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Biodegradation of Phenol by a Trichloroethylene-cometabolizing Bacterium

PARK, GEUN-TAE, HONG-JOO SON, JONG-GOO KIM, AND SANG-JOON LEE*

Department of Microbiology, College of Natural Science, Pusan National University, Pusan 609-735, Korea

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Abstract A microorganism which degrades phenol and co-metabolizes trichloroethylene (TCE) was isolated from Yangsan stream after enrichment in a medium containing phenol as the sole carbon source. The isolate EL-43P was identified as the genus *Rhodococcus* by its morphological, cultural and physiological characteristics. Phenol-induced cells of *Rhodococcus* sp. EL-43P degraded TCE. Toluene and nutrient broth could not replace the phenol requirement. The optimal conditions of initial pH and temperature of media for growth were 7.0~9.0 and 30~50°C, respectively. *Rhodococcus* sp. EL-43P could grow with phenol up to 1,000 ppm. Growth was inhibited by phenol at a concentration above 1,500 ppm. It was observed that *Rhodococcus* sp. EL-43P was able to degrade 90% of phenol (1,000 ppm) after 40 h in a culture. Phenol-induced cells of *Rhodococcus* sp. EL-43P degraded 95% of 5 μM TCE in 6 h. *Rhodococcus* sp. EL-43P hardly degraded TCE above 100 μM.

Key words: Trichloroethylene, *Rhodococcus*, phenol, cometabolism, degradation

Volatile chlorinated aliphatic hydrocarbons (CAHs) are a major concern as potential health hazards in drinking water [7]. One of the most prevalent CAHs is trichloroethylene (TCE). TCE is an industrial chemical which is used extensively as a degreasing agent and is suspected as a carcinogenic agent [3, 5, 11]. Besides, TCE is most commonly detected in groundwater because of its persistance [15, 21].

There have been extensive efforts to document the biodegradation of TCE by microorganisms. TCE has been shown to be degraded under anaerobic and aerobic conditions [7, 11, 14, 15]. Anaerobic TCE biodegradation produces harmful metabolites, such as dichloroethylene and vinyl chloride [17]. Complete mineralization of TCE was obtained by a methane-utilizing bacterium and an aromatic compound degrading bacterium under aerobic condition [2]. The most effective biodegradation of TCE is aerobic cometabolism [15].

Several aerobic bacteria that oxidize hydrocarbons degrade TCE, and the use of these strains in TCE bioremediation has been proposed [16, 17]. Nelson *et al.* [14] have shown that certain strains of bacteria can degrade TCE after growth with mononuclear aromatic compound such as toluene and phenol. These observations suggest that an inducible enzymatic component of the aromatic degradative pathway is involved on the gratuitous degradation of TCE [8, 11]. Further studies by Nelson *et al.* [15] and Wackett *et al.* [20, 21] showed that mutants of *Pseudomonas putida* F1 in which toluene dioxygenase activity was absent failed to degrade TCE. In contrast, mutants defective in other enzymes of the toluene degradative pathway could still degrade TCE [18]. Haker and Kim [7] showed that phenol hydroxylase from *Alcaligenes eutrophus* JMP 134 had performed degradation of TCE. Recently, due to its widespread contamination in soil and water and its bioremediation, TCE has received much attention [8, 5, 19].

In this paper, we report on the isolation and characterization of a phenol-degrading bacterium which metabolizes TCE. We also describe the condition of phenol degradation.

MATERIALS AND METHODS

Chemicals

All chemicals for preparation of media and analyses were extra pure-grade and were purchased from Junsei, Aldrich, Sigma Chemical Co..

*Corresponding author
Phone: 82-51-510-2268; Fax: 82-51-518-1688;
E-mail: Sangjoon@hyowon.pusan.ac.kr

Media and Culture Conditions

The isolation medium of phenol-degrading bacteria was composed of 10 mM KH_2PO_4, 10 mM $Na_2HPO_4 \cdot 12H_2O$, 18 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4 \cdot 7H_2O$, 100 μM $CaCl_2 \cdot 2H_2O$ and 1 μM $FeCl_3$, and its initial pH was adjusted to 7.0 ± 0.2. Phenol (500 ppm) was supplied as a carbon source by addition of filter-sterilized solution to autoclaved isolation medium. All isolates were grown on isolation media supplemented with the appropriate carbon sources (500 ppm phenol and 30 μM TCE). The effect of temperature, pH, phenol concentration, and aeration on the rate of phenol degradation by a selected isolate were investigated. Temperatures were controlled by submersion of the culture vessel (250-ml Erlenmeyer flask) in a circulating water bath. pH effects in mineral salts medium adjusted with HCl or NaOH to the appropriate pH values were determined. Phenol concentration effects were investigated using mineral salt medium containing appropriate concentration of phenol as the carbon source. Different levels of aeration were obtained by varying the amount of medium in 250-ml Erlenmeyer flasks and keeping the agitation constant, i.e. 150 rpm.

Isolation and Identification of Phenol-degrading Bacterium

Samples collected from the Yangsan stream area in Yangsan Gun were inoculated into 10 ml of the liquid isolation medium in test tubes, before the samples were incubated at 30°C in a shaking incubator (150 rpm) for 5 days. The cultures with positive bacterial growth were diluted and spreaded on the isolation media plates. The plates were then incubated at 30°C for 5 days. Single colonies were isolated from the solid media, and were used to further study the degradation of phenol. The isolate was identified according to the Bergey's Manual of Systematic Bacteriology [9] and Manual of Methods for General Bacteriology [6] based on its morphological, cultural and physiological characteristics.

Phenol Analyses

Rates at which microorganisms degraded phenol were determined by using a modified colorimetric assay [4]. At defined intervals, 1-ml samples were transferred to 1.5-ml microcentrifuge tubes containing 50 μl of 2 N NH_4OH and 25 μl of 2% 4-aminoantipyrene. The tubes were closed and the contents were mixed. Then 25 μl of 8% $K_3Fe(CN)_6$ was added and the contents were mixed again and centrifuged ($15,000 \times g$) for 2 min. A_{510} of the supernatant was measured. Phenol concentrations were calculated by reference to a standard curve.

TCE Analyses

Rhodococcus sp. EL-43P strains were grown to an optical density at 660 nm of 1.0 to 1.2 in mineral salts medium with phenol (1,000 ppm) as the sole carbon source. Culture was harvested by centrifugation at $8,000 \times g$ for 10 min. Cell pellets were suspended in mineral salts medium to an optical density of 1.0 at 660 nm. TCE degradation experiments were carried out in 100-ml serum bottles sealed with teflon-lined rubber septa and crimp cap. Each bottle contained 20 ml of cell suspension and TCE (5~100 μM). TCE was added as an aqueous stock by syringe through the septa. Unless otherwise indicated, experiments were terminated 36 h after incubation at 30°C and 150 rpm. TCE degradation was monitored by measuring TCE concentrations in the aqueous phase of the test bottles by pentane extraction and gas chromatography [12]. Cell-free controls were run in parallel to assess the abiotic loss of TCE from the system.

Gas Chromatography

The amounts of TCE in pentane extracts were determined by using a gas chromatograph (Hewlett-Packard 5890A) equipped with a type 60 m HP Vocol capillary column and an electron capture detector system. Peak intergrations were obtained with a HP 3390A intergrator. The operating conditions were; sample volume, 1 μl; carrier gas, nitrogen; nitrogen flow rate, 10 ml/min; injector temperature, 150°C; oven temperature, 45°C; detector temperature, 250°C [13].

RESULTS AND DISCUSSION

Isolation and Identification of Phenol-degrading Bacterium

The microorganisms which degrade phenol were isolated from soil and water samples in Yangsan stream. From these samples, 20 strains of phenol-degrading bacteria were isolated and tested for the degradation of TCE. Among them, strain EL-43P showed the highest degrading activity of phenol and ability to co-metabolize TCE. Therefore, strain EL-43P was selected for further characterization in this work. Table 1 shows that phenol-induced cells of EL-43P degraded TCE, but nutrient broth grown cells of EL-43P did not degrade TCE. Toluene would support the cell growth, but not TCE degradation when substituted for phenol. These results implicate that phenol acts as an inducer for the enzymes involved in TCE degradation. Two strains of *Pseuomonas putida* that degrade TCE by a pathway containing a toluene dioxygenase also co-metabolize TCE [14, 15]. The ability of *A. eutrophus* JMP134 to degrade TCE by a phenol-dependent pathway was reported by Montgomery *et al.* (Montgomery *et al.* 1989. Abstr. *Annu. Meet. Am. Soc. Microbiol.* K-68, p. 256.). Therefore, our results suggest phenol-dependent pathway in TCE metabolism.

We are now investigating the enzymatic experiment in order to confirm the relationship between TCE metabolism and phenol hydroxylase.

Strain EL-43P is a nonmotile, gram-positive, short rod-shaped bacterium which grows to branched substrate mycelium. The isolate was oxidase positive, catalase positive, and strictly aerobic. The cell wall peptidoglycan contained major amounts of *meso*-diaminopimelic acid, arabinose, and galactose (Table 2). The biological and biochemical analysis of EL-43P showed indicative matching characteristics to *Rhodococcus* sp.. Therefore, EL-43P was tentatively designated as *Rhodococcus* sp. EL-43P.

Table 1. TCE degradation by *Rhodococcus* sp. EL-43P grown using different carbon sources.

Media	TCE degradation (%)
Pre-induction in phenol[a] minimal medium	79
Pre-induction in toluene[a] minimal medium	0
Pre-incubation in nutrient broth	0

[a]Each substrate concentration was 500 ppm, respectively.

Table 2. Taxonomical characteristics of the isolated strain EL-43P

Characteristics	Results
Morphological characteristics	
Cell Shape	Filamentous to coccobacilli
Gram stain	Positive
Spore	None
Motility	None
Cultural characteristics	
Colony Shape	Circular, entire, convex
Surface	Smooth
Color	Cream to pink
Opacity	Opaque
Biochemical characteristics	
Cytochrome oxidase	−
Catalase	+
Oxidation/Fermentation	Oxidation
β-Galactosidase	−
Arginine dehydrolase	−
Lysine decarboxylase	−
Ornithine decarboxylase	+
Citrate utilization	−
H_2S production	−
Urease	+
Tryptophan deaminase	−
Indole production	−
Voges-Proskauer	+
Gelatin liquefaction	−
Nitrate reduction	−
Whole-cell hydrolysate	*meso*-Diaminopimelic acid
Whole-cell sugar	Arabinose, galactose

Characterization of Phenol Degradation

In order to increase the degradation of phenol, growth conditions were investigated. The effect of temperature on phenol degradation by *Rhodococcus* sp. EL-43P is shown in Fig. 1. The growth was monitored at a range of temperature between 10 and 60°C. All measurements were carried out after 48 h of cultivation. Maximum growth and phenol degradation had occured at 40°C, which is 1.8 times as high as that at 20°C. The strain is also capable of degrading phenol at 50°C.

The effect of the initial pH of medium on the growth and phenol degradation by *Rhodococcus* sp. EL-43P was also studied (Fig. 2). The growth was monitored after 48 h of culture in the media containing phenol whose pH

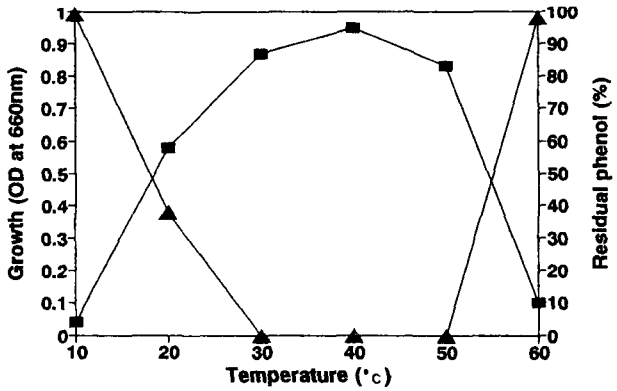

Fig. 1. The effect of temperature on cell growth and phenol degradation by *Rhodococcus* sp. EL-43P.
Cells were cultivated in mineral salts medium containing phenol 500 ppm for 2 days at pH 7.0. —■—, cell growth; —▲—, residual phenol.

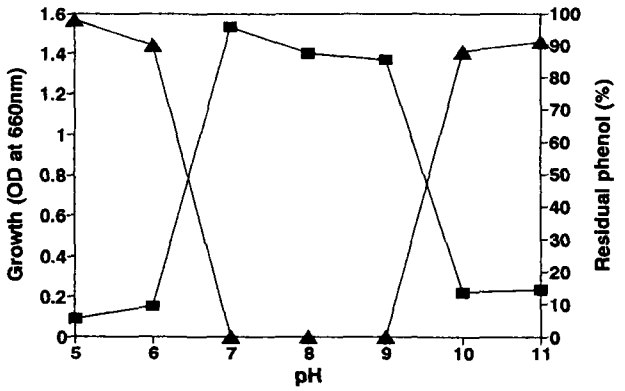

Fig. 2. The effect of pH on cell growth and phenol degradation by *Rhodococcus* sp. EL-43P.
Cells were cultivated in mineral salts medium containing phenol 500 ppm for 2 days at 40°C. —■—, cell growth; —▲—, residual phenol.

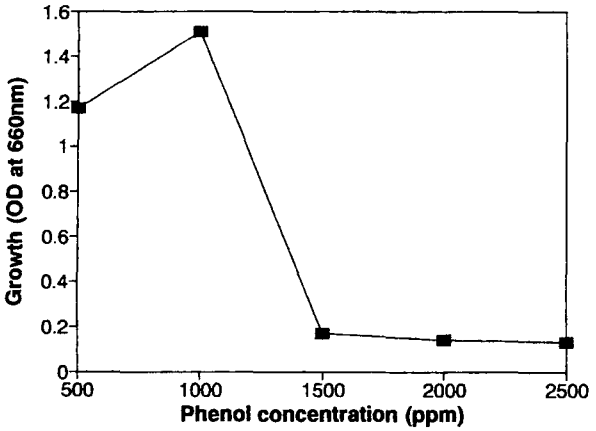

Fig. 3. The effect of phenol concentration on cell growth of *Rhodococcus* sp. EL-43P.
Cells were cultivated for 2 days at 40°C and pH 7.0.

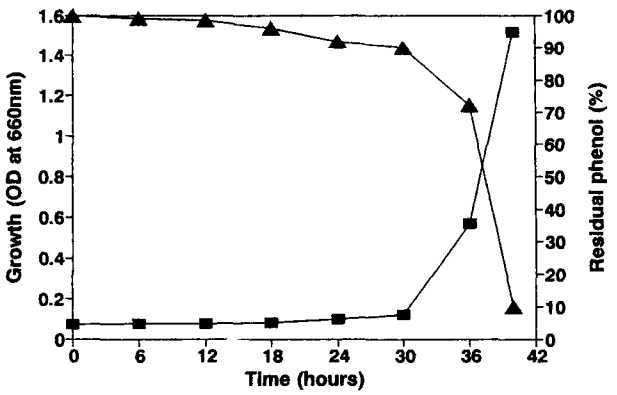

Fig. 4. Time course of cell growth and phenol degradation by *Rhodococcus* sp. EL-43P.
Cells were cultivated in mineral salts medium containing phenol 1,000 ppm at 40°C and pH 7.0. —■—, cell growth; —▲—, residual phenol.

Table 3. The effect of aeration on growth and phenol degradation of *Rhodococcus* sp. EL-43P.

Medium volume[a] (ml)	Growth (OD at 660 nm)	Degradation efficiency[b] (%)
25	0.813	92
50	1.492	100
75	1.484	100
100	0.897	98
150	0.402	55
200	0.214	57

[a]Obtained by varying the amount of medium in 250-ml Erlenmeyer flasks and the keeping the agitation constant, i.e. 150 rpm. [b]Cells were cultivated in mineral salts medium containing phenol 1,000 ppm for 2 days at 40°C.

was adjusted to a range between 5.0 and 11.0. The strain showed the maximum growth and phenol degradation at pH 7.0. But the degradation of phenol at pH 9.0 was also excellent. There was little degradation when the pH of the media was below 6.0. There are no researches published on the isolation and characterization of thermophilic and alkalophilic phenol-degrading bacterium. These results indicate that *Rhodococcus* sp. EL-43P is a novel phenol-degrading bacterium which can be applied to the treatment of alkaline and thermal wastewater.

To confirm the effect of phenol concentration on the growth, *Rhodococcus* sp. EL-43P was cultured in different media ranging from 500 to 2,500 ppm of phenol. We measured cell growth after 48 h of cultivation. As shown in Fig. 3, the maximum growth was obtained in a culture containing 1,000 ppm of phenol. At higher phenol concentrations than 1,000 ppm, the growth was greatly inhibited.

Different levels of aeration were obtained by varying the amount of medium in 250-ml Erlenmeyer flasks and keeping the agitation constant, i.e. 150 rpm. The effect of aeration on the growth and phenol degradation of *Rhodococcus* sp. EL-43P is presented in Table 3. Maximum growth and phenol degradation were obtained with medium volume of 50 ml in 250-ml Erlenmeyer flask. Increasing the medium volume up to 150 ml did reduce the growth and phenol degradation. It suggests that critical concentration of dissolved oxygen determining the growth and phenol degradation of *Rhodococcus* sp. EL-43P exists.

Figure 4 shows the relationship between cell growth and the degradation of phenol during culture of *Rhodococcus* sp. EL-43P in the medium containing 1,000 ppm phenol. After 40 h, this strain was able to degrade 90% of initial phenol and possessed a shorter lag period (30 h) than the isolation medium (36 h). Phenol is a wide-spread environmental pollutant found in the effluent of refinery plants, petro-chemical plants and phenolic resin plants. Wastewaters from phenolic resin plants and coking plants often contain very high concentrated phenol, which cannot be treated by normal activated sludge system [1]. If the detailed degradation conditions and the development of material capable of immobilizing *Rhodococcus* sp. EL-43P are established, the treatment of thermal and alkaline phenol wastewater using *Rhodococcus* sp. EL-43P would be possible.

TCE Cometabolism

To determine the effect of TCE concentrations on degradation activity, the rates of TCE disappearance by phenol-induced cells were determined for concentrations of 5, 10, 20, 50, and 100 μM. As shown in Fig. 5, the time course of TCE degradation illustrates that at all concentraions, an apparent lag period was not observed, and thus the initial degradation rate rapidly increased. Uninoculated controls demonstrated no significant loss of TCE from system. In cases of TCE 5~20 μM, *Rhodococcus* sp. EL-43P degraded 78~95% of the initial

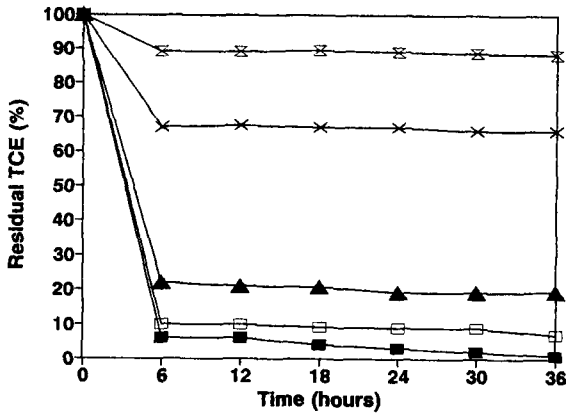

Fig. 5. Time course of TCE degradation by *Rhodococcus* sp. EL-43P.

Cells were preincubated with 1,000 ppm phenol aerobically for 2 days at 40°C. The residue of TCE was determined by comparing differences in the TCE concentrations in the cell suspensions with those of an uninoculated control. —■—, 5 μM; —□—, 10 μM; —▲—, 20 μM; —×—, 50 μM; —⧖—, 100 μM.

TCE after 6 h. When cell suspensions of *Rhodococcus* sp. EL-43P were incubated with increasing concentrations of TCE (50 and 100 μM), cells degraded 32 and 11% of initial TCE, respectively. This occurred probably due to the TCE toxicity. TCE is hydrophobic and can potentially express toxic or inhibitory effects on cells by partitioning into cellular components, such as lipids and polysaccharides [3]. The initial rate of degradation of TCE by *Rhodococcus* sp. EL-43P rapidly reflects the maximum biodegradative potential of this organism under the conditions used.

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