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Catabolic Plasmid-Mediated Heavy Metal Resistance in Herbicide Diuron-Degrading *Pseudomonas* species

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Abstract Three *Pseudomonas* strains (Bk8, Bk9, Bk10) selected from soil for their ability to degrade herbicide diuron were tested for their heavy metal resistance. The growth of these catabolic strains on a minimal medium with various concentrations of Cd^{2+}, Zn^{2+}, Ni^{2+}, and Hg^{2+} revealed a minimal effect on the carbon source for the inhibitory effect of the metals. One of these strains, namely, Bk8, exhibited a high resistance to the heavy metals as compared to the two other strains. This strain harbors plasmid pBk8 (110 kb) and contains at least four determinants encoding heavy metal resistance. Nickel and zinc resistance are encoded by genes located on the chromosome, while cadmium and mercury resistance are on plasmid pBk8. Accordingly, the characteristics of strain Bk8 suggest that it would be useful in the bioremediation of aromatic compounds in the presence of toxic heavy metals as co-contaminants.

Key words: Heavy metals resistance, herbicide diuron, inducible and constitutive genes

The contamination of soil, groundwater, surface waters and air with hazardous and toxic chemicals is one of the major problems facing the industrialized world today. The need to restore these sites has led to the development of new technologies that emphasize the detoxification and destruction of contaminants rather than the conventional approach of disposal. Biodegradation, the use of microorganisms or microbial processes to detoxify and degrade environmental contaminants, is one of these new technologies. Microbial degradation has been proposed as an inexpensive and efficient method to remove xenobiotic compounds from the environment. However, heavy metals often occur as co-contaminants and reportedly have adverse effects on biodegradation [7]. These effects include extended acclimation periods, reduced biodegradation rates, and the failure of target compound biodegradation [11, 12, 19]. However, indigenous microflora respond to this challenge by the evolution of resistant populations that sustain microbial processes in the presence of toxic metals. Although the evolution of resistant populations and biodegradation have been studied independently, not much study has been carried out concerning the biodegradation in the presence of toxic metals as co-contaminants. Barbieri *et al.* [1] observed the resistance of catabolic strains to different heavy metals in the presence of aromatic compounds as the only source of carbon and energy. The degradation of naphthalene in the presence of cadmium has been previously described [14]. Moreover, Kuo and Genthner [12] studied the effect of toxic metals on the biodegradation of 2-chlorophenol and 3-chlorobenzoate in an anaerobic bacteria consortia.

The present study focused on heavy metal resistance in catabolic strains, Bk8, Bk9, and Bk10, in the presence of diuron herbicide, whose catabolic genes are often located on the plasmid [6, 18]. The aim of this study was to evaluate heavy metal resistance in diuron-degrading *Pseudomonas* strains and to obtain preliminary information on the role of the catabolic plasmid pBk8 in heavy metal resistance.

Materials and Methods

Bacterial Strains and Plasmids

The isolated *Pseudomonas* strains, mutants, and transconjugants plus the plasmid used in this study are all listed in Table 1. The *Pseudomonas* sp. strains Bk8, Bk9, and Bk10, which were isolated using enrichment cultures from soil, were selected for their ability to utilize diuron herbicide as a carbon and energy source [6]. Rifampin-resistant (rif^r) mutant of Bk8M was isolated by plating dense culture of this strain onto LB plates containing 50 μg

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

Strains		Phenotype	Source
Pseudomonas sp. Bk8	Wild-type diuron degrader, pBk8	D^+ Rif^S	[6]
Pseudomonas sp. Bk9	Wild-type diuron degrader	D^+	[6]
Pseudomonas sp. Bk10	Wild-type diuron degrader	D^+	[6]
Pseudomonas sp. Bk8M	Cured derivatives of Bk8	D^- Rif^r	This study
Pseudomonas sp. Bk8TC	Transconjugants between Bk8 and Bk8M, pBk8	D^+ Rif^r	This study

D^+, ability to degrade diuron; D^-, inability to degrade diuron; Rif^S, Rifampin sensitive; Rif^r, Rifampin resistant.

of rifampicin per ml. The plates were incubated for 2 to 4 days at 28°C. Single-mutant colony was restreaked onto the same medium for final purification. The mutant was used as primary recipients in the mating assays.

Growth Media

Analytical-grade salts of $CdCl_2 \cdot H_2O$, $ZnCl_2$, $NiCl_2$, and $HgCl_2$ were used to prepare a 1.0 M stock solution (for $HgCl_2$, the concentration was 0.1 M), which was sterilized by filtration. The liquid cultures were prepared with a mineral medium [8]. This medium was buffered with 50 mM Tris-HCl (Trizma, Sigma), pH 7, instead of a phosphate buffer to avoid the precipitation of insoluble metal phosphates. Phosphorus was added to the medium in the form of sodium β-glycerophosphate. Filter-sterilized diuron (dissolved in acetone) was added to the sterile cooled medium to give the final concentration of 30 µg/ml. This medium was then used for testing the metal resistance. The solid medium contained a 1.5% (w/v) agar.

Determination of Minimal Inhibitory Concentration (MIC)

To determine the MIC of Cd^{2+}, Zn^{2+}, and Ni^{2+} (the lowest concentration of metal salts in which no colony was observed), a drop of the cell suspension (10^7 cells/ml) was applied to LB plates, plus Tris-glucose agar plates containing 0.5 to 3 mM $CdCl_2$, 0.8 to 10 mM $ZnCl_2$, or 0.6 to 10 mM $NiCl_2$. The plates were incubated at 30°C and the growth was evaluated after 72 h. Furthermore, a liquid Tris-medium was used to evaluate the levels of Cd^{2+}, Zn^{2+}, and Ni^{2+} tolerated by the *Pseudomonas* strains when grown on diuron or glucose as the only source of carbon and energy. The cultivation was performed at 30°C for 48 h. The intensity of growth was then compared to the control (growth in the absence of metal salts). The MIC was defined in the liquid medium as the lowest concentration of metal above which no growth was observed. The level of resistance to mercury was evaluated in NEM medium plates [16] which contained increasing concentrations of $HgCl_2$ (4 to 50 µM). The plates were incubated at 30°C and the growth was evaluated after 72 h. A liquid M9 medium was used to determine the level of $HgCl_2$ tolerated by the *Pseudomonas* strains when grown on diuron or glucose as the only source of carbon and energy. The growth was ensured as described above.

Inducibility of Heavy Metal Resistance of Strain Bk8

A Tris-glucose medium was used to study the inducibility of cadmium, nickel, and zinc. The pre-cultures were prepared by the inoculation of Bk8 in 10 ml of the Tris-glucose medium, and left to grow overnight at 30°C on a rotary shaker (100 rpm). For the induction, 0.1 ml of this culture was then added to a 100-ml Erlenmeyer flask containing 10 ml of the Tris-glucose medium with or without heavy metals (e.g. 0.1 mM Cd^{2+}, 0.2 mM Zn^{2+}, or 0.1 mM Ni^{2+}) and incubated at 30°C on a shaker for 18 h. These cultures were then used to inoculate 50-ml media containing sub-inhibitory concentrations of heavy metals (0.8 mM Cd^{2+}, 1 mM Zn^{2+}, or 0.8 mM Ni^{2+}) and incubated at 30°C with shaking. The growth of bacteria was monitored periodically by measuring the optical density at 600 nm. For the mercury induction, 200 µl of an overnight culture grown in the NEM medium in the absence or presence of a sublethal concentration of Hg^{2+} (2 µM $HgCl_2$) were inoculated in 50 ml of fresh medium that contained 40 µM of $HgCl_2$, and incubated at 30°C with shaking. The growth was evaluated as described above.

Plasmid Curing

The plasmid curing experiment was performed according to a procedure described by Carlton and Brown (1981). In this experiment, a series of mitomycin C concentrations ranging from 0.1 µg to 10 µg/ml were inoculated with a loopful of cells from a 24 h LB culture of *Pseudomonas* sp. Bk8. After incubation with gentle shaking for 48 h, the tubes with cells containing the highest concentration of curing agent were harvested, washed and diluted with a 0.9% saline solution. One-hundred µl of an appropriate dilution was spread onto LB plates and incubated at 30°C for 48 h. Single colonies from the LB plates were then screened for heavy metal resistant-deficient mutants using microtiter Tris-glucose medium plates and the respective metal salts. These mutants, which were sensitive to one or more heavy metals, were then screened for the presence of the plasmid. The plasmid was isolated from strain Bk8 and its derivatives as described by Kado and Liu [9].

Conjugation

Mating was carried out by biparental conjugation. The donor (strain Bk8) and recipient (strain Bk8M) were grown at 30°C in a nutrient broth. The agar mating was performed as described previously [13]. The plates were incubated at 25°C overnight. The cells were suspended in saline solution and spread on a selective Tris-glucose medium (NEM in case of $HgCl_2$) containing appropriate concentrations of metals.

RESULTS AND DISCUSSION

Heavy metals resistance strains are widespread among Gram-negative and -positive bacteria [20]. The *Pseudomonas* sp. strains Bk8, Bk9, and Bk10 were able to utilize diuron as a carbon and energy source [6]. In the preliminary screening, the ability of these strains to form confluent growth in the presence of increasing concentrations of different metal salts was evaluated. An examination of growth of the tested strains on different liquid medium compositions containing various concentrations of heavy metals showed variable degrees of resistance to one or more of the four metals: cadmium, zinc, nickel, and mercury (Table 2). Strain Bk8 exhibited the highest resistance to cadmium, zinc, and mercury in comparison with the two other strains, and the MICs of Ni^{2+} for the tested strains were similar (Table 2).

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 overestimation of the MIC [15]. Therefore, Tris-medium was used as the minimal medium. Tris-buffer was not utilized as carbon or nitrogen sources by the tested strains (data not shown). In this study, the levels of cadmium, zinc, and nickel resistance in strains BK8, Bk9, and Bk10 were determined on the Tris-medium, amended with glucose as the sole carbon and energy sources. Table 2 showed that the MIC of Cd^{2+} for the sensitive strain Bk10 was 0.3 mM, whereas the MICs for the Bk8 and Bk9 strains were 1 mM and 0.6 mM, respectively. Furthermore, strain Bk8 exhibited the highest level of resistance to zinc (3 mM), whereas strain Bk9 seemed to be less tolerant, although it was much more resistant than that of the sensitive strain Bk10 (Table 2). No significant difference in the MICs of Ni^{2+} among the three strains were observed, and the MICs of Ni^{2+}, Cd^{2+}, and Zn^{2+} in the Tris-medium were lower than those observed in the rich medium (Table 2). In a parallel study, Mergeay *et al.* [15] studied the metal tolerence level of *Alcaligens eutrophas* CH34 in Tris-medium and found that the MICs were lower than those observed in a rich medium. On the other hand, the MICs of Cd^{2+}, Zn^{2+}, and Ni^{2+} for strains Bk8, Bk9, and Bk10 in the presence of diuron were only slightly lower than those evaluated in the presence of glucose (Table 2). Similar observations have been previously reported [1, 12, 14].

The ability of the tested strains to grow in an increasing concentration of $HgCl_2$ was evaluated as described in Material and Methods. Although almost all the screenings for Hg^{2+} resistance proceeded in rich media, the possible formation of complexes with organic compounds and in particular with sulfhydryl groups, can lead to the overestimation of the MIC. For this reason, a liquid NEM medium, which was developed by Nelson *et al.* [16] to minimize the titration of Hg^{2+} by potential binding to sulfur-containing compounds was used in this study. Table 2 showed that the MIC of $HgCl_2$ for the sensitive strain Bk10 was 10 μM, while the MIC of $HgCl_2$ for strain Bk9 was 15 μM. Strain Bk8 exhibited the highest level of resistance (45 μM) to $HgCl_2$. As expected, the MICs of $HgCl_2$ for the tested strains in the M9 medium supplemented with glucose were lower than those evaluated in the rich medium (Table 2). Additionally, the MICs of $HgCl_2$ for the tested strains in the presence of diuron were less significant than those observed in the rich medium, yet slightly lower than those evaluated on the minimal medium replaced with glucose. These results are consistent with the findings of Barbieri *et al.* [1] that the MICs of $HgCl_2$ 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.

Genetic Localization of Cadmium, Zinc, Nickel, and Mercury

To localize the genes determining the heavy metal resistance in the herbicide-degrading bacteria, only the plasmid-containing strain was further investigated. *Pseudomonas*

Table 2. Minimal inhibitory concentrations (MIC) of heavy metals for different *Pseudomonas* strains in different liquid medium.

Strains of *Pseudomonas* sp.	Cd^{2+} (mM)			Zn^{2+} (mM)			Ni^{2+} (mM)			Hg^{2+} (μM)		
	Tris-diuron (30 μg/ml)	Tris-glucose	LB medium	Tris-diuron (30 μg/ml)	Tris-glucose	LB medium	Tris-diuron (30 μg/ml)	Tris-glucose	LB medium	M9-diuron (30 ug/ml)	M9 + glucose	NEM medium
Stain Bk8	0.9	1	2	1.50	2	3	0.8	1	1.6	22	30	45
Stain Bk9	0.4	0.6	0.8	1.30	1.5	2.5	0.8	0.9	1.4	8	10	15
Stain Bk10	0.2	0.3	0.6	0.45	0.6	0.8	0.7	0.8	1.2	4	5	10

sp. strain Bk8 is a plasmid (pBk8)-harboring organism which is capable of the complete degradation of the diuron herbicide [6]. The molecular size of the pBk8 plasmid is about 110 kb and is involved in the diuron degradation [6].

The involvement of plasmid in heavy metal resistance has been widely reported [20]. Silver and Misra [21] demonstrated that metal resistance among diverse bacteria is rather a plasmid-mediated property than a chromosomal-coded function. In many cases, heavy metal resistance and the capacity for xenobiotic degradation are known to be determined by plasmid genes [10]. In particular, this is the case with the Oct plasmid in the octane degrader *Pseudomonas oleovorans* [3], pWW17 in the phenylacetate degrader *Pseudomonas putida* MT14 [17], and pJP4 in the 2,4-D degrader *Alcaligenes eutrophus* JM134 [4, 5]. To examine the correlation between the resistance to cadmium, zinc, nickel, and mercury and the presence of pBk8, mitomycin C was used to cure strain Bk8 from its plasmid pBk8. Loss of the cadmium, zinc, nickel, or mercury resistant property was used for selection. Four-hundred clones were screened for heavy metal resistance deficient mutants. All the mutants obtained were sensitive to both cadmium and mercury yet retained their resistance to both zinc and nickel (Table 3). The frequency of loss of the cadmium and mercury resistance phenotype in strain Bk8 during successive growth on the LB medium was less than 0.1%, and it increased up to 6% when mitomycin C was present during growth. Agarose gel electrophoresis revealed that each cadmium- and mercury-sensitive mutant lost the plasmid pBk8 (Fig. 1, Lane 2). Moreover, these mutants lacking pBk8 also lost the ability to utilize the herbicide diuron as the sole source of carbon (data not shown). These results indicate that pBk8 encodes the Cd^{2+} and Hg^{2+} resistance while the genetic information for Zn^{2+} and Ni^{2+} resistance may be located on the chromosome. One of these mutants was designated Bk8M.

Conclusive evidence that the cadmium and mercury resistance is located in pBk8, however, requires the resistance phenotype to be restored upon the reintroduction of this plasmid in the mutant cadmium- and mercury-sensitive strain Bk8M. Accordingly, to test this possibility, strain Bk8M was marked as rifampin resistant, as described in Materials and Methods, and used as the recipient strain. The wild-type strain Bk8 was used as the donor of pBk8 plasmid. The matings were made between the donor and the recipient strains as described in Material and Methods. The transconjugants obtained were all rifampin resistant, thereby indicating that they represented a true transconujant. Moreover, when either the donor or the acceptor strain was omitted, no rifampin-, cadmium-, or mercury -resistant colonies were observed, indicating that the spontaneous rates of mutation toward cadmium and mercury resistance or toward the used-marker antibiotics were of an order of magnitude lower than the observed transfer frequencies. All the transconjugants that acquired a resistance to Cd^{2+} and Hg^{2+} contained a plasmid which was shown (by agarose gel electrophoresis) to be identical to pBk8 (Fig. 1, lane 3). The frequency of the plasmid transfer from Bk8 to Bk8M ranged from 10^{-4} to 10^{-3} per donor cell. This provides additional evidence that pBk8 functionally mediates cadmium and mercury resistance. One of the transconjugants was designated as Bk8 TC.

Table 3. Minimal inhibitory concentrations (MIC) of heavy metals for wild-type strain Bk8 and its derivatives.

Phenotype	Wild-type strain Bk8	Mutant strain Bk8M	Transconjugant strain Bk8TC
Cd^{2+}	1 mM	0.3 mM	1 mM
Zn^{2+}	2 mM	2 mM	2 mM
Ni^{2+}	1 mM	1 mM	1 mM
Hg^{2+}	45 μM	3 μM	45 μM

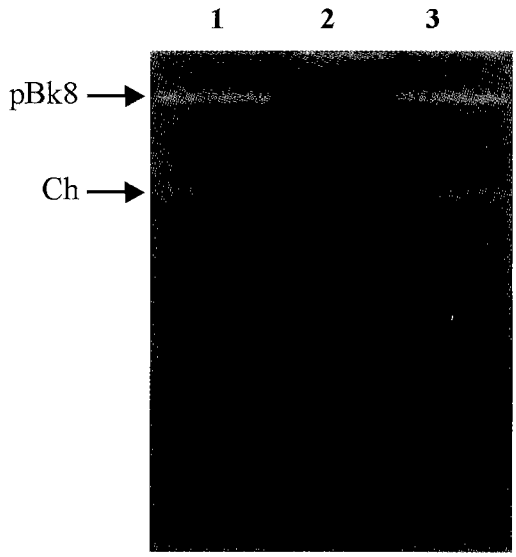

Fig. 1. Plasmid composition of strain Bk8 and its derivatives. Lanes: 1, wild-type strain Bk8; 2, Bk8M mutant from mitomycin-C-treated culture; 3, Bk8TC transconjugant obtained by mating of Bk8 and Bk8M, ch; chromosomal DNA.

Induction of Heavy Metal Resistance of Strain Bk8

Whether the cadmium, nickel, mercury, or zinc resistance property in strain Bk8 is inducible or constitutive was determined by examining the growth curves. The cells were grown in the liquid Tris-glucose medium (NEM in the case of $HgCl_2$) containing a sub-inhibitory concentration (0.8 mM Cd^{2+}, 1 mM Zn^{2+}, or 0.8 mM Ni^{2+}) of heavy metals. The media were inoculated with cells grown with or without 0.1 mM Cd^{2+}, 0.2 mM Zn^{2+}, or 0.1 mM Ni^{2+}. Figure 2 shows that, in the case of cadmium, zinc, and nickel, the induced and uninduced cells passed a lag period of 8 h indicating that the nickel, cadmium, and zinc resistance in strain Bk8 is constitutively expressed. In contrast, the cells

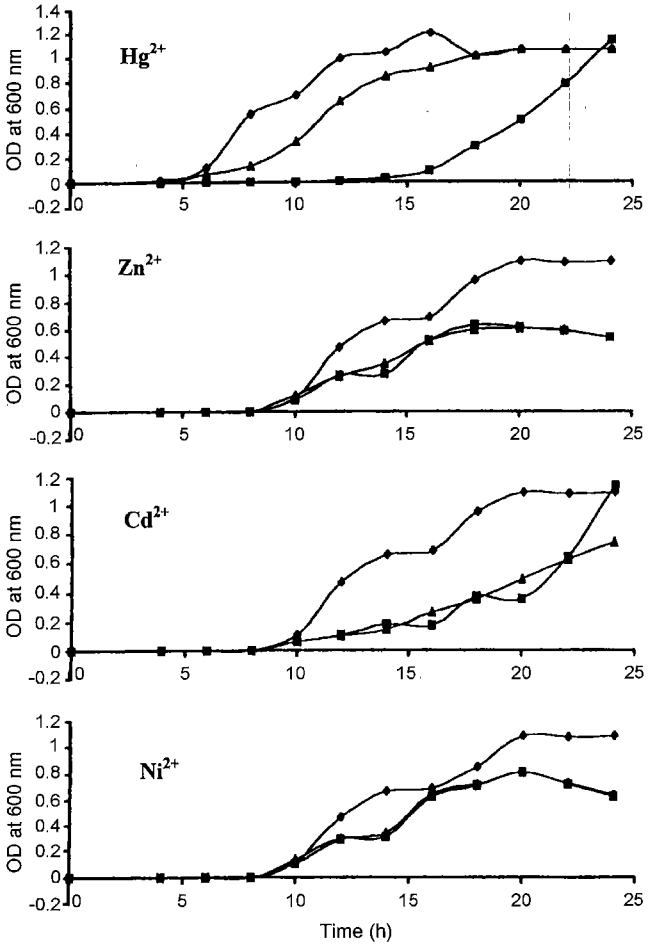

Fig. 2. Growth of *Pseudomonas* sp. strain Bk8 in the presence of 0.8 mM $CdCl_2$, 1 mM $ZnCl_2$, 0.8 mM $NiCl_2$, or 40 μM $HgCl_2$. Cells were inoculated into a Tris-glucose medium (NEM in the case of $HgCl_2$). Symbols: (◆) no metal; (■) uninduced cells; (▲) induced cells. Cells were induced by overnight growth in the presence of 0.1 mM Cd^{2+}, 0.2 mM Zn^{2+}, 0.1 mM Ni^{2+}, or 2 μM Hg^{2+}.

of this strain pre-grown in the presence of 2 μM $HgCl_2$ started to grow after a shorter lag phase on 40 μM $HgCl_2$ than the uninduced cells (Fig. 2). This suggests that the mercury resistance is inducibly expressed in strain Bk8. Similar observations have been reported by Barbieri *et al.* [1] in which mercury resistance genes were induced in the aromatic compound degrader strains.

In conclusion, the study of bacterial resistance to heavy metals, especially when associated with xenobiotic degradation activities, has been increased since both metals and organic compounds pollution are of environmental concern in industrial areas. The ability of the strain Bk8 to utilize diuron as the sole carbon and energy sources in the presence of toxic metals suggests that bioremedation can be carried out even in the presence of toxic metals. Bacteria accomplish this feat through harboring plasmids which are small, mobile pieces of DNA that carry necessary genetic information, such as metal resistance and the ability to degrade xenobiotic compounds. Because a plasmid can be transferred from one bacterium to another, and even to bacteria of different species, there is a potential to create efficient biodegrading microbial communities, suited to unique environments and removal of a particular hazardous waste.

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