates or (B) five selected substrates. The degradative capacity for lignin-derived mono-aromatics was measured by HPLC, measuring the percentage of residual substrate over time (C).

carboxylic acid (benzoic acid) and carboxylated 2-methoxy phenols (ferulic acid and vanillic acid). The concentration of each substrate in the culture supernatant began to decrease sharply after 6-day incubation, with the concentrations of benzoic acid and vanillic acid nearing zero after 8 days. The concentrations of phenol and coniferyl alcohol, however, hardly decreased during the same time indicating that these phenolic compounds are not adequate substrates of the HA-enriched microbes.

Discussion

Arctic tundra is characterized by extremely low temperatures and a short growing season, resulting in permafrost soils that contain enormous stocks of organic carbon (Schuur *et al.*, 2015). The Arctic permafrost is overlaid by an active layer, which is a hotspot of microbial abundance and activity that is subjected to seasonal freeze-thaw cycles. Rising temperatures in this region would thaw the permafrost, leading to an increase in the thickness of the active layer and thus enhance the availability of SOM containing thousands of different organic compounds for microbial degradation. Increased microbial degradative activity may lead to greater SOM mineralization and accelerated carbon dioxide and methane emissions, and therefore, many studies of Arctic tundra soils have focused on microbial decomposition in the active layer.

Until recently, the impact of warming on microbial degradative processes has been a matter of considerable debate, although soil microbial degradative activity is known to be temperature sensitive (Mikan *et al.*, 2002; Biasi *et al.*, 2005). For example, the small and labile fractions of SOM are degraded by heterotrophs in a temperature-sensitive manner, whereas the larger fractions of older and more recalcitrant (resistant to microbial decomposition) SOM are not affected by temperature changes (Liski *et al.*, 1999). However, contrasting evidence has suggested that the older, recalcitrant fraction is even more sensitive to temperature changes than the younger, more labile fraction (Fierer *et al.*, 2005). Additionally, in support of these contrasting data, additional experiments involving aerobic incubation of tundra soil cores from the Gdansky Peninsula, Russia showed that recalcitrant compounds were preferentially degraded by arctic soil microbes at higher temperatures, suggesting that a large portion of tundra SOM is degraded and mineralized by tundra soil microbes when soil temperatures increase (Biasi *et al.*, 2005). According to the common kinetic theory of enzymatic decomposition, organic carbon pools with slower turnover, including large and complex HS, are thought to respond more sensitively to climate warming than pools exhibiting rapid turnover, including simple and small organic compounds (Davidson and Janssens, 2006; Lehmann and Kleber, 2015).

When preferred nitrogen and carbon sources are scarce in cold tundra soils, cold-adapted microbes will enhance their use of less favorable sources, such as heteropolymeric HS, promoting functional efficiency via adaptation of their catabolic enzymes and pathways to low temperatures. In addition to cold adaptation, rising temperatures would further enhance the enzymatic degradation of HS in Arctic tundra soil. HS would be depolymerized into monomers or oligomers by extracellular enzymes, with degradative intermediates subsequently catabolized after uptake by surrounding microbes. It is assumed that cold-adapted soil bacteria play a major role in HS degradation and mineralization in tundra soils owing to their higher abundance and functional diversity. However, few reports have focused on their direct involvement in HS degradation and the associated catabolic pathways.

In this work, microcosm experiments were performed with subarctic tundra soil (AK 1-75) and confirmed that HA (a major constituent of HS) was biologically degraded at temperatures as low as 5°C by AK 1-75 soil microbes adapted to cold tundra soils. HA, however, was not degraded at 25°C, the optimum temperature for temperate plant photosynthesis. The indigenous microbes in AK 1-75 were enriched at 15°C in mineral medium containing $HA_{AK\ 1\text{-}75}$ to develop a better understanding of HS degradation pathways in a microbial consortium, where two or more microbial groups live symbiotically. Following enrichment culture, the HA-enriched microbial consortium (HEMC) was examined to evaluate the changes in community-level physiological activity with EcoPlates. Because the AMR reflects the ability of the HEMC to utilize the substrates, the higher AMR values in the HEMC possibly indicate an increase in metabolic activity and functional diversity in the AK 1-75 microbial consortium. In previous AK 1-75 enrichment culturing using the same mineral medium containing $HA_{AK\ 1\text{-}75}$ cultured

at 5°C for 21 days, the relative abundance of phylum Proteobacteria increased to 79.0% (after enrichment) from 60.2% (before enrichment), with class Betaproteobacteria being highly enriched. At the genus level within Betaproteobacteria, the abundance of the soil-dwelling genus *Janthinobacterium* and of the chitinolytic *Collimonas* increased, indicating that Proteobacteria strains in AK 1-75 soil are involved in HS depolymerization at low temperatures (Park *et al*., 2015).

Comparative Py-GC/MS analysis showed that $HA_{AK\ 1\text{-}75}$ and lignin are structurally similar, due to their common main compositional units, phenols and 2-methoxy phenols, assumed to derive from the microbial degradation processes of both $HA_{AK\ 1\text{-}75}$ and lignin. As shown in Fig. 3C, HEMC almost completely degraded benzoic acid, ferulic acid, and vanillic acid. Lignin-derived benzoic and ferulic acids are abundant in nature, whereas vanillic acid is one of the intermediates formed by the microbial degradation of ferulic acid. This enrichment experiment indicates that the metabolic state of AK 1-75 soil microbes can be actively induced by HA, leading to HS degradation via bacterial lignin catabolic routes.

In general, it is known that lignin is aerobically depolymerized primarily by lignolytic fungi, with some bacteria playing supportive roles in initial lignin breakdown. However, during the recent decade, lignolytic dye-decolorizing peroxidases have been discovered and the catabolic mechanisms (i.e., genes and pathways) for lignin-derived small aromatics have been further characterized in several bacteria (Kamimura *et al*., 2017). Contrary to previous perception, these new findings suggest that bacteria might play major roles in lignin and HS up to mineralization.

Based on these results with AK 1-75 enriched microbes and the general concept of bacterial lignin degradation pathways (Bugg and Rahmanpour, 2015; Salvachúa *et al.*, 2015; Kamimura *et al*., 2017), we propose that the AK 1-75 microbial consortium, containing diverse bacteria of the Proteobacteria phylum, degrades HS via catabolic processes similar to the lignin degradation pathways used by bacteria (Fig. 4). HS is depolymerized by bacterial extracellular oxidoreductases, such as dye-decolorizing peroxidases, and the resulting HS-derived ferulic acid is funneled into a main metabolite, vanillic acid, which is *O*-demethylated to produce protocatechuic acid. Through another route, vanillic acid is decarboxylated into guaiacol, which is subsequently *O*-demethylated to catechol (Alvarez-Rodriguez *et al.*, 2003). HS-derived benzoic acid is converted to catechol via the hydroxylation of the aromatic ring. Protocatechuic acid and catechol are further degraded to TCA cycle intermediates through the *meta*- or *ortho*-cleavage pathway.

In summary, we investigated the capacity and pathways of HS, natural aromatic heteropolymers, degradation by the

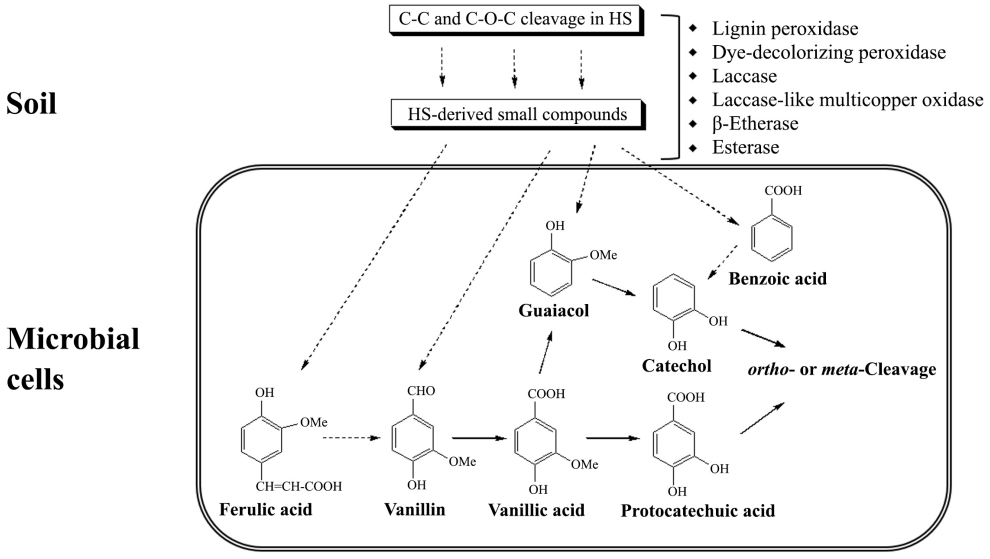

Fig. 4. Proposed HS-degradation pathway (vanillic acid route) of AK 1-75 soil microbes. Dotted and solid lines represent multi-step reactions by different enzymes and one-step reactions by one enzyme, respectively.

microbial communities present in Arctic tundra soil using enrichment culturing methods. We found that the cold-adapted microbes (possibly diverse bacterial strains in Proteobacteria phylum) symbiotically degraded HS, via at least one (i.e., vanillic acid route) of the lignin-catabolic pathways. To the best of our knowledge, this is the first report to describe a HS-degradative pathway based on a microbial consortium, which was actively induced and enriched by HA, a main component of HS. We anticipate that our novel findings will contribute to a better understanding of the ability of cold-adapted microbial communities to metabolize available soil organic matter, including large and complex HS, at both low temperature (at present) and higher temperatures (in progress) resulting from global warming.

적 요

극지역 저온환경 내 토양 유기물의 핵심 구성물질인 부식질(humic substances)은 천연의 aromatic heteropolymer이며 리그닌(lignin)과 구조적으로 유사하다. 2011~2012년 미국 알래스카 툰드라 지역 토양의 여름철 평균 온도는 5.6°C였으며, 이 곳 토양을 5°C 저온배양하는 환경모사실험 동안에 토양 내 미생물군집은 부식질의 주요 구성성분인 부식산(humic acids)을 미배양 대조군 대비 약 29% 분해하였다. 반면에, 식물성장에 적합한 25°C 중온 배양 시에는 부식산을 거의 분해하지 못했다. 부식산을 유일한 탄소원으로 포함하는 미네랄 최소배지에 토양 시료를 접종하고 15°C 액체배양하였을 때, 부식산-농화배양된 미생물 컨소시엄은 대표적인 토양 유기탄소물질(예, 4-hydroxybenzoic acid and D-cellobiose)에 대한 물질대사능력이 향상되었으며, 리그닌 미생물 분해대사산물인 2-methoxy phenol류 화합물(예, ferulic acids and vanillic acids)를 완전히 분해할 수 있었다. 이번 실험은 미생물군집 수준에서 부식질 분해대사 경로를 연구하는 첫 사례로서 결과를 정리하면, 알래스카 툰드라 토양 내 미생물군집은 저온에 잘 적응되어 있고 상호협력하여 부식질을 분해하는데, 이미 알려져 있는 세균의 리그닌 분해경로(vanillic acid가 주요 분해대사산물로 축적)를 통해서 부식질을 분해하리라 추정한다.

Acknowledgements

This work was supported by a grant, 'Modeling responses of terrestrial organisms to environmental changes on King George Island (PE19090)', funded by the Korea Polar Research Institute.

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