

Characterization of Aromatic Hydrocarbon Degrading Bacteria Isolated from Pine Litter

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Using a novel pine needle agar, fifteen bacterial species were isolated from pine litter. These bacteria were able to degrade aromatic hydrocarbons derived from lignin and utilize the *ortho*-cleavage of the β -keto adipate pathway to degrade protocatechuate or catechol. A different utilization array of aromatic hydrocarbons by these bacteria was also determined. This study provides the information on bacterial species living in pine litter and suggests that these bacteria have metabolic abilities to utilize aromatic hydrocarbons derived from lignin biodegradation.

Key words: Bioremediation, biodegradation, biogeochemical cycling, lignin, aromatic hydrocarbons, β -keto adipate pathway

Introduction

In plants, lignin comprises about 25% of the land-based biomass on earth, and the recycling of lignin is critical in the earth's carbon cycle [13]. However, the biological degradation of lignin is not fully understood [19]. Lignin, a complex polymer of aromatic hydrocarbons, is very difficult to degrade because it lacks of a standard, repeating covalent bond. Fungi are proposed to play a major role in lignin biodegradation by producing a variety of extracellular enzymes such as laccase, lignin peroxidase (LIP), and manganese peroxidase (MNP) [8, 13, 23]. These enzymes are capable of forming radicals inside lignin to destabilize bonds and to lead to the breakdown of the macromolecule. On the other hand, roles of bacteria in lignin biodegradation are not well characterized. Bacteria may play a less important role in lignin biodegradation because they may not be able to perform the initial depolymerization step to degrade lignin [13]. Instead, bacteria can utilize aromatic monomers derived from fungal biodegradation of lignin [8, 13, 24].

The distortion of an aromatic ring is very difficult because of stability imparted by the high resonance energy of electrons. The degradation of aromatic hydrocarbons

seems to be restricted to microorganisms [11]. The aerobic degradation of aromatic hydrocarbons by microorganisms has been investigated extensively [9]. In the presence of air, an oxygenase system breaks aromatic rings [12]. In addition, microorganisms, that can degrade aromatic compounds anaerobically by a reductive enzyme system, have been reported [9, 10].

Under aerobic conditions, degradation pathways of a variety of aromatic hydrocarbons converge at either protocatechuate or catechol which is further degraded via β -keto adipate pathway to succinyl-CoA and acetyl-CoA (Fig. 1) [11, 22]. The β -keto adipate pathway is widely distributed in soil bacteria and fungi [11]. In the β -keto adipate pathway, either protocatechuate or catechol is cleaved between hydroxyl groups of the ring (*ortho*-cleavage) [11, 22]. The *ortho*-cleavage is catalyzed by either protocatechuate-3,4-oxygenase or by catechol-1,2-oxygenase [22]. Another pattern of ring cleavage also occurs in which the ring is broken adjacent to one of the hydroxyls (extradiol cleavage or *meta*-cleavage) [22]. In *meta*-cleavage, aromatic hydrocarbons are degraded via muconic semialdehyde rather than β -keto adipate. The *meta*-cleavage is catalyzed by either protocatechuate-4,5-oxygenase or by catechol-2,3-oxygenase [22]. Several strains of *Bacillus* species are able to degrade aromatic hydrocarbons by a novel *meta*-cleavage pathway catalyzed by protocatechuate-2,3-oxygenase [3, 4]. Yet, another mechanism, called the gentisate pathway, appears when some aromatic hydrocarbons are

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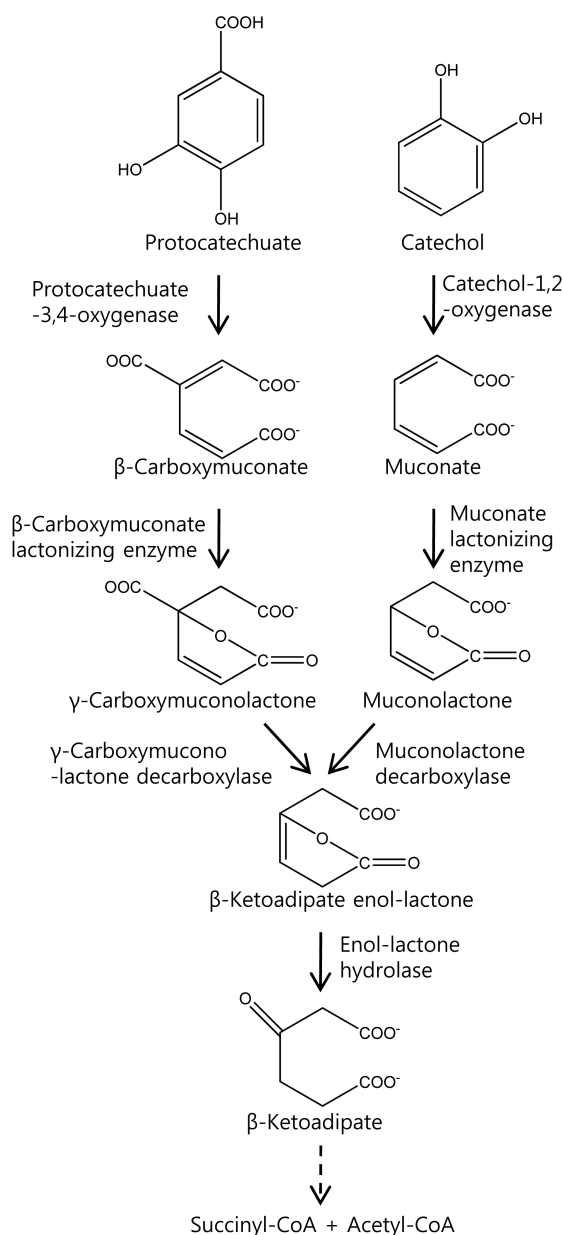


Fig. 1. The β -ketoadipate pathway [11, 12].

converted into gentisate. For the gentisate pathway, the cleavage occurs between the carboxyl-substituted carbon and the adjacent hydroxylated carbon [6].

Pine tree have large amounts of lignin in their needles. Bacteria living in pine litter may be involved in lignin recycling by utilizing low molecular weight aromatic hydrocarbons derived from fungal digestion of lignin. In this study, bacterial flora from pine litter were isolated and characterized for their metabolic capacities to utilize aromatic monomers derived from lignin biodegradation.

Materials and Methods

Collection of samples

Pine litter samples were collected at Hartmann's Creek State Forest, west of Waupaca, Wisconsin. The collection site was about 1.5 km into the forest and about fifty meters from a nature trail. Sterile forceps were used to place 5.0 g of pine litter into each 250 mL Erlenmeyer flask that contained 100 mL of sterile Phosphate Buffered Saline (PBS). Flasks were shaken vigorously at 150 rpm overnight at room temperature.

Isolation of bacteria

Glucose Yeast Extract Agar (GYEA) and Pine Needle Agar (PNA) were used to isolate bacteria. GYEA contained 0.5% glucose, 0.1% peptone, 0.1% yeast extract, 0.0005% FeSO_4 , and 1.5% agar. For PNA, 12.5 g of pine needles were placed in a blender with 250 mL distilled water and ground at high speed for 15 min. The slurry was filtered through cheese cloth, and the ground needles were returned to the blender. Pine needles are primarily composed of cellulose and lignin, and chemical constituents of pine needles are described previously [20]. Following low molecular weight-monomeric aromatic compounds were found in pine needles: limonene, pinene, abietic acid, borneol, protocatechuate, catechol, pyrogallol, resorcinol, gallic acid, hydroquinone, *p*-amino benzoic acid, sodium salicylate, gentisate, vanillate, ferullic acid, sodium benzoate, *p*-hydroxybenzoate, *o*-amino benzoic acid and phenyl acetic acid [1, 2].

This extraction was repeated three more times, and agar (1.5% for final concentration) was added to the filtrate. The pH of PNA was approximately 4.5. After autoclaving, the medium was cooled to 60°C and cycloheximide (150 $\mu\text{g}/\text{mL}$) was added to inhibit fungal growth. The pine litter suspensions in PBS were serially diluted and plated onto both PNA and GYEA. The plates were incubated at room temperature until colonies could be observed (usually after 3-7 days). Well isolated colonies from GYEA or PNA were re-streaked onto the same medium, and incubated at room temperature until pure cultures were obtained as judged by microscopic observation with Gram staining.

Growth with protocatechuate or catechol

Each isolate was inoculated into MR-1 broth, and incubated at room temperature with shaking at 150 rpm. MR-

1 broth contained per liter: KH_2PO_4 2.0 g, K_2HPO_4 7.0 g, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ 0.1 g, and Vitamin-free Casein Hydrolysate 2.5 g (pH 7.2). After 4-7 days of incubation, 0.3 mL of cell suspension was transferred to Defined Mineral Medium (DMM) with 17 mM protocatechuate or catechol (16). DMM contained 25 mM KH_2PO_4 , 25 mM Na_2HPO_4 , 0.1 % $(\text{NH}_4)_2\text{SO}_4$, and 10 mL/l Hutner's mineral base (pH 6.8). Hutner's mineral base of DMM contained per liter: Nitrioloacetic acid 10.0 g, MgSO_4 14.45 g, Ammonium molybdate 3.335 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.099 g, and 50 mL stock salt solution [7]. Stock salt solution for Hutner's mineral base contained per liter: EDTA 2.5 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 10.95 g, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 5.0 g, $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 1.54 g, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 0.392 g, $\text{CO}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ 0.248 g, and sodium borate 0.177 g. After 5-7 days of incubation, visible turbidity was taken as evidence of growth.

Classification of isolates

Taxonomic identification of isolates capable of utilizing aromatic compounds was done by application of the 16S rDNA sequencing. Bacterial DNA was isolated as described [14, 21]. Isolated DNA was amplified by the polymerase chain reaction (PCR) using the universal 16S rDNA primers [25]. 16S rDNA sequences were compared with available 16S rDNA sequences deposited in the GenBank databases (National Center for Biotechnology Information, Bethesda, MD). In addition to 16S rDNA sequencing, BIOLOG's Bacteria & Yeast Identification system was applied to determine the identification of the isolates. The procedure and more information about the BIOLOG system is available at www.biolog.com.

Rothera test

To determine the mode of ring cleavage of protocatechuate or catechol, rothera test was performed as described [18]. A loopful of an isolate was inoculated into 50 mL of DMM with 17 mM benzoic acid or *p*-Hydroxybenzoic acid and incubated at room temperature with shaking at 150 rpm for two days. The cells were harvested by centrifugation at $8,000 \times g$ for 20 min after further sub-culturing 2-3 times. A large loopful of the cell pellet was resuspended in 2.0 mL 0.02 M Tris-Cl (pH 8.0), then 0.5 mL toluene and 2.5 mL 4 mM protocatechuate were added. A green yellow color within 3 min after vigorous shaking of the tube indicated a positive meta-cleavage. If no reaction occurred, the tube was incubated by rotation for 18 hr at room temperature,

then, 1 g of $(\text{NH}_4)_2\text{SO}_4$, five drops of a freshly prepared aqueous 1% sodium nitroprusside ($\text{Na}_2(\text{Fe}(\text{CN})\text{NO}) \cdot 2\text{H}_2\text{O}$) solution, as well as 0.5 mL concentrated ammonia ($d=0.91$) were added. A deep purple color appearing within 3 min indicated a positive *ortho*-cleavage of the substrates.

Protocatechuate-3,4-oxygenase and catechol-1,2-oxygenase assays

Each isolate was inoculated into 50 mL of MR-1 broth, and incubated at room temperature with shaking at 150 rpm. After 2-3 days of incubation, the cells were harvested and resuspended in 10 mL of DMM with 17 mM *p*-hydroxybenzoate or benzoate, and 0.3 mL of the cell suspension was inoculated into 50 mL of DMM with 17 mM of *p*-hydroxybenzoic acid for the protocatechuate-3,4-oxygenase assay or with 17mM of benzoic acid for the catechol-1,2-oxygenase assay. After subculturing 2-3 times in the same medium, the cells were harvested and re-suspended in 5 mL of cold 0.1 M phosphate buffer (pH 7.0), and these suspensions were passed twice through a French pressure cell (American Instrument Co., Silver Spring, MD) at $10,000 \text{ lb/in}^2$ (3). Disrupted cell suspensions were centrifuged at $8,000 \times g$ for 20 min, and the supernatant were saved. The amount of protein in each sample was determined by Bio-Rad's Coomassie Blue Protein Assay according to the manufacturer's directions. Protocatechuate-3,4-oxygenase and catechol-1,2-oxygenase assays were performed as described [5, 17]. For enzyme assays, 2.0 mL of 0.1 M of phosphate buffer (pH 7.0), 100-200 mg of protein from cell extract, 0.4 mL of 0.01 M ethylenediaminetetraacetate (EDTA), and distilled water to make the final volume to 2.7 mL were added in a cuvette with a 1 cm light path. The reaction was initiated by the addition of 0.5 mL of 0.001 M protocatechuate or 0.3 mL of 0.001 M catechol. Readings are taken at 290 nm (for protocatechuate-3,4-oxygenase) or at 260 nm (for catechol-1,2-oxygenase) for every 30 sec for 5 min. A reference cuvette contained all components except substrate. Concentration of absorbing material in the sample was calculated by using the Beer-Lambert law. The coefficient was obtained by preparing a standard curve and was found to be $4,230.77 \text{ liters moles}^{-1} \text{ cm}^{-1}$ (for protocatechuate-3,4-oxygenase) or $16,900 \text{ liters moles}^{-1} \text{ cm}^{-1}$ (for catechol-1,2-oxygenase). For protocatechuate-3,4-oxygenase assay, one unit enzyme activity oxidized protocatechuate at an initial rate of $0.075 \text{ } \mu\text{mole per minute}$. For catechol-1,2-oxygenase assay, one unit of

enzyme activity oxidized 1 μ mole of catechol to cis,cis-muconate per minute. Specific activity was defined as number of units per mg of protein.

Growth with low molecular weight aromatic hydrocarbons

Each isolate was inoculated into 50 mL of MR-1 broth, and incubated at room temperature with shaking at 150 rpm. After 2-3 days of incubation (Klett units between 100-150), the cells were transferred into 18mm diameter tubes containing DMM with 17 mM of a selected low molecular weight aromatic hydrocarbons. The aromatic hydrocarbons used for this test were: protocatechuate, catechol, pyrogallol, resorcinol, gallic acid, hydroquinone, *p*-aminobenzoic acid, salicylate, gentisic acid, vanillate, *p*-hydroxybenzoic acid, and phenyl acetate. After 10-15 days of incubation, visible turbidity was taken as evidence of growth.

Results

Isolation and classification of bacteria isolated from pine litter

To isolate bacteria, the pine litter suspensions were serially diluted and plated onto both PNA and GYEA. Well isolated colonies were re-streaked several times until pure cultures were obtained. Forty three bacteria were isolated from pine litter. More colonies appeared on GYEA than on PNA. GYEA showed 4.4×10^7 colony forming units (CFU) per mL of suspension on average compared to PNA at 5.1×10^6 CFU per mL (data not shown). Since a variety of aromatic hydrocarbons are degraded by catabolic pathways that converge upon protocatechuate or catechol under aerobic conditions, the ability of these isolates to grow with protocatechuate or catechol was determined. Among the forty three isolates initially obtained, fifteen isolates were able to utilize both protocatechuate and catechol as an energy source or a carbon source. Doubling times ranged from 2.24 to 4.78 hr on protocatechuate, and from 2.39 to 6.49 on catechol at room temperature (data not shown). The isolates grew slower in catechol than in protocatechuate.

To classify these isolates, a 16S rDNA sequencing was performed (Table 1). A BIOLOG analysis was also used as an independent method to identify the isolates. The bacterial isolates were identified as *Pseudomonas marginalis*, *Rhodococcus fascians*, *Pseudomonas fragi*, *Pseudomonas*

Table 1. Bacterial isolates from pine litter identified by 16S rDNA sequencing.

| Taxonomic Identification | GenBank Number |
|---------------------------------------|----------------|
| <i>Pseudomonas marginalis</i> | AJ851206 |
| <i>Rhodococcus fascians</i> | AY730713 |
| <i>Pseudomonas fragi</i> | AF094733 |
| <i>Pseudomonas corrugata</i> | AF348508 |
| <i>Serratia marcescens</i> | AF124036 |
| <i>Flavimonas oryzihabitans</i> | AF288732 |
| <i>Klebsiella pneumoniae ozaenae</i> | AF009170 |
| <i>Pseudomonas putida type B1</i> | FM208189 |
| <i>Pantoea dispersa</i> | AB273743 |
| <i>Burkholderia cepacia</i> | GQ359110 |
| <i>Serratia liquefaciens</i> | AY253924 |
| <i>Pseudomonas fluorescens type B</i> | U63900 |
| <i>Pseudomonas fluorescens type F</i> | AJ308306 |
| <i>Pseudomonas fluorescens type G</i> | AJ308307 |
| <i>Microbacterium esteraromaticum</i> | Y17231 |

corrugata, *Serratia marcescens*, *Flavimonas oryzihabitans*, *Klebsiella pneumoniae ozaenae*, *Pseudomonas putida type B1*, *Pantoea dispersa*, *Burkholderia cepacia*, *Pseudomonas fluorescens Type B, F and G*, *Serratia liquefaciens*, and *Microbacterium esteraromaticum*. Most of these isolates were members of the genera *Pseudomonas*.

Bacterial isolates from pine litter utilize the *ortho*-cleavage to degrade protocatechuate or catechol

To determine whether the bacterial isolates from pine litter degrade protocatechuate or catechol by the *ortho*- or *meta*-cleavage, the Rothera test was performed as described in materials and methods. All isolates that were able to utilize either protocatechuate or catechol converted substrates to β -keto adipate by the *ortho*-cleavage (data not shown). Since the *ortho*-cleavage is catalyzed by either protocatechuate-3,4-oxygenase or by catechol-1,2-oxygenase [22], the specific activities of these enzymes in these isolates were further determined (Fig. 2). *Pseudomonas fluorescens species* had higher levels of protocatechuate-3,4-oxygenase activities than other isolates, and *Microbacterium esteraromaticum* had a higher level of catechol-1,2-oxygenase activity than other isolates.

Bacterial isolates from pine litter utilize an array of low molecular weight aromatic hydrocarbons

To determine whether the bacterial isolates from pine litter have metabolic capacities to utilize aromatic hydrocarbons, the growth of the isolates with low molecular weight

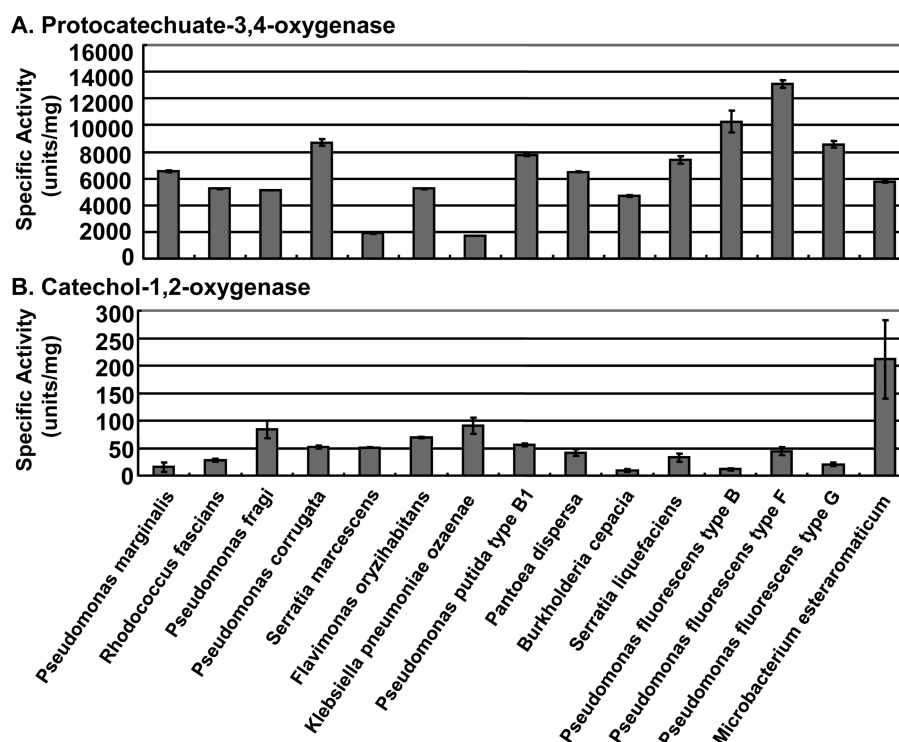


Fig. 2. Specific activity of protocatechuate-3,4-oxygenase or catechol-1,2-oxygenase. Fifteen bacterial isolates from pine litter, that were able to utilize both protocatechuate and catechol, were tested for (A) protocatechuate-3,4-oxygenase and (B) catechol-1,2-oxygenase activities as described in Materials and Methods.

Table 2. The growth of bacterial isolates from pine litter with low molecular weight aromatic hydrocarbons.

| | Pca | Cat | Hq | Phl | Phe | Sal | Gen | Pyr | Pab | Van | Gal | Res | Ben | Phb |
|---------------------------------------|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| <i>Pseudomonas Marginalis</i> | + | + | - | - | + | + | - | - | + | + | - | - | + | + |
| <i>Rhodococcus Fascians</i> | + | + | - | - | + | + | - | - | - | + | + | - | + | + |
| <i>Pseudomonas Fragi</i> | + | + | - | - | + | + | - | - | - | + | - | - | + | + |
| <i>Pseudomonas Corrugata</i> | + | + | - | - | + | + | - | - | - | + | - | - | + | + |
| <i>Serratia Marcescens</i> | + | + | - | - | + | + | - | + | - | + | - | - | + | + |
| <i>Flavimonas Oryzihabitans</i> | + | + | + | - | + | - | - | - | + | - | - | - | + | + |
| <i>Klebsiella pneumoniae Oz.</i> | + | + | - | - | + | - | - | - | - | - | - | - | + | + |
| <i>Pseudomonas putida B1</i> | + | + | - | + | + | - | - | - | - | - | - | - | + | + |
| <i>Pantoea Dispersa</i> | + | + | - | - | + | + | - | - | - | + | - | - | + | + |
| <i>Burkholderia cepacia</i> | + | + | - | - | + | + | + | - | + | + | - | + | + | + |
| <i>Serratia liquefaciens</i> | + | + | - | - | + | + | - | - | - | + | - | - | + | + |
| <i>Pseudomonas Fluorescens B</i> | + | + | - | - | + | + | - | - | - | + | - | - | + | + |
| <i>Pseudomonas fluorescens F</i> | + | + | - | - | + | + | - | + | - | + | + | - | + | + |
| <i>Pseudomonas fluorescens G</i> | + | + | - | + | + | + | - | - | + | + | - | - | + | + |
| <i>Microbacterium esteraromaticum</i> | + | + | - | - | + | - | - | - | + | - | - | - | + | + |

*Pca, protocatechuate; Cat, catechol; Hq, hydroquinone; Phl, phloroglucinol; Phe, phenyl acetate; Sal, salicylate; Gen, gentisate; Pyr, pyrogallol; Pab, *p*-aminobenzoic acid; Van, vanillate; Gal, gallic acid; Res, resorcinol; Ben, *p*-hydroxybenzoic acid; Phb, phenyl benzoate.

aromatic hydrocarbons derived from lignin was tested (Table 2). The aromatic hydrocarbons tested in this study were: protocatechuate, catechol, hydroquinone, phloroglucinol, phenyl acetate, salicylate, gentisate, pyrogallol, *p*-aminobenzoic acid, vanillate, gallic acid, resorcinol, *p*-

hydroxybenzoic acid and phenyl benzoate [15]. Table 2 lists the result of growth tests of the isolates with these aromatic hydrocarbons. In this experiment, an aromatic hydrocarbon was the only organic molecule in the medium. Thus, the growth meant that the isolates could use the

molecule as a growth substrate. All isolates utilized protocatechuate, catechol, phenyl acetate, *p*-hydroxybenzoic acid and phenyl benzoate. Hydroquinone was utilized only by *Flavimonas oryzihabitans*, and gentisate and resorcinol were utilized only by *Burkholderia cepacia*.

Discussion

The purpose of this research was to isolate bacteria from pine litter and to examine the evidence for their involvement in lignin biodegradation. Pine Needle Agar (PNA) was used as a selective medium to isolate bacteria from pine litter. In the present study, forty three bacteria were initially isolated from pine litter. Following the initial isolation, the ability of these bacterial isolates to utilize aromatic hydrocarbons was studied. Fifteen out of forty three isolates were able to utilize both protocatechuate and catechol for the growth. Most of isolates, that were able to utilize both protocatechuate and catechol, were members of the genus *Pseudomonas*. The others were *Rhodococcus*, *Serratia*, *Flavimonas*, *Klebsiella*, *Pantoeba*, *Burkholderia*, *Serratia*, and *Microbacterium spp.*

Pine Needle Agar (PNA) was proved to be a good selective medium for isolating aromatic hydrocarbon degrading bacteria from pine litter. Although more organisms grew on GYEA than PNA, the percentage of aromatic degraders on PNA (84.6% utilized both protocatechuate and catechol) was higher than on GYEA (40.0% for protocatechuate, and 36.7% for catechol) (data not shown). Two isolates from PNA could not utilize any of the aromatic hydrocarbons tested (data not shown), and these isolates might grow on PNA using cellulose or other carbohydrates extracted from pine needles. Interestingly, PNA seemed to inhibit the growth of fungi (data not shown). The bacterial isolates from pine litter in this study (except *Rhodococcus fascians*, Gram positive) were Gram negative (Table 1). Thus, Gram negative bacteria might be the predominant flora in pine litter.

According to the result of Rothera test, all isolates from pine litter, that were able to grow in the presence of aromatic hydrocarbons, degraded protocatechuate or catechol by the *ortho*-cleavage of the β -keto adipate pathway. To confirm the results of the Rothera tests, protocatechuate-3,4-oxygenase and catechol-3,4-oxygenase assays were performed. Protocatechuate-3, 4-oxygenase and catechol-3,4-oxygenase are significant enzymes in biodegradation of

aromatic hydrocarbons because these enzymes catalyze the initial cleavage of double bonds in an aromatic ring. All isolates exhibited protocatechuate-3,4-oxygenase and catechol-1,2-oxygenase activities.

The bacterial isolates from pine litter were able to metabolize low molecular weight aromatic hydrocarbons derived from lignin as a sole organic substrate. When offered as a sole source of carbon and energy, aromatic hydrocarbons derived from lignin biodegradation supported the growth of one or more of the isolates. Protocatechuate, catechol, phenyl acetate, *p*-hydroxybenzoic acid and phenyl benzoate were utilized by all isolates. Only one isolate could utilize hydroquinone (*Flavimonas oryzihabitans*), gentisate (*Burkholderia cepacia*), or resorcinol (*Burkholderia cepacia*). The distribution of metabolic capacities to utilize different aromatic substrates suggested that each isolate could grow at the expense of a different utilization array of potential substrates. Thus, combined metabolic activities of the bacterial flora in pine litter may play a role in the lignin recycling by degrading aromatic hydrocarbons derived from lignin biodegradation.

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국문초록

솔잎 퇴적물에서 추출한 방향족 탄화수소물질 분해 박테리아의 동정

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새로운 pine needle agar를 이용하여 15종의 박테리아를 솔잎퇴적물에서 추출하여 동정하였다. 이들 박테리아는 lignin biodegradation에서 주로 유도되는 방향족 탄화수소물질을 β -ketoadipate pathway의 ortho-cleavage를 이용하여 분해하는 것으로 밝혀졌다. 나아가서 이들 박테리아에 의한 여러 종의 방향족 탄화수소물질 분해에 관해서도 조사하였다. 본 연구는 솔잎 퇴적물에 존재하는 박테리아 종들이 방향족 탄화수소물질을 분해할 수 있는 대사능력을 가지고 있다는 것을 검증하였다.