Natural combination of genetic systems for degradation of phenol and resistance to heavy metals in phenol and cyanide assimilating bacteria

El-Deeb, B.

Biological Science Department, Taif University, Taif, Saudia Arabia.
E-mail: bahig56@yahoo.com

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ABSTRACT

Combination of genetic systems of degradation of xenobiotic compound and heavy metal resistance is one of the approaches to the creation of polyfunctional strains for bioremediation of soil after co-contamination with organic pollutants and heavy metals. A bacterial strain *Pseudomonas putida* PhCN (pPhCN1, pPhCN2) has been obtained. This bacterium contains two plasmids, a 120-kb catabolic plasmid that encodes for breakdown of phenol (pPhCN1) and pPhCN2 plasmid that codes for cadmium and copper resistant. Cyanide assimilation by this bacterium is encoded by chromosomal genes. The inhibitory effect of cadmium (Cd^{2+}) or copper (Cu^{2+}) on the degradation of phenol by *P. putida* strains PhCN and PhCN1 (contained pPhCN1) were investigated in the presence of phenol and cyanide as a sole carbon and nitrogen source, respectively. The resistant strain PhCN showed high ability to degrade phenol and cyanide in presence of Cd^{2+} or Cu^{2+} as compared to the sensitive strain PhCN1. In addition, Cd^{2+} or Cu^{2+} was also found to exert a strong inhibitory effect on the C23O dioxygenase enzyme activity in the presence of cyanide as a nitrogen source. However, the presence of heavy metal resistance plasmid alleviated the inhibitory effect of metals on the enzyme activity in resistant strain.

Keywords: bacteria, cadmium, copper, plasmid, phenol degradation

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INTRODUCTION

Forty percent of hazardous wastes on the environmental protection agency's (EPA) national priority list (NPL) are co-contaminated with organic and heavy metals pollutants that pose health hazards to human and wildlife (Norena-Barrosa *et al.*, 2004). Common organic pollutants at these sites include phenol, polycyclic aromatic hydrocarbons (PAHs), chlorinated solvents, cyanide, herbicide and pesticides, while common heavy metal contaminants include arsenic, cadmium, chromium, copper, lead, mercury, nickel, and zinc. Isolation of bacterial strains that are able to degrade more than one organic pollutants such as phenol and cyanide, is becoming increasingly important for decontaminating polluted soil, sledges, and ground water (Jain and Sayler, 1987; Chen *et al.*, 2005). The use of these microorganisms may face various problems, including poor survival, substrate accessibility (Lin *et al.*, 2006), or the presence of inhibitory compounds. Heavy metals are known to be powerful inhibitors of biodegradation activities (Said and Lewis, 1991; Roane and Pepper, 1997; Roane *et al.*, 2001; Amor *et al.*, 2001; Hong *et al.*, 2007; Silva *et al.*, 2007), thus, their presence may impair the biodegradation of aromatic compounds in polluted sites (Springael *et al.*, 1993; Kuo and Genther, 1996; Benka-Coker and Ekundayo, 1998; White and Knowles, 2003; Roane *et al.*, 2001; Sokhn *et al.*, 2001; Riis *et al.*, 2002; Lin *et al.*, 2006; Silva *et al.*, 2007; Siunova *et al.*, 2007). Simultaneous contamination by

heavy metals and organic compounds may also occur and such contamination cases had been reported before at industrial areas (Al-Saleh and Obuekwe, 2005). For this reason, there is an increasing interest (Wasi *et al.*, 2008) in bacterial strains that are able to degrade aromatic compounds and tolerant to toxic metals. It has previously been shown that strains of *Alcaligenes eutrophus* bearing plasmids of metal resistance and plasmids of biodegradation of polychlorinated biphenyls and 2,4-dichlorophenoxyacetic acid degrade these xenobiotics more effectively in the presence of nickel or zinc as compared to sensitive strain (Springael *et al.*, 1993; Collard *et al.*, 1994). However, there have been no data on the interaction of genetic systems of aromatic compounds degradation, cyanide assimilation, and metal resistance and their effect on physiology, biodegradation efficiency, and the activity of the key enzyme in multifunctional strains.

The objectives of this work are to examine the physiological and biochemical features of phenol degradation in variants of the soil strain *P. putida* PhCN sensitive and resistant to heavy metals, in the presence of three toxicants, cadmium (Cd^{2+}), copper (Cu^{2+}) and cyanide, as a nitrogen source.

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*Corresponding author

MATERIALS AND METHODS

Chemical

Phenol, agarose, and ethidium bromide were purchased from Sigma (St. Lous, MO, USA). Sodium cyanide, cadmium chloride ($CdCl_2$), nickel chloride ($NiCl_2$), zinc chloride ($ZnCl_2$) and copper chloride ($CuCl_2 \cdot 2H_2O$) were procured from Merck (Darmstadt, Germany). Bacteriological media were purchased from Difco (Difco Laboratories, Detroit, Michigan) and Oxoid (Oxoid Inc., Nepean, Ontario). Reagents compounds used were of the highest grade commercially available.

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *P. putida* strain PhCN was isolated from soil contaminated with pesticides and was assimilated the phenol and cyanide as a sole source of carbon and nitrogen respectively (El-Deeb, 2006). PhCN1 (pPhCN1), PhCN2 (pPhCN2) and PhCNM (plasmidless) strains were the cured derivatives of wild type strain *P. putida*. PhCN (El-Deeb, 2006). All isolates were grown aerobically at 30 °C on a rotary shaker at 150 rpm in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989) or Tris-buffered (MSM), which was prepared according to Mergeay *et al.* (1985) and supplemented with phenol and KCN as the carbon and nitrogen sources, respectively. All isolates were maintained either on nutrient agar slants at 4 °C or in 20% (v/v) glycerol solution at – 80 °C.

Effect of the presence of heavy metals on the growth of *P. putida* PhCN and its derivatives

Bacterial cells were pre-grown in MSM with phenol (1 mM) and cyanide (0.5 mM) in Erlenmeyer flasks at 28 °C (150 rpm) until an optical density (OD_{600}) of about 0.5 was reached. This culture (2.2×10^5) was used to inoculate Erlenmeyer flasks containing 1 mM phenol and ammonium and/or cyanide as the sole source of carbon and nitrogen, respectively. The concentrations of studied heavy metals were adjusted to 10 or 100 µM. The initial OD_{600} was adjusted to about 0.05. Control flasks contained free metals medium inoculated with the same studied strains. All cultures were incubated on an orbital shaker (150 rpm) at 28 °C. OD_{600} nm was determined periodically and used as the measure of growth to construct growth curves and assess heavy metal effects on biodegradation as described by Malakul *et al.*, (1998) and White and Knowles (2003). All tests were done in triplicate.

Analytical Methods

The phenol degradation was determined by monitoring the change in phenol concentration (Folsom *et al.*, 1990). At different intervals of incubation, 1 mL samples were taken and transferred to an Eppendorff 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 curve.

Determination of minimal inhibitory concentration (MIC) of Cd^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} against studied bacterial variants

Minimum inhibitory concentrations (MIC) of metals for each isolate was determined by the plate-dilution method as adopted by Mergeay *et al.* (1985). The metals Cd^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} were used as $CdCl_2$, $CuCl_2 \cdot 2H_2O$, $NiCl_2$, and $ZnCl_2$, respectively, in various concentrations ranging from 0.001 to 8 mM. The plates were incubated at 28 to 30 °C for 72 to 96 h. The concentration of metal in which the toxicity begins to manifest was considered as the MIC of the metal against the strain tested. Carbon free Tris-salt medium (MSM) with 1.5% agar, containing phenol and ammonium or cyanide as a sole source of carbon and nitrogen respectively, was also used for detection of tolerance against these metals. The concentration of added phenol and cyanide in the media was 1 mM and 0.5 mM, respectively.

Preparation and Crude Enzyme Extract

The effects of Cd^{2+} or Cu^{2+} were studied at the same concentration, as previously mentioned in the study of effect on growth and phenol degradation. *P. putida* strain PhCN and its derivatives were 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

Table 1: Bacterial strains and plasmids

Strains or plasmids	Relevant characteristic(s)	Reference or source
Pseudomonas putida		
PhCN	Ph[+], Cd[r], Cu[r], wild type pPhCN1, pPhCN2 carrier	El-Deeb (2006)
PhCN1	Ph[+] a cured mutant of PhCN, pPhCN1 carrier	El-Deeb (2006)
PhCN2	Cd[r], Cu[r] a cured mutant of PhCN, pPhCN2 carrier	This study
PhCNM	Ph[-], Cd[-], Cu[-] a cured mutant of PhCN, Plasmidless	El-Deeb (2006)
Plasmids		
pPhCN1	120 Kb Ph[+] catabolic plasmid of *P. putida* PhCN	El-Deeb (2006)
pPhCN2	100 Kb Cd[r], Cu[r], heavy metal resistant plasmid of *P. putida* PhCN	This study

nitrogen source and 0.5 mM phenol as a carbon source. Cells were harvested by centrifugation at 10,000 x 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). The enzymes activity were measured by incubating the crude enzymes 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. The activity of catechol-2,3-dioxygenase was determined from the rate of α-oxymuconic semialdehyde formation (α = 375 nm, E = 33.4 µM/cm). The activity of catechol-1,2 dioxygenase was determined from the rate of cis-cis muconate formation (α = 260, E = 16.9 µM/cm). The specific enzyme activity was expressed in nano moles of the cofactor consumed or the product formed in 1 min per 1 mg of total bacterial protein. The protein concentration was determined spectrophotometrically according to Lowery *et al.* (1951).

Plasmid analysis

Plasmids were analyzed from cells grown in liquid media under selective conditions given by either metals or the carbon source. Plasmids of *P. putida* PhCN strains and its derivatives were analyzed according to the method of Kado and Liu (1981). Standard DNA techniques were used as described previously (Sambrook *et al.*, 1989). For plasmids analysis, 0.7% (wt/vol) agarose gels with Tris borate-EDTA buffer were employed. The size estimate of the isolated plasmid was obtained by comparing relative mobility on agarose gel with standard molecular markers.

Stability, curing and conjugation

The stability of degradation and heavy metal resistance properties were monitored by the growth of PhCN and its derivatives on glucose or LB broth. For chemical curing, culture was grown on LB broth containing various concentrations of mitomycin C (5, 10, and 20 µg/mL), on a rotary shaker at 30 °C for 48 h. Appropriate dilutions were spread on LB agar plates. Isolated colonies were replicated on LB plus metals and Tris-medium plus phenol and heavy metal plates. Ph$^-$ and Cd$^-$, Cu$^-$ were scored and reported as percent of curing. The conjugation between donor PhCN and recipient PhCNM was performed by liquid mating technique. Donor strains were grown overnight in LB broth in the presence of one selective metal. PhCNM recipients were usually cultivated until reach the late-logarithmic growth phase. Cells were harvested, washed gently and suspended in sterile LB broth (cell density 6.4 x 10^8 cell/mL). Equal volume of cell suspensions were mixed together without shaking at 30 °C for 24 h. Transconjugants were selected on heavy metal-Ph double selection plates and confirmed by growing them in MSM containing Ph in case of PhCN1 or heavy metals in case of PhCN2.

RESULTS AND DISCUSSION

Native plasmids in *P. putida* PhCN

Plasmid DNA analysis of *P. putida* strain PhCN cultures grown on phenol and cyanide indicated the presence of two large plasmid elements (El-Deeb, 2006). Plasmid curing experiments produced a plasmid-free strain named as PhCNM, a variant containing the 120 kb plasmid (pPhCN1) named as PhCN1 and a strain harboring 100 kb plasmid (pPhCN2) named as PhCN2. Based on the fact that subsequent plasmid curing experiments 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 (El-Deeb, 2006). However, the function of the second plasmid (pPhCN2) still unknown.

The present study showed that the pPhCN2 plasmid had no role in phenol degradation (Figures 1 to 4, Table 2). To investigate the correlation between the resistance to Zn^{2+}, Cu^{2+}, Ni^{2+}, and Cd^{2+} in relation to the presence of second large plasmid pPhCN2, mitomycin C was used to cure strain PhCN2 from its plasmid. Loss of the cadmium, copper, zinc, and nickel property was used for selection. Several hundreds clones were screened for heavy metals resistance deficient mutants. Most of the obtained mutants were sensitive to both cadmium and copper yet retained their resistance to other two metals of Ni^{2+} and Zn^{2+} (Table 2).

Conjugation studies also indicated that pPhCN2 (100 kb) was expressed for Cd^{2+} and Cu^{2+} resistance. Transfer of Cu^{2+} and Cd^{2+} from strain PhCN or PhCN2 to strain PhCNM occurred at frequency of 10^{-7} to 10^{-8}, which was comparable to the frequency reported previously when a plasmid-free derivative of strain PhCN was used as the recipient strain (Mergeay *et al.*, 1985).

Stability of plasmids

The stability of phenol degradation and heavy metal resistance properties of PhCN strain and its variants was monitored as described in material and methods. Different variants, PhCN1 (contained pPhCN1) and PhCN2 (contained pPhCN2) were obtained. Each plasmid in these variants remained stable and retained the determined phenotype phenol degradation (ph$^+$) and/or heavy metal resistance (Cd^r/Cu^r) after several passages in non selective medium. In the wild type strain PhCN (contained pPhCN1, pPhCN2), both plasmids were also maintained stably and retained the determined phenotype of ph$^+$ and Cd^r/Cu^r after several passages in a non selective medium.

Minimal inhibitory concentration (MIC) of Cd^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} against studied bacterial variants

In the preliminary screening, the ability of *P. putida* PhCN and its derivatives; PhCN1, PhCN2, and PhCNM,

Table 2: MICs of heavy metals for *P. putida* PhCN and its derivatives in different cultural conditions

Bacterial strains	MIC of Cd^{2+} (mM)				MIC of Cu^{2+} (mM)				MIC of Zn^{2+} (mM)				MIC of Ni^{2+} (mM)			
	LB	MS + G	MS + Ph	MS + CN + Ph	LB	MS + G	MS + Ph	MS + CN + Ph	LB	MS + G	MS + Ph	MS + CN + Ph	LB	MS + G	MS + Ph	MS + CN + Ph
PhCN	2.00	1.50	1.00	0.800	1.80	1.20	1.00	0.85	1	0.9	0.60	0.50	1.2	0.9	0.60	0.40
PhCN1	0.05	0.04	0.03	0.015	0.04	0.03	0.02	0.01	1	0.8	0.02	0.01	1.2	0.8	0.60	0.40
PhCN2	2.00	1.50	0.00	0.000	1.80	1.20	0.00	0.00	1	0.8	0.00	0.00	1.2	0.9	0.00	0.00
PhCNM	0.04	0.04	0.00	0.000	0.04	0.04	0.00	0.00	1	0.7	0.00	0.00	1.2	0.8	0.00	0.00
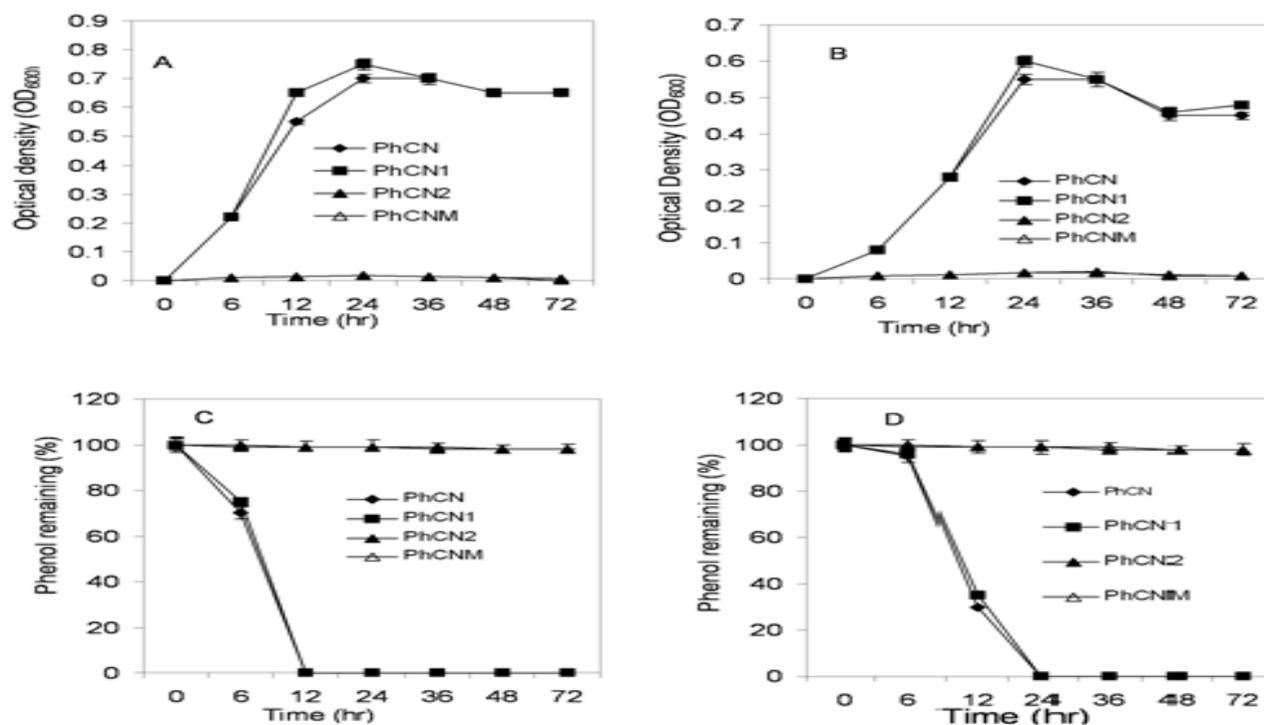
Figure 1: Bacterial growth and phenol utilization by *P. putida* PhCN and its derivatives PhCN1, PhCN2, PHCNM in minimal medium amended with ammonium ions (A, C) and/or cyanide (B, D) as a sole nitrogen source, in absence of metals ions

to form confluent growth in the presence of incremental concentrations of studied metal salts was evaluated. On complete medium (LB), the wild type strain PhCN and its variant PhCN2 exhibited the highest resistance to Cd^{2+} and Cu^{2+} in comparison with the other strains, PhCN1 and PhCNM (Table 2). However, the MICs of Zn^{2+}, and Ni^{2+} for tested variants were very close.

It has been previously demonstrated that the interference of a high phosphate content in the usual minimal medium with a metal effect can lead to the over estimation of the MIC (Mergeay et al., 1985). Therefore, in the present work, levels of resistance in wild type strain PhCN and its cured derivatives to Zn^{2+}, Cu^{2+}, Ni^{2+}, and Cd^{2+} were determined on Tris-medium supplemented with glucose as the sole carbon and energy source. As presented in Table 2, for the resistance variants PhCN and PhCN2, MIC of Cd^{2+} (1.5 mM) and Cu^{2+} (1.2 mM) were much higher than in sensitive strain PhCN1 (0.03 mM and 0.02 mM for Cd^{2+} and Cu^{2+}, respectively). No significant difference in the MICs of Zn^{2+} and Ni^{2+} among the four strains was observed. Furthermore, the MICs of Cd^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} for tested strains in the Tris-medium were lower than those observed in the LB medium (Table 2). This result was in harmony with those mentioned by Mergeay et al. (1985). They stated that MICs of heavy metals in Tris-medium were lower than those observed in a rich medium. On the other hand, the MICs of Cd^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+} in the presence of phenol and cyanide as sole carbon and nitrogen sources, respectively, for tested strains were lower than those observed in the Tris-medium supplemented with ammonium as a nitrogen source (Table 2). These results are consistent with findings of Barbieri et al. (1996) that the MICs of metals for catabolic strains in the presence of aromatic compounds as the only source of carbon are only slightly lower than those evaluated in the presence of glucose or malate.

Growth kinetics

Growth on phenol in the absence of heavy metals

Preliminary assays were performed to evaluate the growth rate of wild type strain PhCN and its derivatives; PhCN1, PhCN2, and PhCNM (plasmidless) on MSM medium free from heavy metals and supplied with phenol and ammonium or cyanide as a sole source of carbon and nitrogen, respectively. The results presented in Figure 1 (A-D) indicated that both strains which harboured a catabolic plasmid pPhCN1 were able to degrade the compound at similar rate. However, strains grown on MSM medium free from heavy metals and supplied with phenol and cyanide revealed a decrease in the growth rate as well as the rate of phenol consumption comparing to the strains supplied with ammonium salt as a nitrogen source. Similar findings were reported by Arutchelvan et al. (2005) for *Pseudomonas* sp. grown on phenol and cyanide.
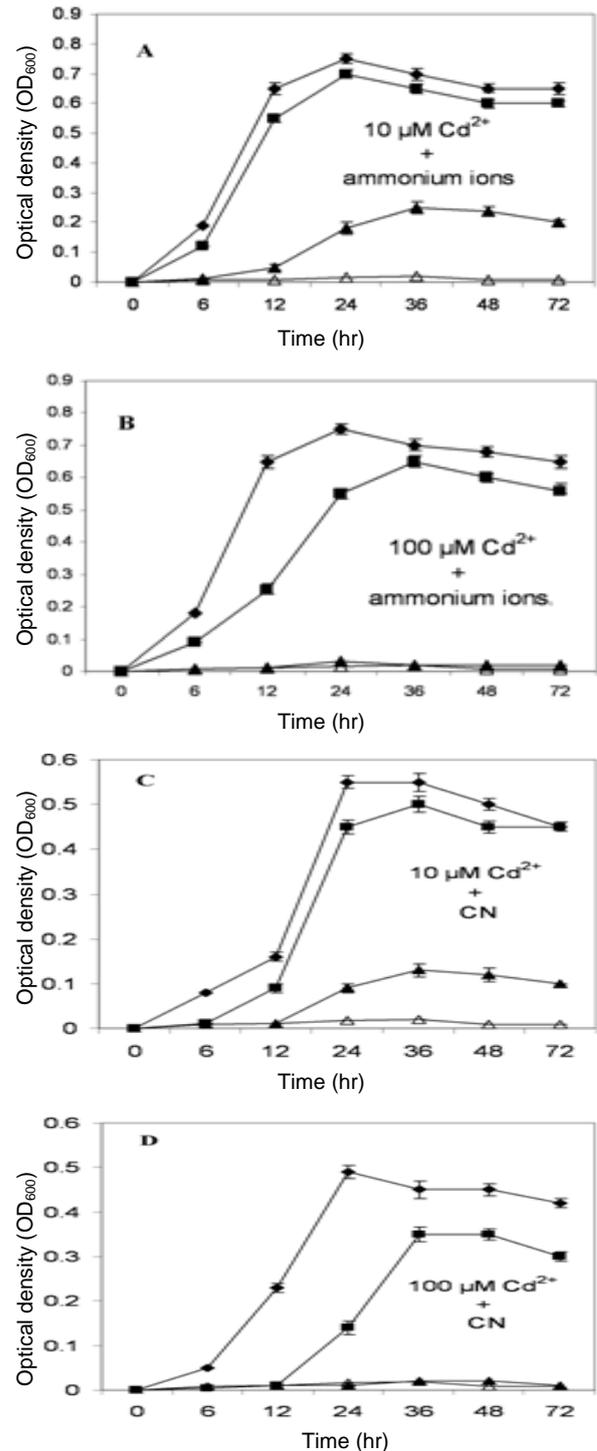
Figure 2: Effect of cadmium concentrations on the growth of *P. putida* PhCN and its derivatives on phenol and ammonium ions/or cyanide as a sole carbon and nitrogen source respectively. Strains symbols: (♦)PhCN without Cd^{2+} (control); (■), PhCN ; (▲) PhCN1 and (△), PhCN2
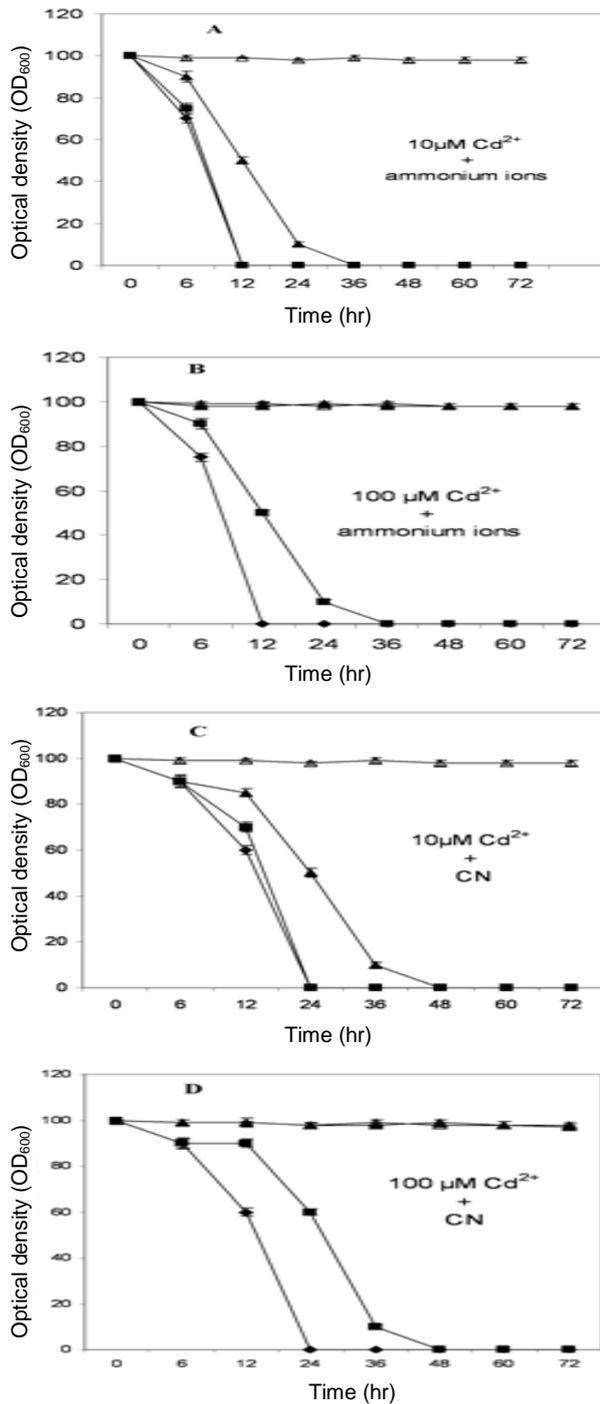
Figure 3: Effect of cadmium concentrations on the phenolutilization by *P. putida* PhCN and its derivatives in minimal medium amended with ammonium ions/or cyanide as a nitrogen source respectively. Strains symbols: (♦) PhCN without Cd²⁺ (control); (■), PhCN ; (▲) PhCN1 and (△), PhCN2

Growth on phenol in the presence of the cadmium

The effect of cadmium on growth parameters and phenol consumption by resistant strain PhCN (pPhCN1, pPhCN2) and sensitive strain PhCN1 (pPhCN1) was investigated. Cadmium at concentration of 10 µM in MSM medium with phenol and ammonium as a sole source of carbon and nitrogen, respectively, did not affect the growth rate of PhCN, whereas, the growth of sensitive strains was significantly reduced comparing with that of the cadmium free medium (Figure 2A). Phenol consumption by sensitive strain PhCN1, was significantly reduced compared to that of the free-cadmium MSM (Figure 3A) (Amor *et al.*, 2001; Siunova *et al.*, 2007). However, the addition of cadmium at a concentration of 10 µM did not change the rate of phenol consumption by resistant strain PhCN as compared to that of the free-cadmium MSM (Figure 3A) (Siunova *et al.*, 2007).

Increasing concentration of cadmium by ten-fold (100 µM) caused a complete inhibition of growth in sensitive strain PhCN1 (Figure 2B), whereas, the growth and phenol consumption by resistance strain PhCN was slightly reduced as compared to that of the free-cadmium MSM (Figure 2B and 3B). Previously a similar finding has been reported for the multifunctional strains of *Alcaligenes eutrophus* that bear plasmids of resistance to bivalent cations, pMOL28 (*cnr*-Coʳ Nʳ) and pMOL30 (*czc*-Coʳ Znʳ Cdʳ), and catabolic plasmids of biodegradation of polychlorinated biphenyls, pSS50 (BpH⁺/Cbp⁺), and 2,4-dichlorophenoxyacetic acid, pJP4 (Tfd⁺) are effectively degraded the above pollutants in the presence of metals (Collard *et al.*, 1994; Mergeay, 1995) .

The combination effects of heavy metals and cyanide on bacterial growth and phenol degradation were investigated. As presented in Figure 2C, addition of cadmium (10 µM) to MSM containing phenol and cyanide as the sole source of carbon and nitrogen, respectively, showed a slightly decrease in the growth rate and phenol consumption by resistant strain PhCN (Figure 3A and 3C), whereas, induced a significant decrease in bacterial growth and retarded phenol consumption by sensitive strain PhCN1 (Figure 2C and 3C).

A ten-fold increase of cadmium (100 µM) combined with cyanide as a nitrogen source caused a 12 h lag-phase (Figure 2D) and phenol consumption was less active by resistant strain PhCN as compared to that of the MSM free cadmium. Under the same conditions, the growth of sensitive strain PhCN2 was completely inhibited (Figure 2B and 2D). This may be due to the fact that when various toxicants were added together, the growth of almost all bacterial isolates was very poor or absent, suggesting that the tolerance level of heavy metal resistance strains is affected by many xenobiotics (De *et al.*, 2003).

Growth on phenol in the presence of the copper

Growth response of the PhCN and its variants in the presence of cupric chloride was presented in Figure 4. At low concentration (10 µM) in MSM supplemented with

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phenol and ammonium as a sole source of carbon and nitrogen, respectively, the growth rate of resistant strain PhCN was not affected, however the growth rate of the sensitive strain PhCN1 was significantly reduced (Figure 4A). Phenol consumption by resistance strain PhCN was less active as compared to the MSM free from copper (Figure 5A), whereas, phenol consumption by sensitive strain was significantly reduced (Figure 5A). This result was in agreement with previous results of Lin *et al.* (2006) which showed that copper exhibited high toxicity at relatively low concentrations of 10 mg/L.

The addition of copper at a concentration 10 μM to the MSM containing phenol and cyanide as a sole source of carbon and nitrogen respectively, produced no appreciable affect on the growth and phenol consumption by resistance variant, while the growth of sensitive strain was significantly reduced (Figure 4C and 5C).

A ten-fold increase of copper (100 μM) in MSM supplemented with phenol and cyanide as a sole source of carbon and nitrogen source respectively, caused a long lag-phase (12 h) and required long time (60 h) for complete phenol consumption by resistance strain PhCN (Figure 4D and 5D). However, the addition of copper at a concentration of 100 μM to MSM was completely inhibited the growth of sensitive strain (Figure 3D and 4D) (Sokhn *et al.*, 2001).

By comparing the obtained results in Figure 1 to 4, it can be concluded that, low concentration of both Cu^{2+} and Cd^{2+} (10 μM) did not exhibit any significant inhibitory effect on microbial growth of resistant variants in presence of ammonium or cyanide as a nitrogen source. However, both metals at low concentration significantly reduced the growth of sensitive strain (Siunova *et al.*, 2007). Ten-fold increase of both metals concentration caused slightly increased a lag phase in the case of resistant variant PhCN whereas completely inhibited the growth of the sensitive strain PhCN1. However, in the presence of KCN as a nitrogen source, the overall bacterial growth and phenol consumption was less than that observed when the bacteria were grown in the presence of ammonium as a nitrogen source. Medium composition affects the degree and pattern of metals inhibition of organic pollutants biodegradation (Hoffman *et al.*, 2005).

Effect of metal ions on the phenol degradation enzyme activity

The C23O pathway for catechol degradation during phenol metabolism in *Pseudomonas* sp. PhCN was previously demonstrated (El-Deeb, 2006). In order to study whether phenol degradation by the strain PhCN and its variants affected by different nitrogen source in presence and absence of metal ions, the variants were grown in LB medium and phenol – MSM media amended with different nitrogen sources in presence of metal ions. When the activity of C23O enzyme was measured (Table 3), no C23O activity was detected in LB medium in the absence of phenol. In the presence of phenol, the activity of C23O was negligible.
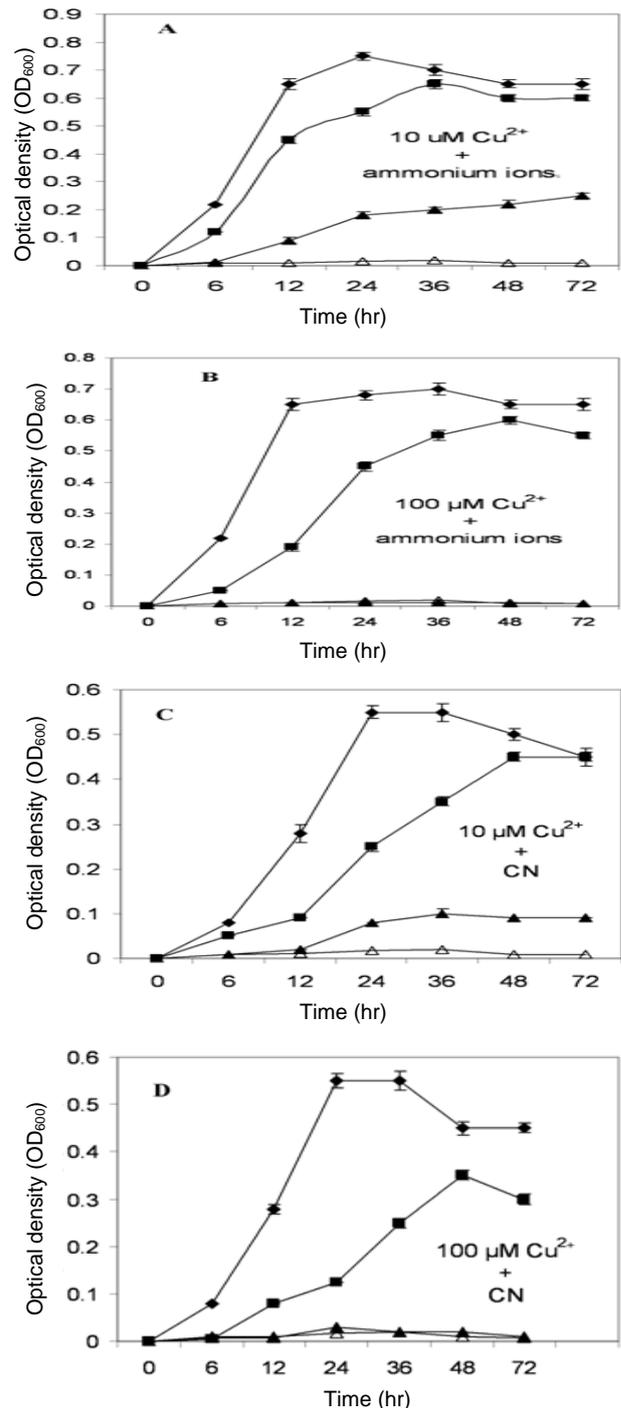
Figure 4: Effect of copper concentrations on the growth of *P. putida* PhCN and its derivatives on phenol and ammonium ions/or cyanide as a sole carbon and nitrogen source respectively. Strains symbols: (♦)PhCN without Cu^{2+} (control); (■), PhCN ; (▲) PhCN1 and (Δ), PhCN2
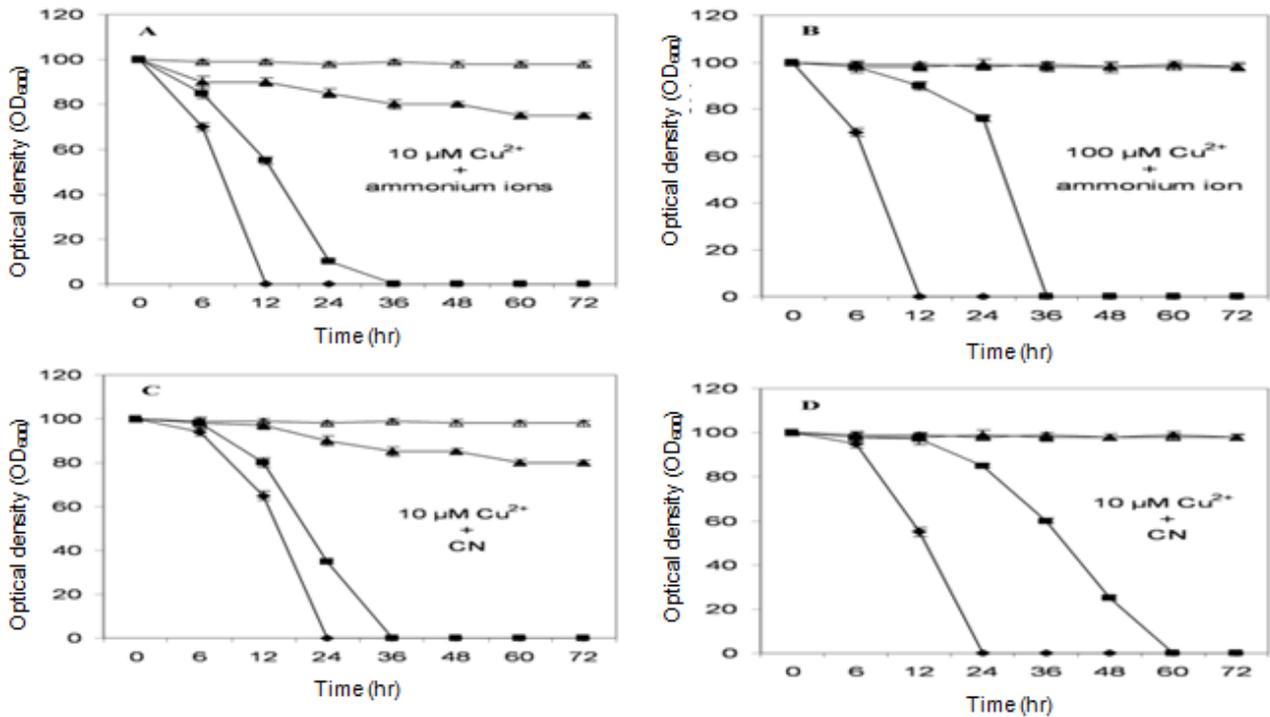
Figure 5: Effect of copper concentrations on the phenol utilization by *P. putida* PhCN and its derivatives in minimal medium amended with ammonium ions/or cyanide as a nitrogen source respectively. Strains symbols: (♦)PhCN without Cu^{2+} (control); (■), PhCN ; (▲) PhCN1 and (∆), PhCN2

In the absence of heavy metals, the C23O activity was high when the cells were grown in the presence of phenol and ammonium salt as a sole source of carbon and nitrogen respectively (Table 3). In the presence of KCN as a nitrogen source, the overall enzyme activity was less than that observed in the presence of ammonium as a nitrogen source (Table 3).

The effect of Cd^{2+} or Cu^{2+} on the 2,3-dioxygenase enzyme activity in the MSM containing phenol and ammonium as a sole source of carbon and nitrogen respectively, was investigated. The results in Table 3 showed that both the metals had a severe inhibitory effect on the 2,3-dioxygenase activity in the sensitive strain while they had slight effect on the enzyme activity in resistant strain PhCN. (Nakazawa and Nakazawa,

1970; Yeom and Yoo, 1997). The effect of Cd^{2+} or Cu^{2+} was also found to exert a strong inhibitory effect on the enzyme activity in the presence of cyanide as a nitrogen source (Table 3). This could be attributed to enzymes produced into the extracellular environment often face higher concentration of metals, since they are not protected by cell-associated metal-detoxification mechanisms (Yeom and Yoo, 1997)

CONCLUSIONS

Multifunctional strain of *P. putida* strain PhCN that bear plasmids of resistance to bivalent cations, pPhCN2 (Cd^r, Cu^r) and catabolic plasmids of biodegradation of phenol pPhCN1 (Ph^+) are effectively degraded the above

Table 3: Specific activities of *meta*-cleaving enzyme C23O dioxygenase in a crude extract of parent strain PhCN and its derivatives grown on different substrate, enzyme was assayed as described in Materials and Methods

	Substrate							
Nitrogen source	In presence of ammonium ions				In presence of cyanide			
Strains	TSM +G	TSM + Ph	TSM + Ph + Cd	TSM + Ph + Cu	TSM + G	TSM + Ph	TSM + Ph + Cd	TSM + Ph + Cu
PhCN (pPhCN1,pPhCN2)	0.01	14.2	12.2	11.4	0.01	12.8	11.6	10.8
PhCN1(pPhCN1)	0.01	14.6	0.4	0.2	0.01	10.8	0.09	0.02
PhCN2 (pPhCN2)	ND	ND	ND	ND	ND	ND	ND	ND
PhCNM(Plasmidless)	ND	ND	ND	ND	ND	ND	ND	ND

Enzyme activity is given in U/mg of protein.
ND, not detected; G, glucose; Ph, phenol; Cd, cadmium; Cu, copper

pollutants in the presence of metals as compared with sensitive strain. The effect of Cd^{2+} or Cu^{2+} was also found to exert a strong inhibitory effect on the C23O dioxygenase enzyme activity in the presence of cyanide as a nitrogen source. However, the presence of heavy metal resistance plasmid alleviated the inhibitory effect of metals on the enzyme activity in resistant strain. These findings demonstrate the possibility of combining the plasmids of phenol catabolism, plasmids of resistance to heavy metals, and systems of chromosomal gene encoded for cyanide assimilation. This approach can be used for the creation of polyfunctional bacterial strains effective for bioremediation of soil polluted by phenol in combination with cyanide and heavy metals.

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