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Effects of Intermediate Metabolites on Phenanthrene Biodegradation

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Abstract Stimulatory effects of three different intermediate metabolites (1-hydroxy-2-naphthoate, salicylate, and catechol) as potential inducers on phenanthrene degradation were investigated using two different bacteria (*Pseudomonas putida* ATCC 17484 and *Burkholderia cepacia* PB12). The relative induction capacity was high in the sequence of 1-hydroxy-2-naphthoate, salicylate, and catechol in both strains. The highest of up to 12 times increase of the induction was obtained by the addition of 1-hydroxy-2-naphthoate in the strain PB12, compared with the control where no exogenous inducer was added. The induction capacity of the potential inducers was closely related with the number of oxygenations required per electron equivalents in one mole of the inducer.

Key words: Biodegradation, bioremediation, induction, intermediates, phenanthrene

Polycyclic aromatic hydrocarbons (PAHs) are released to the environment as a result of incomplete combustion of fossil fuels or by accidental discharge during the transport, use, and disposal of petroleum products [3, 6]. Remediation of sites contaminated with these compounds is a major environmental concern because of their toxic and carcinogenic properties [3, 7, 9]. Bioremediation appears to be the most attractive means because of its cost effectiveness and use of natural degradation process [1, 8, 20]. However, the intrinsic biodegradation in a natural system is very slow, because of the stability of the condensed ring structure in PAHs. Furthermore, their hydrophobicity and tendency to sorb to organic matters greatly decrease the fraction of PAHs available to microorganisms in liquid phase [12, 19]. Therefore, the natural degradation process of PAHs is very slow and they persist in the environment for a long period of time [3].

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Recently, various strategies using supplements have been tried to enhance the intrinsic biodegradation rate of target compounds by stimulating the growth and metabolism of microorganisms. One of the strategies proposed to enhance the degradation of specific PAHs is to add one or more known pathway inducers to the bacteria [4]. The potential pathway inducers, which are produced as intermediates during PAH degradation, include salicylate, salicylaldehyde, 1-hydroxy-2-naphthoate, catechol, phthalate, gentisate, and cinnamate, and some of these pathway inducers have shown to stimulate biodegradation of PAHs [4, 10, 11, 13, 16]. These inducers affect not only enzymatic expression, but also cell growth. However, the factor of cell growth in much of these researches has not been intensively studied. In this study, we investigated the biodegradation of phenanthrene and induction by exogenously providing three different metabolic intermediates (1-hydroxy-2-naphthoate, salicylate, and catechol) to two different bacteria (*Pseudomonas putida* ATCC 17484 and *Burkholderia cepacia* PB12). Furthermore, we introduced the concept of induction capacity to compare the effects of metabolic inducers and cell growth. We suggest here specific relationships between induction capacity and the type of metabolic inducers during phenanthrene degradation.

We isolated *Burkholderia cepacia* PB12 from a PAH-contaminated soil by enrichment on phenanthrene as a sole carbon source [18]. *Pseudomonas putida* (ATCC 17484) was purchased from KCTC (Korean Culture Type Collection) and acclimated on mineral salt medium (MSM) containing phenanthrene as a sole carbon source with several subcultures for 1 month. Cultures of PB12 and ATCC 17484 were preserved in 50% glycerol at −70°C. To recover a frozen culture, a sterilized toothpick was stabbed into the cryostat vial and inoculated into 5 ml of NB (nutrient broth, Difco, U.S.A.) for 24 h. The cultures were harvested by centrifugation and washed twice with MSM. This was resuspended in MSM and used as inoculum for all biodegradation experiments. The mineral salt medium for biodegradation experiments

contained 50 mM NH_4Cl, 5 mM NaH_2PO_4, 11.3 mM Na_2HPO_4, 10 mM KCl, 2 mM Na_2SO_4, 1.25 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $CaCl_2$, 0.1 M $Na_2MoO_4 \cdot 2H_2O$, 1 mM EDTA, and 5 ml per 1 l of a trace solution containing 0.12 mM HCl, 5 mM $ZnSO_4 \cdot 7H_2O$, 20 mM $FeSO_4 \cdot 7H_2O$, 10 mM $MnSO_4 \cdot H_2O$, 1 mM $CuSO_4 \cdot 5H_2O$, 2 mM $CoCl_2 \cdot 6H_2O$, and 0.8 mM H_3BO_3. The final pH was 7.0.

Biodegradation experiments were carried out with completely soluble phenanthrene in MSM containing 1 wt% Triton X-100. Thus, phenanthrene crystals were solubilized in the surfactant medium at a concentration of 50 mg/l, and then the medium was autoclaved at 121°C for 20 min. Eight ml of the culture medium and microbial inoculum to final OD of 0.03 were placed to a 15-ml glass tube. The tubes were shaken in a rotary shaker at 150 rpm at 25°C. The effect of three metabolic inducers (1-hydroxy-2-naphthoate, salicylate, and catechol) on biodegradation of phenanthrene and cell growth was examined under the same culture conditions. Various concentrations of each inducer, ranging from 0 to 1,000 mg/l, were examined for the microorganisms (PB12 and ATCC 17484). Three controls were also included: (1) tests done with phenanthrene as a single carbon source, (2) tests on the growth of microorganisms in the control medium without phenanthrene, and (3) tests to assess abiotic loss of phenanthrene. Separate triplicate runs were performed for all the experiments.

Liquid samples were taken at appropriate times from the tubes and used for the measurement of phenanthrene. Cell growth was determined by measuring the optical density of culture broth samples at 600 nm (OD_{600}) with a DR/2010 spectrophotometer (HACH, U.S.A.). Phenanthrene, salicylate, and 1-hydroxy-2-naphthoate were analyzed by HPLC (high-performance liquid chromatography, Dionex, U.S.A.) using a UV detector at 254 nm. The analytical column was a reversed-phase column, Supelcosil^{TM} LC-PAH column (150 mm×4.6 mm). The column was eluted with mobile phase (75% acetonitrile, 24% deionized water, and 1% of acetate) at a flow rate of 1.5 ml/min. The minimum detectable concentration was approximately 0.1 mg/l of phenanthrene, 1-hydroxy-2-naphthoate, and catechol and 1 mg/l of salicylate. For analysis of the compounds, approximately 0.5 ml of a liquid was withdrawn with a disposable glass Pasteur pipette and placed into a 1.5-ml borosilicate glass vial. This was immediately centrifuged at 6,000 ×*g* for 10 min, and the supernatant was injected directly into the HPLC system.

The bacterial strains, *Pseudomonas putida* ATCC 17484 and *Burkholderia cepacia* PB12, were able to degrade phenanthrene as a single carbon and energy source. Both strains could also degrade 1-hydroxy-2-naphthoate, salicylate, and catechol as a single carbon source. For example, Fig. 1 shows the time courses of cell growth of the strain ATCC 17484 and phenanthrene biodegradation with or without exogenous salicylate as a potential inducer. The cell growth with only phenanthrene was not appreciable, and the

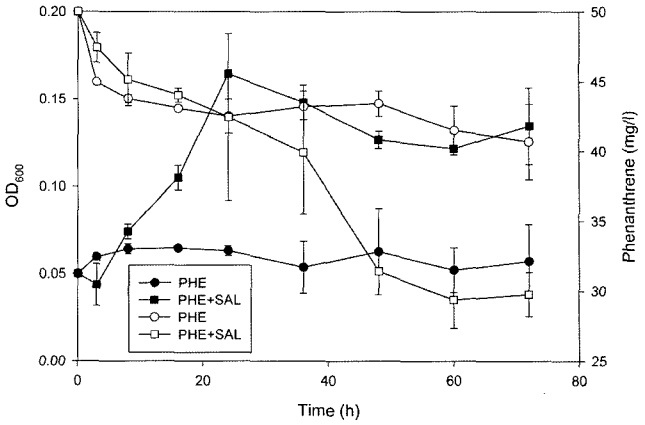

Fig. 1. Time courses of cell growth (filled symbols) and phenanthrene (PHE) degradation (open symbols) during the culture of *Pseudomonas putida* ATCC 17484 with (square) or without (circle) addition of salicylate (SAL) at 100 mg/l.
The error bars indicate the standard deviations of triplicate tests.

amount of phenanthrene degraded was only 18.6 % during 72 h of cultures. In separate control tests, phenanthrene degradation in the absence of bacterial inoculation was negligible (<5%). When 100 mg/l of salicylate was supplemented, phenanthrene degradation was greatly enhanced, and 40.4% of the initial phenanthrene was degraded and the OD value by cell growth was also increased from 0.05 to 0.17. The salicylate was completely consumed within 24 h (data not shown). The stimulatory effect of exogenously provided metabolic inducers on the degradation of various PAHs has been demonstrated by several previous studies: The stimulatory metabolic inducers include intermediates produced during biodegradation of phenanthrene, which are salicylate, salicylaldehyde, 1-hydroxy-2-naphthoate, catechol, phthalate, gentisate, and cinnamate [4, 11, 13]. However, induction by the pathway inducers was not always effective in some cases and was dependent on environmental conditions [2].

Cell growth and phenanthrene degradation were examined at various initial concentrations of three different metabolic inducers (1-hydroxy-2-naphthoate, salicylate, and catechol). Figure 2 shows the results on the growth and phenanthrene degradation by the strain ATCC 17484. In all cases of inducers, OD values higher than that of the control (0.045) were observed, since all the inducers were used as additional carbon substrates. Whereas the cell growth on catechol and salicylate was significantly higher than control, 1-hydroxy-2-naphthoate was not notably effective on the cell growth. Salicylate showed significant inhibition of cell growth at a high concentration, 500 mg/l. The stimulating effect on phenanthrene degradation was observed in all the inducers added. However, the effect of the inducers on phenanthrene degradation was quite different from that on the cell growth. The stimulating effects of 1-hydroxy-2-naphthoate and salicylate were higher than that of catechol. The phenanthrene

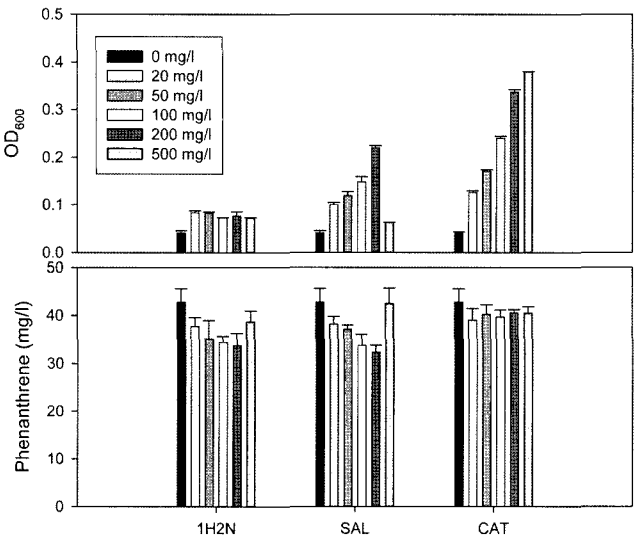

Fig. 2. The effects of initial concentrations of three potential inducers [1-hydroxy-2-naphthoate (1H2N), salicylate (SAL), and catechol (CAT)] on cell growth and phenanthrene degradation after 2 days of culture of *Pseudomonas putida* ATCC 17484.
The initial phenanthrene concentration was 50 mg/l. The error bars indicate the standard deviations of triplicate tests.

degradation increased with increasing initial concentrations of salicylate and 1-hydroxy-2-naphthoate up to 200 mg/l, but it did not increase with increasing initial concentrations of catechol.

Figure 3 shows the cell growth and phenanthrene degradation for the strain PB12. The results were somewhat

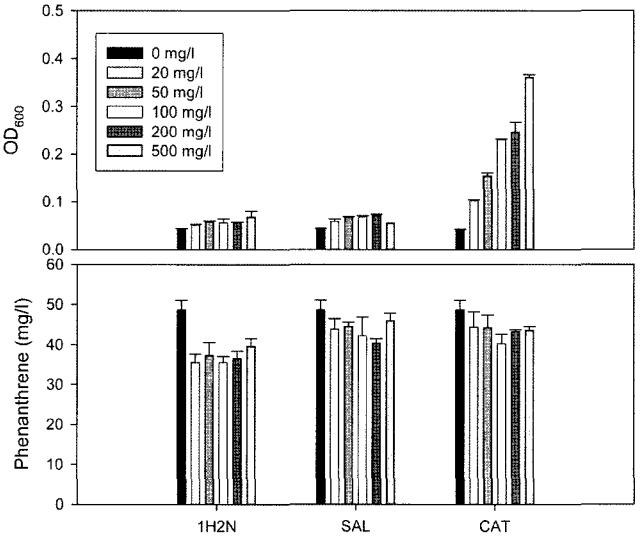

Fig. 3. The effects of initial concentrations of three potential inducers [1-hydroxy-2-naphthoate (1H2N), salicylate (SAL), and catechol (CAT)] on cell growth and phenanthrene degradation after 2 days of culture of *Burkholderia cepacia* PB12.
The initial phenanthrene concentration was 50 mg/l. The error bars indicate the standard deviations of triplicate tests.

different from those of the strain ATCC 17484. In the absence of exogenous inducer, the cell growth was similar between the two strains, but phenanthrene degradation was lower with PB12 (3% degraded) than with ATCC 17484 (15% degraded). The results of cell growth on catechol and 1-hydroxy-2-naphthoate were very similar with the strain ATCC 17484. However, unlike the results of the strain ATCC 17484, the cell growth on salicylate was not much higher than the control. The stimulating effect of catechol on phenanthrene degradation for the strain PB12 was somewhat higher than the strain ATCC 17484. The stimulating effect of salicylate was lower than that of the strain ATCC 17484, which might result from low cell growth on salicylate. The stimulating effect of 1-hydroxy-2-naphthoate was the highest among the inducers, particularly at low initial concentrations.

We introduced the concept of induction capacity, which was defined as the quantity of target chemicals degraded per unit mass of microbial cells grown on target chemicals and/or inducers at a given time. The capacity would be different, depending on the type and amount of inducers initially added.

$$I_C = \frac{\text{Target chemicals degraded [mg/l]}}{\text{Cell mass [mg/l]}} \quad (1)$$

Without exogenous inducers added, self-induction capacity ($I_{C,\,self}$) could be defined as a basal case. The relative induction capacity can be represented by the ratio of I_C to $I_{C,\,self}$ as the following:

$$I_{C,\,rel} = \frac{I_C\ [-]}{I_{C,\,self}\ [-]} \quad (2)$$

When $I_{C,\,rel} > 1$, it means an increased degrading power of the given mass of microorganisms in the presence of inducers.

The self-induction capacity ($I_{C,\,self}$) was 0.1 for the strain ATCC 17484 and 0.2 for the strain PB12 during 2-day degradation tests. Relative induction capacity ($I_{C,\,rel}$) obtained by adding each inducer was compared for each microbial strain (Fig. 4). The relative induction capacity in both strains was high in the sequence of 1-hydroxy-2-naphthoate, salicylate, and catechol. For each inducer, the strain PB12 had a higher relative induction capacity than the strain ATCC 17484, indicating that the strain PB12 was more effectively induced for phenanthrene degradation by the addition of these metabolic inducers. In particular, the $I_{C,\,rel}$ value was dramatically increased up to 12 times by the addition of 1-hydroxy-2-naphthoate to the strain PB12, compared with the control in the absence of an exogenous inducer. In the case of catechol, the $I_{C,\,rel}$ value was similar to the control, indicating that catechol is not an effective inducer. The strain ATCC 17484 showed a lower $I_{C,\,rel}$ value than the control when catechol was added, indicating decreased phenanthrene degradation per unit cell mass during higher cell growth on catechol.

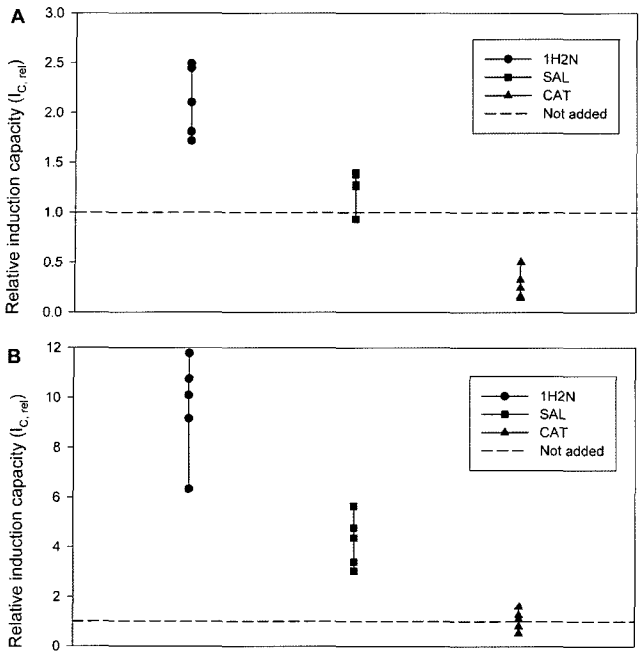

Fig. 4. Comparisons of relative induction capacity by the addition of different intermediate metabolites [1-hydroxy-2-naphthoate (1H2N), salicylate (SAL), and catechol (CAT)] during phenanthrene degradation.

The five symbols for a single metabolite represent data from the tests using different initial concentrations. The individual data point represents the mean of three separate tests. **A.** *Pseudomonas putida* ATCC 17484. **B.** *Burkholderia cepacia* PB12.

Among three intermediates (1-hydroxy-2-naphthoate, salicylate, and catechol), 1-hydroxy-2-naphthoate showed the highest induction capacity per unit cell on both microorganisms. The unique result is that the induction capacity was higher as the inducer was located closer to the upper pathway of phenanthrene degradation: A possible reason could be that a compound in the lower pathway has a chemically simpler structure and can be more easily degraded by bacteria. Therefore, higher cell growth was obtained when an inducer in the lower pathway, such as catechol, was used. This rapid cell growth might shift cellular metabolism to cell synthesis from the phenanthrene degradation machinery [5, 15]. Second, the rapid cell differentiation can also cause plasmid loss, mainly by segregation, which is the division of the cell into two daughter cells when only one plasmid is present [14]. Such plasmid loss would be prominent during the growth using a simpler inducer. Third, a compound in the upper pathway requires more oxygenation reactions for its complete degradation, possibly inducing more phenanthrene oxygenation reactions. The number of oxygenation reactions required per electron equivalents in one mole of compound can be a useful mathematical indicator for induction capacity of the compound. The value has been reported to be 4/46 (=0.087), 2/28 (=0.071), and 1/26 (=0.038) for 1-hydroxy-2-naphthoate, salicylate, and catechol, respectively [17]. This value represents how many oxygenase reactions are required for the degradation of substrate with equivalent energy source. Thus, microorganisms should require more oxygenase reactions in the system, when exogenously provided with inducers with a higher value. Our observations suggest that the induction capacity per unit cell would be superior when an inducer was closer to the original compound. The induction capacity of potential inducer might be related with oxygenation requirements per electron equivalents of the inducer, which could be calculated from its chemical structure.

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