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Plasmid- and Chromosome-Mediated Assimilation of Phenol and Cyanide in *Pseudomonas* sp. Strain PhCN

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Summary. *Pseudomonas* sp. PhCN strain, which has the potential to utilize phenol and cyanide as a sole carbon and nitrogen source, was isolated. A comparison of the effect of cyanide on phenol degradation and *vice versa* by strain PhCN showed that the degradation time was significantly delayed by an increase in either phenol or cyanide concentration, and the greatest activities were obtained in basal medium containing a low concentration of cyanide and phenol. This strain contained two plasmids of approximately 120 kb (pPhCN-1) and 110 kb (pPhCN-2). Plasmid curing experiments produced a plasmid-free strain as well as strains containing either the 120- or the 110 kb plasmid. The strains were tested for their ability to utilize phenol and KCN. The results demonstrated that the ability to utilize phenol was encoded by the 120 kb plasmid, whereas the ability to utilize cyanide appeared to be encoded by the chromosome.

Key words: Biodegradation, cyanide, phenol, plasmid, *Pseudomonas*

Phenolic wastes are major environmental pollutants from industrial processes, such as oil refineries, cooking plants, industrial resin manufacturing and petroleum-based processing plants, pharmaceuticals, and plastic industries, etc. [38]. Biodegradation of phenolic wastes by bacteria has been studied extensively, and a large number of phenol-degrading bacteria have been isolated and characterized at the physiological and genetic levels [18, 20, 27, 36, 37, 41]. Cyanide compounds are also toxic and carcinogenic pollutants [8, 44] that have been widely used in industries involving metal-plating, pharmaceuticals, synthetic fibers, coal gasification, coal coking, ore leaching, gold mining, and electroplating [28, 43, 44]. It has been shown that KCN is rarely a direct carbon and energy source for sustaining microbial growth [40], whereas a variety of microorganisms are able to degrade cyanide and use it as a sole nitrogen source [12, 17, 21, 25, 32].

Although individual biodegradation of phenol and cyanide is well proven, biological removal of individual compounds can be affected by the presence and concentration of other pollutants, when wastewater contains combinations of these compounds [1, 3, 11]. Therefore, the strains used for decontamination should be not only highly active on one of the contaminants, but also resistant to the remainder of the pollutants or possess different biodegradation abilities.

The purpose of the present study was to investigate the substrate removal pattern of a phenol-degrading *Pseudomonas* in the presence of a secondary toxic compound and the role of plasmid in degradation of these compounds.

Materials and Methods

The strains and plasmids used in this study are described in Table 1.

Chemicals and Culture Media

All chemicals, including phenols and potassium cyanide, were of the highest purity and were purchased from Sigma or Aldrich. Analytical reagent grade materials were used for the preparation of all other assay solutions and culture media. The mineral salt medium (MSM) for enrichment and degradation tests contained per liter: H_2KPO_4, 1 g; KH_2PO_4, 1 g; $(NH_4)_2SO_4$, 0.6 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $FeSO_4$, 0.1 g; and 1% ml trace elements solution. The pH after sterilization was 7.0. $MgSO_4$ and $FeSO_4$ were autoclaved separately and added to the medium. $(NH_4)_2SO_4$ was omitted from the MSM if cyanide was used as the sole source of nitrogen. Concentrated stock solutions of phenol, cyanide, and glucose were used after sterilization with 0.2-μm pore size filters. Cultures in liquid MSM were grown aerobically at 28°C in Erlenmeyer conical flasks

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Table 1. Bacterial strains and plasmids used in this study.

Strains	Relevant characteristics	Phenotype	Source
Pseudomonas sp. strain PhCN	Wild-type phenol degrader, pPhCN-1, pPhCN-2	$Ph^+CN^+Rf^s$	This study
Pseudomonas sp. strain PhCN1	Cured derivatives of PhCN, phenol degrader, pPhCN-1	$Ph^+CN^+Rf^s$	This study
Pseudomonas sp. strain PhCN2	Cured derivatives of PhCN pPhCN-2	$Ph^-CN^+Rf^s$	This study
Pseudomonas sp. strain PhCNM	Cured derivatives of PhCN (free plasmid)	$Ph^-CN^+Rf^r$	This study
Pseudomonas sp. strain PhCNT	Transconjugants between PhCN or PhCN1 and PhCNM (pPhCN-1)	$Ph^+CN^+Rf^r$	This study

(250 ml). LB medium contained 13 g of nutrient broth (Difco) per liter. For solid medium, 15 g/l of agar was added.

Enrichment and Isolation of Phenol-Degrading Bacteria

Samples of soil were obtained from agriculture soil that had been exposed to different pesticides for several years but had not been exposed to phenol directly. Five g of soil samples was inoculated in a 250-ml Erlenmeyer flask containing 100 ml of mineral salt medium with 5 mM phenol and 4 mM potassium cyanide as a sole source of carbon and nitrogen. The enrichment of phenol- and cyanide-degrading microorganisms was conducted by subculturing every 5 days for one month with a 5% (v/v) inoculum in a rotary shaker (150 rpm) at 28°C. The subculture was diluted and plated on agar MSM containing phenol and cyanide as a sole source of carbon and nitrogen. After about 3 days of incubation, three morphologically distinctive colonies were obtained, and they were separately maintained in the liquid and agar media containing phenol and KCN. All the colonies were tested for their ability to grow on phenol and cyanide as sole carbon and nitrogen sources. Among the three colonies, a single strain was selected on the basis of the greatest efficiency of phenol and cyanide degradation (better growth), and it was designated as strain PhCN.

The identification of the isolated bacteria was performed according to standard procedures in *Bergey's Manual* [33]. After the color and shape of the colonies were determined, Gram staining was performed on the isolates. Based on the Gram stain and shape, several physiological and biochemical tests were performed for the isolate. The strain was further identified with API 20 NE strips (bioMerieux, Marcy l'Etoile, France).

Optimal Growth and Conditions to Degrade Phenol

To study the effect of phenol and cyanide as the sole carbon and nitrogen sources, the initial concentration of these compounds in growth medium were varied. Then, the strain PhCN was precultured in nutrient broth for 24 h and harvested by centrifugation (15,000 ×*g* for 10 min at 4°C). The cells were washed twice in a sterile sodium phosphate buffer (50 mM, pH 6.8) and resuspended in a fresh phosphate buffer (A_{600} of 1.0) for use as an inoculum in subsequent experiments. One ml of bacterial suspension was inoculated into 100 ml of MSM in separate 250-ml Erlenmeyer flasks containing ammonium sulfate or cyanide (3 mM) and 0, 1, 2.5, 5, 7.5, and 10 mM phenol. The same suspension was transferred into separate flasks containing 5 mM phenol and 0, 1, 2, 3, 4, and 5 mM KCN. The cultures in triplicate for each test were incubated with shaking (150 rpm) at 28°C. Optical density of the samples was determined spectrophotometrically at 600 nm and was used as a growth parameter. At different intervals of incubation, samples were transferred and analyzed.

Analytical Method

The phenol degradation was determinated by monitoring the change in phenol concentration [14]. At different intervals of incubation, 1-ml samples were taken and transferred to an Eppendorf tube containing 25 µl of 2% 4-aminoantipyrene and 50 µl of 2 M ammonia. After mixing, 25 µl of 8% potassium hexacyanoferrate (III) was added. The suspension was centrifuged at 5,000 ×*g* for 2 min, and absorbance at 500 nm was measured and compared with phenol standards. Alternatively, the concentration of phenol was measured spectrophotometrically at its corresponding absorbance peak, 269 nm, and compared with a standard curve (phenol, A_{269}=1 correspond to 71 mg^{-1}).

Cyanide was assayed by a modified picric acid method [13, 32]. A linear calibration curve was obtained with the standard cyanide solution as follows: 0.05 ml aliquots of cyanide-containing solution (after centrifugation at 15,000 ×*g* for 10 min at 4°C) was added to 0.1-ml aliquots of solution containing 0.5% (w/v) picric acid and 0.25 M Na_2CO_3. The resulting solution was placed in a boiling water bath for 5 min, diluted to 1 ml with 0.85 ml distilled water, and cooled in tap water for 30 min. Absorbance at 520 nm was read against a blank of distilled water and picric acid reagent.

Preparation and Crude Enzyme Extract

Pseudomonas strain PhCN was grown overnight in MSM containing 0.5 mM phenol. Then, the culture was diluted in fresh LB medium, LB medium plus 0.5 mM phenol, or MSM containing either ammonium or KCN as a sole nitrogen source and 0.5 mM phenol. Cells were harvested by centrifugation at 10,000 ×*g* for 10 min at 4°C. The pellets were washed twice in 50 mM phosphate buffer and were disrupted sonically at 4°C. Cellular debris was removed by centrifugation (12,000 ×*g* for 20 min at 4°C)

and the clear supernatant was used immediately for enzyme activity.

Enzyme Activity

Catechol 2,3-dioxygenase activity was measured by incubating the crude enzyme extract with 100 μM catechol or phenol in 50 mM phosphate buffer (pH 7.5 at 25°C) [10]. Catechol 1,2-dioxygenase activity was measured by incubating the crude enzyme extract with 100 μM catechol or phenol in 33 mM Tris-HCl (pH 8.0), containing 1.3 mM EDTA and 3.3 mM 2-mercaptoethanol, at 25°C [2].

Isolation of Plasmid

The following isolation methods of plasmids and their modification were used: alkaline lysis [2], Kado and Liu [22], Brenner *et al.* [5], and Hansen and Olsen [16].

Spontaneous Loss and Plasmid Curing

To monitor spontaneous plasmid loss, cells were grown in nutrient broth for approximately 50 cell divisions with frequent fresh media replacement. Cultures were then diluted and spread on nutrient agar plates, and 600 individual colonies were picked up and checked for growth on phenol and KCN as sole carbon and nitrogen sources.

Plasmid curing experiments were performed according to a procedure essentially described by Lin and Kado [29]. Thus, 10^3 to 10^4 cells from an overnight culture were inoculated in several tubes containing 2 ml of nutrient broth and ethidium bromide at concentrations ranging from 5 to 25 μg/ml. Cultures were shaken at 28°C until visible growth, as evidenced by turbidity. An aliquot from the highest concentration of ethidium bromide that still permitted bacterial growth was diluted and spread on nutrient agar plates. Individual colonies were tested on solid minimal salt media for growth on phenol and KCN as a sole carbon and nitrogen source. Stability of the cured strains was monitored by periodic cycling of cells under nonselective condition and retesting for the ability to grow on phenol. One of these cured strains (free plasmids) was designated as PhCNM.

Isolation of Nalidixic Acid-Resistant Mutant

The nalidixic acid-resistant mutant (Nal^r) of PhCNM was isolated by treatment of the PhCNM strain with *N*-methyl-*N*-nitrosoguanidine [7], and Nal^r mutants were selected on LB medium containing nalidixic acid (100 μg/ml). A single mutant colony was restreaked onto the same medium for final purification. The mutant was then used as the primary recipient in the mating assay.

Plasmid Transfer

Both donor and recipient strains were grown to exponential or late exponential phase in nutrient broth, and then 10 μl of donor cells was mixed with 10 μl of recipient cells on a 0.2-μm membrane filter placed on the surface of a nutrient agar plate, and they were incubated overnight. Cells were scraped from the filter, suspended in saline water, and several dilutions were plated on minimal salt medium supplemented with phenol as a sole carbon source and rifampin. Conjugation frequencies were expressed as number of transconjugants per one donor cell.

Screening for Additional Plasmid-Encoded Properties

To identify additional plasmid-encoded properties, plasmid-bearing strains were tested for their ability to utilize a variety of compounds as a sole source of carbon and nitrogen and for resistance to antibiotic and heavy-metal ions.

RESULTS AND DISCUSSION

Characterization and Identification of Isolate

Three bacterial strains were isolated from soil using the enrichment technique described in Materials and Methods. They were all able to utilize phenol as a sole carbon source, but were different in their ability to use cyanide as a nitrogen source (data not shown). Consequently, the strain PhCN, which exhibited the highest phenol and cyanide degrading activity and also harbored the plasmid, was selected for further studies.

The bacterial cells were rod-shaped, Gram-negative, and motile. Spores were not observed. Colonies were fluorescent. Oxidase, catalase, and arginine dihydrolase reactions were positive. Growth on sodium benzoate, McConkey, and glucose plates was observed, but not on xylose and maltose. The isolate failed to hydrolyze gelatine. On the basis of these characteristics and other biochemical properties determined by API 20NE, the organism was identified as *Pseudomonas* sp., and designated as strain PhCN. The isolate was found to grow well between pH.5.5 and 8.0, and maximum yields were obtained at pH 7.0. No growth occurred below pH 5.0 or above pH 8.6. Attempts have been made to isolate bacteria that can simultaneously degrade cyanide and phenol; however, none has been found [20]. On the other hand, there is at least one paper that describes the isolation of such strains [4].

Phenol Biodegradation

The growth and phenol degradation by the strain PhCN under different conditions were expressed by optical density (OD_{600}) and phenol concentration. Growth kinetics of the strain PhCN in minimal medium containing ammonium salt as a sole nitrogen source and increasing concentration of phenol are given in Fig. 1. The growth of the organism increased with increase of phenol concentration. However, the lag period was extended at a higher concentration of phenol. Shake flask experiments were also conducted to

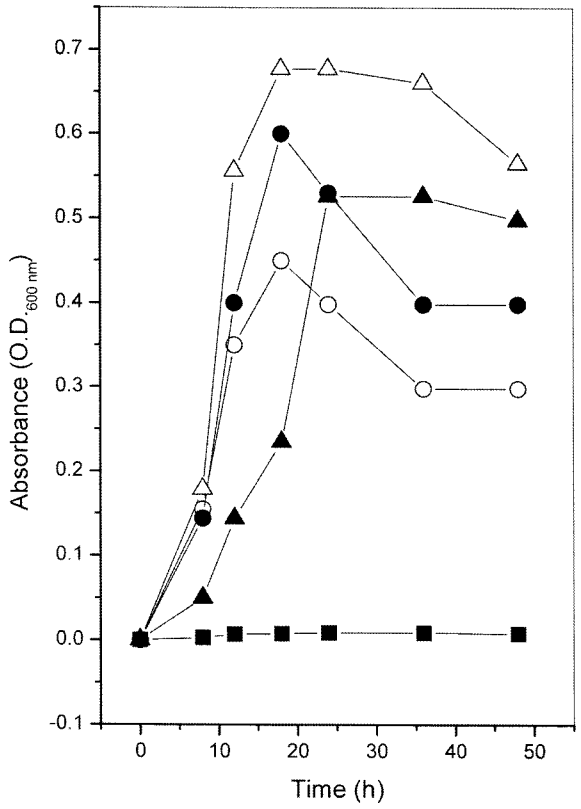

Fig. 1. Growth of *Pseudomonas* sp. strain PhCN with $(NH_4)_2SO_4$ as a sole nitrogen source and various concentrations of phenol.
Concentrations of phenol were (■) no phenol, (○) 1 mM, (●) 2.5 mM, (△) 5 mM, (▲) 7.5 mM.

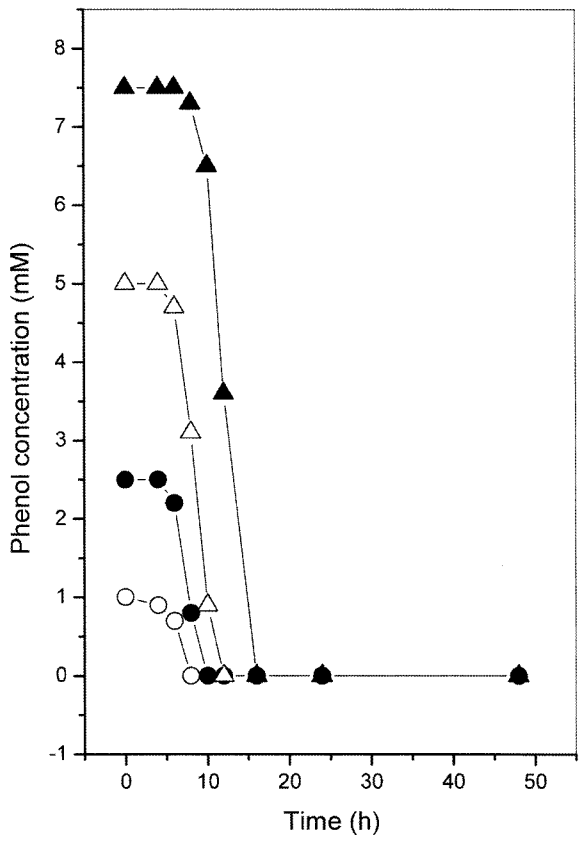

Fig. 2. Utilization of phenol by growing cells of *Pseudomonas* sp. strain PhCN in minimal medium amended with NH_4SO_4 as a sole nitrogen source.
The initial concentration of phenol is indicated by the ordinate values. (○) 1 mM; (●) 2.5 mM; (△) 5 mM; (▲) 7.5 mM.

examine the effect of various initial concentrations of phenol on the degradation behavior of the strain PhCN. Results of these studies show that the higher the initial concentration of phenol, the more time it took to be degraded completely. Figure 2 shows that complete degradation of phenol at low concentration (1 mM and 2.5 mM) was achieved in 8 and 10 h, respectively, whereas complete degradation of 5 and 7.5 mM phenol occurred at 12 and 16 h, respectively. This is a characteristic behavior of the metabolism of toxic substances [19].

The phenol-degrading capacity of the strain PhCN was also tested in the presence of cyanide, since this toxicant is present along with phenol in most industrial wastewaters [3].The degradation of phenol in cultures amended with initial concentration of cyanide (3 mM) as a nitrogen source and various concentrations of phenol was also studied. Figure 3 shows that complete phenol degradation at low concentrations of phenol (1 mM and 2.5 mM) was achieved at 12 and 36 h, respectively, whereas complete degradation of 5 and 7.5 mM phenol occurred at 60 and 108 h, respectively. Comparison of the data in Figs. 2 and 3 indicates that cyanide has a negative influence on the removal of phenol by the strain PhCN. However, the time required for complete removal of phenol at a low concentration (1 mM phenol) in the cyanide culture was only 4 h longer than that required by the culture that received ammonium salt. Similar findings were reported by Arutchelvan *et al.* [3] for *Pseudomonas* sp. grown on phenol and cyanide. They observed that the presence of cyanide reduced the rate of phenol removal.

Biodegradation of phenol by the *Pseudomonas* sp. strain PhCN was also examined in the presence of 1, 2, 3, 4, and 5 mM cyanide together with initial concentration of 5 mM phenol. Figure 4 shows complete phenol degradation in the presence of 1 mM KCN within 24 h, whereas complete phenol degradation in the presence of 2 mM and 3 mM KCN occurred at 48 and 60 h, respectively. In cultures containing 4 and 5 mM KCN, complete phenol degradation was observed after 84 and 108 h, respectively. Arutchelvan *et al.* [3] reported similar observations for the degradation of phenol in the presence of various concentrations of cyanide: They observed that phenol removal was more effective at a low concentration of cyanide, but decreased with increase of cyanide concentration. Many reasons can be attributed to the above phenomena. One of the reasons

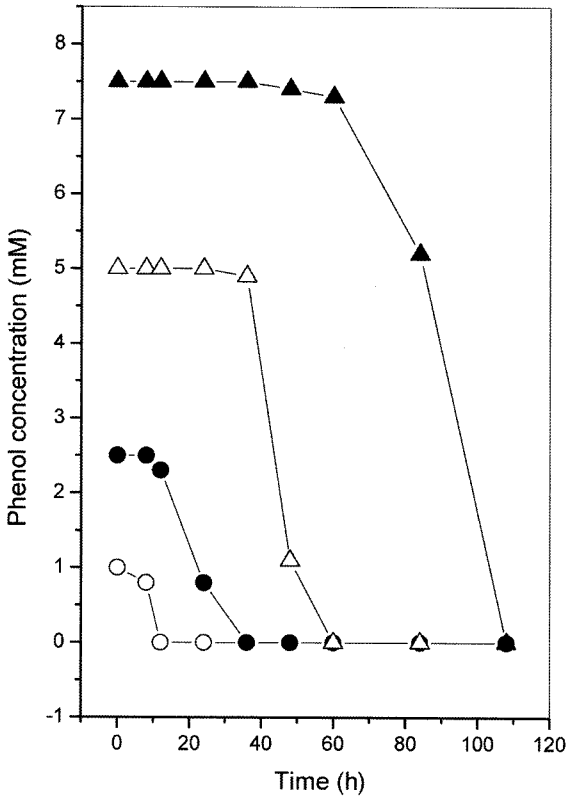

Fig. 3. Utilization of phenol by growing cells of *Pseudomonas* sp. strain PhCN in minimal medium amended with KCN as a sole nitrogen source.
The initial concentration of phenol is indicated by the ordinate values. (○) 1 mM; (●) 2.5 mM; (△) 5 mM; (▲) 7.5 mM.

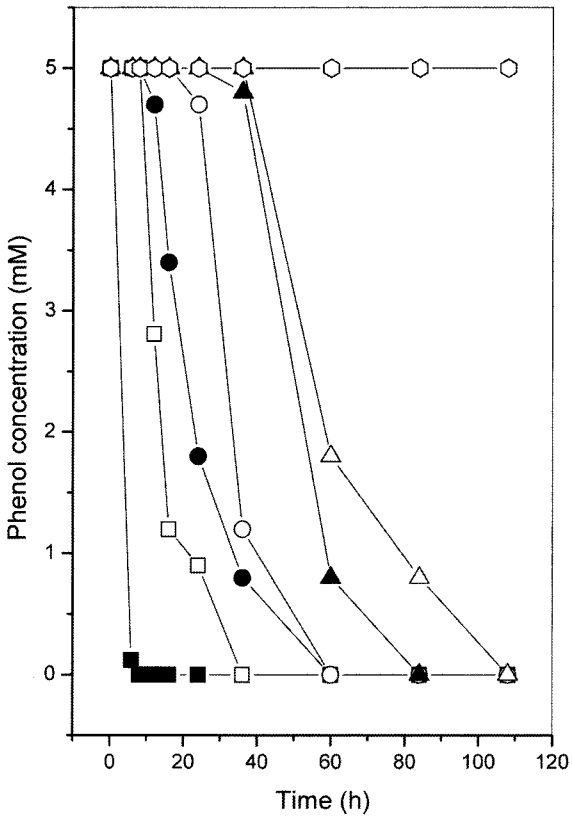

Fig. 4. Effect of cyanide concentration on the biodegradation of phenol by *Pseudomonas* sp. strain PhCN.
Symbols: (□) 1 mM, (●) 2 mM, (○) 3 mM, (▲) 4 mM;,(△) 5 mM, (◇) negative control (no nitrogen source), and (■) positive control $(NH_4)_2SO_4$).

can be that cyanide at a high concentration inhibits one of the enzymes that participate in phenol degradation metabolism. Thus, the enzyme becomes rate limiting, in turn reducing phenol degradation. The second reason may be that cyanide inhibits phenol transportation into the cells, thus affecting the phenol degradation.

Degradation of Cyanide

A number of toxic compounds are formed during industrial processes, giving the multicomponents composition of wastewater. Therefore, the strains used for decontamination should not only be highly active to one of the contaminants, but also resistant to the remainder of the pollutants or possess different biodegradation abilities. In the present study, the abilities of the PhCN strain to assimilate cyanide as a sole nitrogen source was tested. Growth profiles of the strain PhCN in separate cultures supplemented with initial concentration of phenol (5 mM) and various concentrations of cyanide were investigated. The results in Fig. 5 reveal that optimum growth occurred at 1 mM cyanide with a lag period of 8 h. As the concentration of cyanide increased, a longer lag period (accompanied by a slower growth rate) was observed, indicating that cyanide could be used as an alternative nitrogen source for the growth of PhCN strain in a phenol-MSM. Moreover, PhCN strain cannot grow in cyanide-containing culture medium without the addition of other carbon substances (glucose or phenol). There are a number of reports on growth of microorganisms on cyanide as the sole source of nitrogen [30, 42]. However, there are relatively very few studies on growth of microorganisms on cyanide as a sole source of carbon and nitrogen [28, 40].

The cyanide degradation capacity was tested in the presence of various concentrations of phenol. Different effects were observed, depending on the phenol concentration. The highest utilization of 3 mM cyanide occurred within 12 h, in the culture containing 1 mM phenol. Thereafter, the cyanide degradation decreased progressively as the concentration of phenol was raised (Fig. 6). These results are in contrast with that of Shivaraman and Parhad [39], who found that *Pseudomonas* strain could not utilize cyanide in the presence of phenol. However, Kang and Kim [23] reported that phenol had no effect on cyanide degradation by mixed culture.

In conclusion, a comparison of the effect of cyanide on phenol degradation and *vice versa* by the strain PhCN

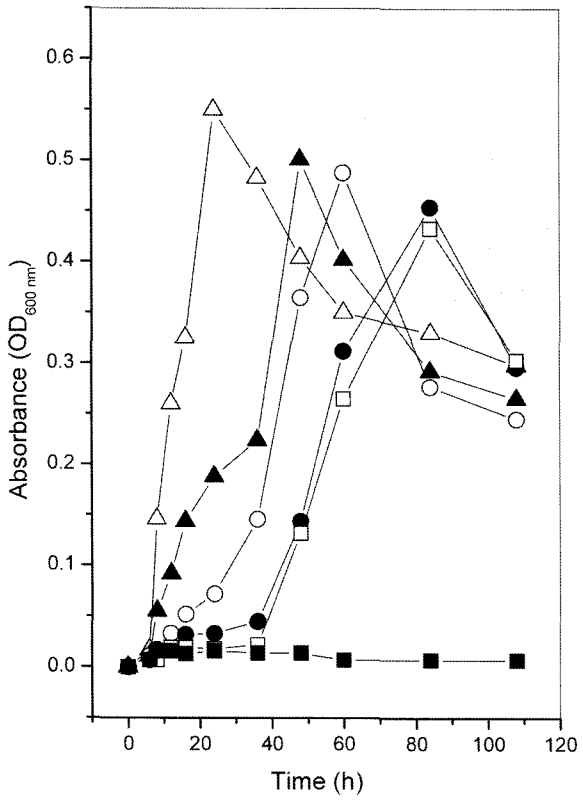

Fig. 5. Growth of *Pseudomonas* sp. strain PhCN in phenol minimal medium (5 mM) supplied with various concentrations of KCN.
Concentrations of KCN are (■) control (no nitrogen source), (△) 1 mM, (▲) 2 mM, (○) 3 mM, (●) 4 mM, and (□) 5 mM.

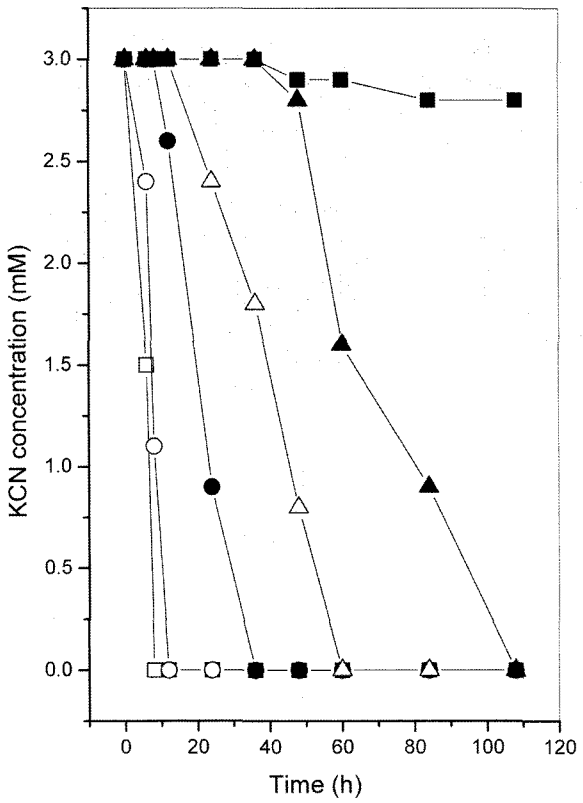

Fig. 6. Effect of phenol concentration on the utilization of cyanide by *Pseudomonas* sp. strain PhCN.
Symbols: (○) 1 mM, (●) 2.5 mM, (△) 5 mM, (▲) 7.5 mM, (■) negative control (no carbon source), and (□) positive control (glucose, 1%, w/v).

showed that degradation time was significantly delayed by either increase of phenol or cyanide concentration, and that the greatest activities were obtained in basal medium containing low concentrations of cyanide and phenol. In the actual wastewater treatment process, the concentration of xenobiotic compounds contained in the influents is relatively low and fluctuates considerably. Therefore, the most desirable characteristics of degrading microorganisms for wastewater treatment are high degradation activity at low substrate concentration and rapid induction of degradative enzymes [15].

Enzyme Activity

Phenol is usually degraded via the catechol degradation pathway. There are two pathways for catechol ring cleavage, the *meta* and *ortho* pathways [36]. The majority of bacteria use the *meta* pathway of catechol degradation, especially when the phenol degradation gene is located on plasmids, like *Pseudomonas* sp. strain CF600 [36] and *Pseudomonas* sp. strain EST1001 [27]. Both *meta* and *ortho* pathways are distinguishable by measuring characteristic enzymes, such as C23O for the *meta* pathway and C12O for the *ortho* pathway. In the present study, the activities of both enzymes were measured in PhCN cells grown in phenol minimal media. Activity of C23O, but no evidence of C12O, could be detected in a crude extract of PhCN cells (Table 2). This conclusion indicates that *Pseudomonas* sp. strain PhCN uses the C23O pathway of catechol degradation during phenol metabolism. Moreover, in order to study whether phenol degradation by the strain PhCN was affected by different nitrogen sources, the strain was grown in LB medium and minimal media amended with different nitrogen sources in the presence of phenol as a sole carbon source. When the activity of C23O enzyme was measured (Table 2), no C23O activity could be detected in LB medium in the absence of phenol. In the presence of phenol, the C23O activity was negligible. The C23O activity was high, when the cells were grown in the presence of phenol and ammonium salt as a sole carbon and nitrogen source. In the presence of KCN, the overall enzyme activity was less than that observed when the bacteria grew in the presence of ammonium as a nitrogen source. This difference in the enzyme activity could explain the higher values of phenol

Table 2. Specific activities of *meta*-cleaving enzyme C23O dioxygenase in a crude extract of strain PhCN and its derivatives on different media. Enzyme was assayed as described in Materials and Methods.

Growth medium	*Pseudomonas* sp. strain PhCN	*Pseudomonas* sp. strain PhCN1	*Pseudomonas* sp. strain PhCN2	*Pseudomonas* sp. strain PhCNM	*Pseudomonas* sp. strain PhCNT
L-Broth	ND	ND	ND	ND	ND
L-Broth+phenol	<0.001	<0.001	<0.001	<0.001	<0.001
Phenol+ammonium	22.1	21.7	1.0	<1.0	21.9
Phenol+KCN	16.9	16.9	<1.0	<1.0	16

Enzyme activity is given in U/mg of protein.
ND, not detected.

degradation and bacterial growth by the strain PhCN, when grown in the presence of ammonium as a nitrogen source.

Plasmid Profiles

Many of the derivative functions in *Pseudomonas* species have been attributed to plasmid-associated genes, including those that degrade phenol [27, 36] and other aromatic compounds [7, 31]. Various isolation procedures of plasmid have been used to demonstrate the presence of plasmids in the strain PhCN. These include the alkaline lysis method [2] as well as methods that have previously been used for isolating large, low-copy biodegradative plasmids [5, 16, 22, 35]. Kado and Liu [22] provided the best results for plasmid visualization (Fig. 7). The molecular size of plasmids was determined by analysis of their digestion patterns after treatment with restriction enzymes, and the total size of plasmids was estimated on the basis of electrophoretic mobility of fragments compared with those of lambda DNA digestion patterns of known sizes. The strain PhCN was found to contain 120- and 110-kb plasmids, designated as pPhCN-1 and pPhCN-2, respectively (Fig. 7).

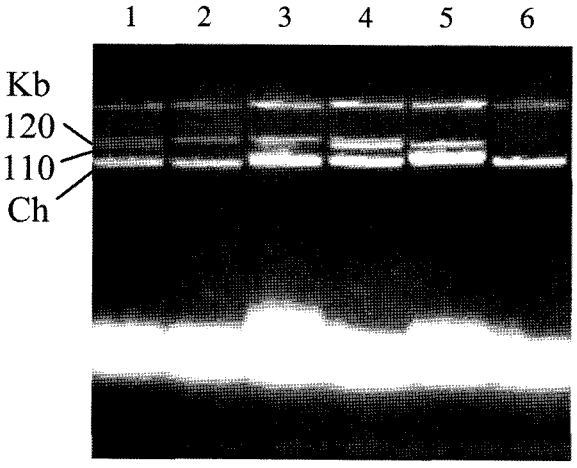

Fig. 7. Agarose gel electrophoresis of plasmids from strain PhCN and its derivatives.
Lanes: 1, 4, PhCN (pPhCN-1, pPhCN-2); 2, PhCN1 (pPhCN-1); 3 PhCNT (pPhCN-1, transconjugant); 5, PhCN2 (pPhCN-2); 6, PhCNM (free plasmid).

Plasmid Loss

The potential spontaneous loss of plasmids was tested by screening individual colonies from cultures grown for approximately 50 generations in nutrient media with no selection. Screening was for the loss of ability to grow on phenol and cyanide as sole carbon and nitrogen sources. No colony was found that had spontaneously lost one of these degradative abilities. The induced curing of plasmid DNA was attempted using ethidium bromide treatment of cultures. Thus, the PhCN strain grew in different concentrations of ethidium bromide, and 15 μg/ml ethidium bromide allowed only weak growth that could be observed after 48 hours of incubation in nutrient broth. Individual colonies were again screened for the ability to grow on phenol and cyanide. All colonies tested were able to grow on cyanide as a nitrogen source. However, four colonies out of 500 lost the ability to grow on phenol as a sole carbon source. In screening single colonies of cured experiments, those that maintained the ability to degrade phenol either had both plasmids or the larger of the two plasmids, pPhCN-1 (Fig. 7). Isolates that could no longer degrade phenol either had no detectable plasmid, designated as PhCNM, or the smaller of the two plasmids, pPhCN-2 (Fig. 7). These results strongly indicated that the larger plasmid endowed the wild-type strain with the ability to degrade phenol.

Plasmid Transfer

In order to test the transfer ability of apparent phenol biodegradative plasmid, we first isolated several rifampin-resistant mutants of the plasmids-free strain described above. The strains PhCN (pPhCN-1, pPhCN-2) and PhCN1 (pPhCN-1) were used in subsequent experiments as the donor strains, whereas strain PhCNM (plasmid-free), which is rifampin resistant, was used as a recipient. Four exconjugants from this mating were analyzed for the presence of plasmid DNA along with donor strain PhCN. Plasmid DNA from all strains exhibited identical mobility on agarose gels to that from the plasmid in PhCN1 (pPhCN-1) or harbored large plasmid (Fig. 7). None of exconjugants exhibited

both plasmids or small plasmid. Strain PhCN1 (pPhCN-1) was also tested as donors of the plasmids in a second round of conjugation. Again, a phenol-positive transconjugant was obtained and designated as PhCNT (Fig 7). Transconjugants were obtained at an average frequency of 1.6×10^{-6} per donor colony formed. The location of the degradative genes on the plasmid can help or promote transfer to other strains, leading to increase of metabolic diversity of soil and sludge microbial populations. New genes combination could also allow the degradation of related compounds, degradation via different pathways, or recombination between related genes, thus generating greater metabolic diversity [26].

Comparison of PhCN and its Cured Derivatives

When sufficient evidence for the presence of the degradative plasmid in *Pseudomonas* PhCN was gathered and strains cured of plasmids were obtained, we searched for differences in the properties of the original strain and cured derivatives, represented by strains PhCN1, PhCN2, and PhCNM. Growth on a range of substrates in liquid media were tested. Table 3 summarizes the difference in the growth abilities on different carbon and nitrogen sources. In addition to phenol, PhCN (pPhCN-1, pPhCN-2), PhCN1 (pPhCN-1), and transconjugant PhCNT (pPhCN-1) were able to grow in liquid media containing *o*-, *m*-, and *p*-cresol and KCN as sole carbon and nitrogen sources. This property indicates that the strains possess the effective *meta*-cleavage pathway genes [31]. Strains PhCN2 (pPhCN-2) and plasmid-free strain PhCNM were not able to grow in liquid media containing phenol, cresols, and KCN as a sole carbon and nitrogen source. However, these strains were able to grow in liquid media containing glucose and KCN as sole carbon and nitrogen sources, respectively (Table 3). These results demonstrated that the ability to utilize phenol was encoded by the 120-kb plasmid, whereas the ability to utilize cyanide appeared to be encoded by the chromosome. Previous study by Watanabe *et al.* [42] demonstrated that KCN dissimilation was encoded by chromosomal genes.

We attempted to find out if there were any heavy-metal resistance genes present on these plasmids. For this, we tested wild-type strain PhCN and its cured derivatives, including PhCN1, PhCN2, and PhCNM, for growth in the presence of different heavy metals. All strains exhibited an identical pattern, in that they were resistant to Pb^{+2} (400 ppm), Zn^{+2} (100 ppm), Cd^{+2} (40 ppm), Cu^{+2} (30 ppm), and Ni^{+2} (100 ppm).

This study demonstrates that *Pseudomonas* sp. strain PhCN has all of the properties of an efficient phenol-degrading microorganism. In contrast, similar to most phenol degrader *Pseudomonads*, the strain PhCN efficiently used two xenobiotics compounds (phenol and cyanide) as sole carbon and nitrogen sources, which is very advantageous for its biotechnological application in bioremediation. Strain PhCN contained two plasmids of approximately 120 kb; (pPhCN-1) and 110 kb (pPhCN-2). The results demonstrated that the ability to utilize phenol was encoded by the 120 kb plasmid, whereas the ability to utilize cyanide appeared to be encoded by the chromosome. This property makes the strain advantageous for biotechnological application with regard to aerobic removal of xenobiotic compounds in the presence of a secondary toxicant (cyanide). It should be mentioned that many industries are hesitant to use anaerobic treatment methods, because of the sensitivity of anaerobic bacteria, especially methanogens, to toxicity [34].

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Table 3. Growth of *Pseudomonas* sp. strain PhCN and its derivatives on different phenolic compounds and cyanide as sole sources of carbon and nitrogen, respectively.

Substrate carbon and nitrogen	*Pseudomonas* sp. strain PhCN (pPhCN-1, pPhCN-2)	*Pseudomonas* sp. strain PhCN1 (pPhCN-1)	*Pseudomonas* sp. strain PhCN2 (pPhCN-2)	*Pseudomonas* sp. strain PhCNM	*Pseudomonas* sp. strain PhCNT (pPhCN-1)
Phenol+ammonium salt	++	++	–	–	++
Phenol+1 mM KCN	++	++	–	–	++
Phenol+1 mM Acetonitrile	–	–	–	–	–
p-Cresol+ammonium	++	++	–	–	++
p-Cresol+1 mM KCN	++	++	–	–	++
m-Cresol+Ammonium	++	++	–	–	++
m-Cresol+1 mM KCN	++	++	–	–	++
Glucose+1 mM KCN	++	++	++	++	++
Glucose+1 mM Acetonitrile	+	+	+	+	+

high strength phenol degrading bacterial strains from phenol-formaldehyde resin manufacturing industrial wastewater. *J. Hazard. Mat.* **B27:** 238–243.
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