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Biodegradation of Phenanthrene by Psychrotrophic Bacteria from Lake Baikal

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Abstract Psychrotrophic phenanthrene-degrading bacteria were identified in the sediment samples collected from Lake Baikal, Russia. Among 70 phenanthrene-degrading isolates, the seven that had the highest phenanthrene-degradation rates were identified by 16S rDNA sequencing. Isolate P25, identified as the Gram-positive rod-shaped organism *Rhodococcus erythropolis*, had the highest growth and degradation rate at 15°C. It could remove 26.0% of 100 mg l^{-1} phenanthrene in 20 days at 15°C, and degradation was less at 5°C and 25°C. The addition of surfactants to enhance degradation was tested. Brij 30 and Triton X-100 inhibited degradation at all surfactant concentrations tested, but Tween 80 stimulated phenanthrene degradation, especially at low concentrations. When 20× CMC (critical micelle concentration) of Tween 80 was added, 38.0% of 100 mg l^{-1} phenanthrene was degraded in 12 days at 15°C. This psychrotrophic phenanthrene-degrading bacterium is a candidate for use in bioremediation of polycyclic hydrocarbon contamination in low temperature environments.

Key words: Biodegradation, phenanthrene, psychrotrophic bacteria, *Rhodococcus erythropolis*, surfactant

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants produced by industrial operations using fossil fuels as well as by natural events such as forest fires. Since some of these PAHs and their metabolites are toxic, mutagenic, and carcinogenic to animals and humans, they must be removed [15, 29]. There have been numerous studies of the biodegradation and bioremediation of PAHs and PAH-containing petroleum, most using mesophilic bacteria [5, 13, 16, 27, 28, 33, 35]. Temperature has a very significant effect on the extent of degradation by microbes, and it also affects the solubility of less soluble hydrophobic compounds and thus their bioavailability [23]. Since large parts of the Earth are at low temperatures, cold-adapted microorganisms play a significant role in removing many recalcitrant contaminants. To date, most biodegradation studies at low temperature have been directed at the removal of crude oil or fuel oils contaminating polar soils [2, 10, 22, 25]. Studies of the effect of temperature on PAH degradation have been restricted to the soil [3, 11, 21], and very little is known about the degradation of recalcitrant PAHs by freshwater bacteria. Although Korea is a temperate area, most freshwater environments (for example, large rivers and lakes and subsurface aquifers) are much below ambient temperature. In this study, psychrotrophic PAH-degrading bacteria were isolated from Lake Baikal and their biodegrading capability was investigated. Lake Baikal in Siberia, Russia, is the world's deepest freshwater lake and contains the largest volume of fresh water on Earth. The average annual temperature of the subsurface water is below 4°C and it might harbor unique microorganisms, in particular, cold-adapted ones. The isolation of psychrophilic or psychrotrophic PAH-degrading bacteria could be very useful in increasing the microbial resources available for industrial purposes.

We isolated one thousand psychrotrophic bacterial strains from the water and sediments of Lake Baikal by incubation on R2A agar medium (Difco Lab.) at 10°C, and differentiated them on the basis of colony appearance and cell morphology after Gram staining. Of these, 70 strains grew on a minimal salts basal medium [30] containing 100 mg l^{-1} of the three-ring PAH, phenanthrene, as sole source of carbon and energy. Seven of these strains that grew most actively and degraded more than 10% of phenanthrene in 6 days at 15°C (data not shown) were identified by sequencing of 16S rDNA (Table 1). Strain P25 was one of the strains that showed the highest rates of growth and phenanthrene degradation. A Gram-positive rod-shaped bacterium, P25 was identified as *Rhodococcus erythropolis*.

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Table 1. Identification of psychrotrophic phenanthrene-degrading bacteria isolated from Lake Baikal by 16S rDNA sequence analysis.

Strain No.	16S rDNA identification	% of similarity	GenBank accession
P1	*Rhodococcus erythropolis*	1373/1377 (99%)	AY833096
P12	*Rhodococcus erythropolis*	1401/1403 (99%)	AY833097
P18	*Bacillus simplex*	1436/1438 (99%)	AY833099
P25	*Rhodococcus erythropolis*	1402/1404 (99%)	AY833095
P37	*Actinobacterium* sp.	1409/1411 (99%)	AY833100
P49	*Arthrobacter nicotinovorans*	1411/1416 (99%)	AY833102
P62	*Rhodococcus erythropolis*	1394/1398 (99%)	AY833103

It was found in the sediment of an area that receives a large amount of pulp mill wastewater from Baikalsk City and contains various aromatic compounds of lignin. The sample site is 300 m deep and its average annual temperature is below 4°C. *Rhodococcus* spp. are well known for their diverse biodegradative capabilities [6, 24], but there has been no reports describing PAH degradation by *Rhodococcus* at low temperature. When incubated at 15°C with 100 mg l^{-1} of substrate, *R. erythropolis* P25 used phenanthrene as a sole source of carbon and energy; however, glucose was a better substrate for growth than phenanthrene (Fig. 1). The addition of readily utilizable compounds such as glucose, organic acids, and yeast extract has been reported to increase the rate of degradation of PAHs [19, 35]. When 100 mg l^{-1} of phenanthrene was present as the sole source of carbon in the minimal salts medium, the growth of *R. erythropolis* P25 was much slower, and the protein concentration measured by the Lowry method [20] was lower than with 100 mg l^{-1} glucose as substrate for growth. The addition of 100 mg l^{-1} phenanthrene to medium containing the same concentration of glucose did not inhibit the growth of *R. erythropolis* P25 (Fig. 1).

To investigate the effect of temperature on phenanthrene degradation by *R. erythropolis* P25, replicate bacterial cultures in minimal salts basal medium containing 100 mg l^{-1} of phenanthrene were incubated at 5°C, 15°C, and 25°C on rotary shakers (150 rpm). At various times, duplicate cultures were extracted three times with methylene chloride in a separating funnel, and the remaining phenanthrene was measured using a gas chromatograph (GC) (Hewlett Packard Co., Model 5890) equipped with a flame ionization detector, as described elsewhere [14]. The rate of degradation of phenanthrene was higher at 15°C than at 5°C and 25°C. At 15°C, the bacteria degraded 26.0% of the phenanthrene in 20 days compared with 17.1% and 16.0% at 5°C and 25°C, respectively (Fig. 2). Although *R. erythropolis* P25 is not a true psychrophile, the faster removal of phenanthrene at 15°C indicates that it is at least psychrotrophic. Other strains of *R. erythropolis* have been shown to degrade hydrocarbons (alkanes, alcohols, and phenol) faster at mesophilic temperatures [6, 24]. It is difficult to compare biodegradation by *R. erythropolis* P25 to that by other PAH-degrading bacteria at low temperature because of the scarcity of similar studies. When enrichment cultures of Antarctic seawater containing cold-adapted hydrocarbonoclastic bacteria were incubated with crude oil (0.1% v/v) at 4°C, 94.4% of the initial amount of PAH remained present after a month [34]. Margesin [22] reported 37–41% removal of diesel oil at 10–20°C after 30 days in a liquid culture of cold-adapted *Yarrowia lipolytica*, and less was removed at 4°C and 25°C. Considering the recalcitrance of phenanthrene

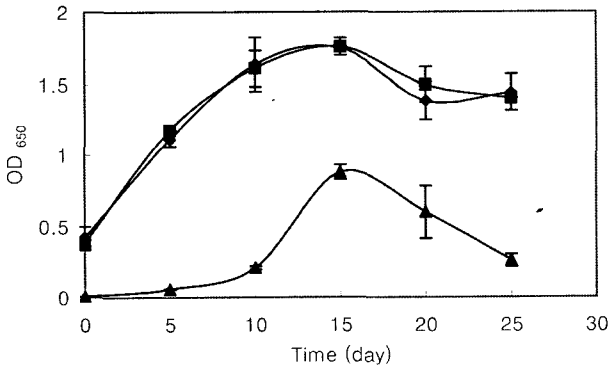

Fig. 1. Growth of psychrotrophic phenanthrene-degrading *Rhodococcus erythropolis* P25 with different C source (100 mg l^{-1}) at 15°C.
Symbols: glucose only (■), glucose+phenanthrene (◆), phenanthrene only (▲).

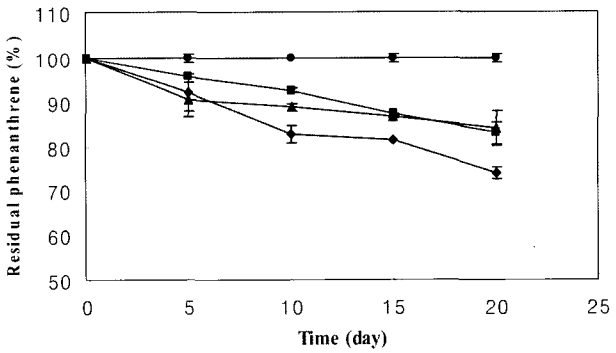

Fig. 2. Degradation of 100 mg l^{-1} phenanthrene by *Rhodococcus erythropolis* P25 at different temperatures.
Symbols: 5°C (■), 15°C (◆), 25°C (▲), un-inoculated control at 15°C (●).

and higher percentages of more readily degradable alkanes and monocyclic aromatic hydrocarbons in crude oil and diesel oil, the degradative capacity of *R. erythropolis* P25 may well not be lower than those of the cold-adapted *Y. lipolytica* [22] and antarctic bacteria [34].

The rate of disappearance of four kinds of PAHs from the soil decreased sharply when the temperature was lowered from 25°C to 10°C, although the effect of temperature on the rate of PAH disappearance depended on the physico-chemical properties of the compounds and of the soil [21]. Due to the low degradation rates of the PAHs, it was suggested that PAHs would persist for longer at low temperatures. Moran and Hickey [26] have raised the question of whether the results of laboratory tests at mesophilic temperatures are representative of those that might be obtained during *in situ* bioremediation of aquifers at low temperatures. Since temperature is probably the main limiting factor for the biodegradation of PAHs under field conditions, the isolation of bacteria with a high degradative capability at low temperature may be the key to the application of degradative microorganisms to bioremediation of PAH-contaminated sites. The effect of temperature on phenanthrene degradation by *R. erythropolis* P25 should be tested further at more temperatures to determine the optimum temperature.

We examined the effect of adding a surfactant on phenanthrene degradation by *R. erythropolis* P25. The solubility of phenanthrene in water is just 1.29 mg l^{-1} at 25°C, and surfactants have been observed to increase the solubility and biodegradation of hydrophobic compounds [4]. The solubility of hydrophobic compounds decreases at low temperature, and they become more resistant to microbial degradation. Moreover, the viscosity of oil increases at low temperature, reducing spreading and the subsequent degradation of the oil [32]. There have been many studies of the effect of surfactants on the degradation of recalcitrant hydrophobic compounds. However, the results have not been consistent [4, 9, 18, 31]. In this study, we added the non-ionic surfactants, Brij 30, Triton X-100, and Tween 80, separately to the minimal salts basal medium. The critical micelle concentrations (CMC) of Brij 30, Triton X-100, and Tween 80 are 9.7, 13.6, and 13.4 mg l^{-1}, respectively [17] and the concentration of each surfactant in the cultures was adjusted to 1, 20, and 100 times CMC. Brij 30 inhibited phenanthrene degradation at all three concentrations, although the extent of inhibition was somewhat less with 1× CMC than with 20× and 100× CMC (Fig. 3A). Although Triton X-100 at 1× CMC did not have a significant effect on degradation, it also inhibited degradation at the higher concentrations (Fig. 3B). These patterns of inhibition of phenanthrene degradation by Brij 30 and Triton X-100 are similar to those observed in an aerobic mixed culture [35]. They confirm the general inhibitory effect of surfactants on PAH degradation due presumably to disruption of membrane lipids [4]. Volkering *et al.* [31] have suggested

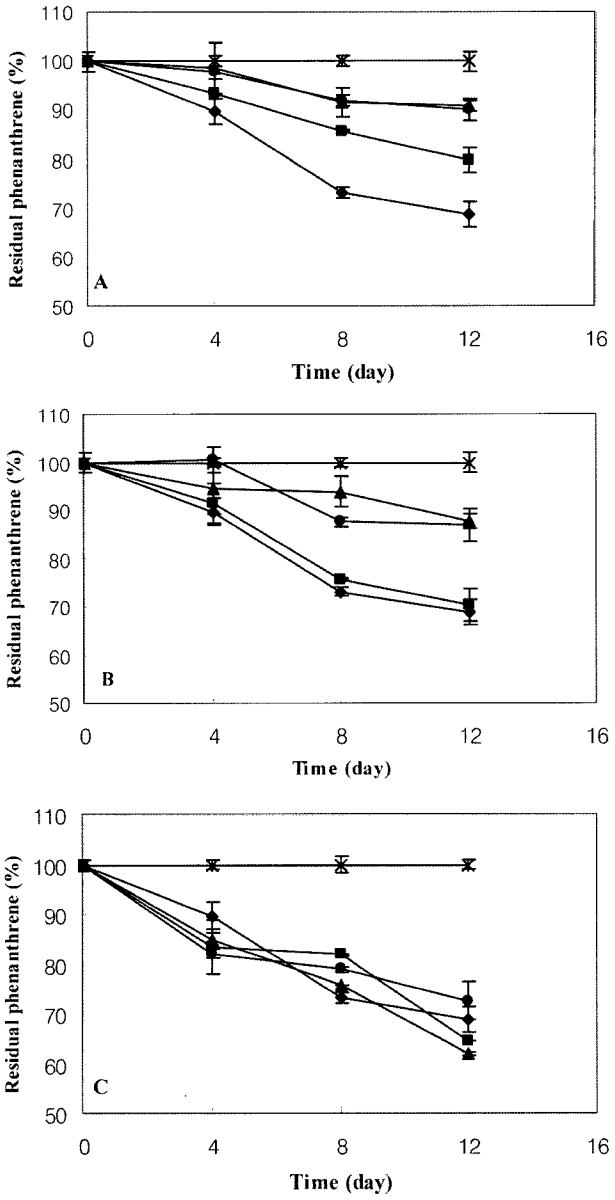

Fig. 3. Effect of surfactants (A: Brij 30; B: Triton X100; C: Tween 80) on the degradation of 100 mg l^{-1} phenanthrene by *Rhodococcus erythropolis* P25 at 15°C.
Symbols: no surfactant (◆), 1 CMC (■), 20 CMC (▲), 100 CMC (●), un-inoculated control (×).

that substrate present in the micellar phase may not be readily available for microbial degradation; micellar PAH is probably a protected reservoir and may replenish the aqueous phase PAH when the latter is depleted by degradation. Triton X-100 inhibited the growth of *Mycobacterium* sp. and *Pseudomonas* sp. on solid anthracene even at low surfactant concentration (0.09 CMC) [8]. In contrast to Brij 30 and Triton X-100, low concentrations of Tween 80 enhanced phenanthrene degradation by *R. erythropolis* P25, and 38.0% of the phenanthrene was degraded at a

concentration of 20× CMC in 12 days (Fig. 3C). Even 100× CMC did not significantly inhibit degradation. This result confirms the classical effect of a surfactant in promoting dissolution of less water-soluble compounds at concentrations exceeding the CMC [31]. Unlike Brij 30 and Triton X-100, Tween 80 had no toxic effect on microorganisms [17]. The authors of that work suggested that this was probably due to the higher molecular weight of Tween 80. Kotterman *et al.* [18] also reported an improvement in PAH bioavailability by adding Tween 80 to fungal cultures. Since the effect of a surfactant on the degradation of hydrophobic compounds may depend upon the type of surfactant, the target compound, the concentration of surfactants, and the environmental conditions, the use of a surfactant in bioremediation of environments contaminated with hydrophobic compounds should be carefully considered and the optimum concentration of surfactant first determined.

Inoculation with degradative microorganisms may appreciably enhance the degradation of persistent contaminants in various types of environment, including those at low temperature [4]. Whyte *et al.* [32] showed that psychrotrophic *Rhodococcus* sp. stimulated the mineralization of alkanes in the soil microcosm at 5°C. Although the degradative capacity of *R. erythropolis* P25 is somewhat lower than those of other mesophilic PAH degraders [1, 29], it may be useful for application to contaminated freshwaters with low temperatures. The biochemical and genetic properties of *R. erythropolis* P25 should be characterized in order to improve its biodegrading ability and industrial utility. It would also be desirable to identify additional psychrotrophic degradative bacteria to obtain improved strains for bioremediation, and for biotechnological applications of cold-adapted enzymes with high catalytic efficiency [7, 12].

Acknowledgments

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