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# Construction and Characterization of Multiple Heavy Metal-Resistant Phenol-Degrading Pseudomonads Strains

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**Abstract** Metal ions contamination may inhibit microorganisms involved in the biodegradation of organic compounds and affect biodegradation rates. Therefore, it is likely that bioremediation of xenobiotics-contaminated soils and waste will require inoculation with efficient biodegrading microbial communities, with capabilities of being resistant to heavy metals as well. Two different transconjugants (*Pseudomonas* sp. KM12TC and *P. aeruginosa* TC) were constructed by conjugation experiments. Results on MIC, induction and growth inhibition strongly indicated that arsenic-resistant plasmid, pKM20, could be mobilized, and the newly acquired phenotype of pKM20 was not only expressed but also well regulated, resulting in newly acquired resistances to $As^{5+}$, $As^{3+}$, and $Sb^{3+}$ in addition to $Cd^{2+}$, $Zn^{2+}$, and $Hg^{2+}$. The phenol-degradation efficiencies of *Pseudomonas* sp. KM12TC were maintained significantly even at high heavy metal concentrations at which these efficiencies of *P. aeruginosa* TC were completely impaired. The results in this study on the effects of heavy metals on phenol degradation, especially after conjugation, are the first ever reported. All the results described in this study encourage to establish a goal of making "designer biocatalysts" which could degrade certain xenobiotics in the area contaminated with multiple heavy metals.

**Key words:** *Pseudomonas* sp. KM12, conjugation, heavy metal resistance, phenol degradation

Volatile organic compounds (VOC) such as phenol have been found in many sites of environmental concern. For cleaning up such sites microorganisms can provide an effective alternative bioremediation, since they are extremely adaptable and able to degrade numerous organic compounds. However, heavy metals often occur as cocontaminants and are toxic to microorganisms. Arsenic [4] is found at 1,014 of the 1,598 National Priority List sites identified by the United States Environmental Protection Agency (U.S. EPA), nickel [2] at 706 of the 1,430 sites, and cadmium [3], mercury [3], lead [3], and zinc [1] in at least 776, 714, 801, and 1,026 of the 1,467 sites, respectively. Ren and Frymier [18] determined the inhibition coefficients (*K*i) of seven metals and observed the 50% bioluminescent repression of bioluminescent bacteria (*Vibrio fisheri* Shk1) at 0.0045 mM $Hg^{2+}$, 0.017 mM $Cd^{2+}$, 0.018 mM $Pb^{2+}$, 0.029 mM $Zn^{2+}$, 0.15 mM $Cu^{2+}$, 0.75 mM $Cr^{6+}$, and 1.64 mM $Ni^{2+}$. It was indicated that Hg was the most toxic and Cd, Pb, and Zn were very toxic compared to the other heavy metals. Falih [10] examined the heavy metal-tolerance of white-root fungus *Phanerochaete chrysosporium* capable of degrading a wide range of pollutants, and reported a large reduction in growth yield at 400 μg/ml (the yield percentages were 33, 35, 30, 27, 18, and 31% in media containing Cd, Cu, Pb, Mn, Ni, and Co, respectively). Therefore, it is likely that bioremediation of xenobiotics-contaminated soils and waste will require inoculation with efficient biodegrading microbial communities which are resistant to heavy metals [8].

Hassen *et al.* [14] found that the dominating bacterial isolates from wastewater, solid waste, and sewage sludge belonged to the genera *Pseudomonas* and *Proteus*. Among the isolates, *P. aeruginosa* was selected as the most important isolate and a bioindicator of toxicity. Pseudomonads, common soil bacteria, have the abilities to utilize many toxic compounds as sources of nutrients by converting them into intermediates of conventional metabolic pathways [6, 7, 19, 21], and also have the ability to be resistant to various heavy metals [9, 15, 22]. Since many heavy metal resistances and the xenobiotic metabolism in bacteria are known to be conferred by plasmids [9, 15, 22, 23], by natural conjugation it may be feasible to construct a new bacteria strain, by which certain desirable heavy metal determinants and biodegradation pathways from different organisms are brought together in a single host with the

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aim of performing specific reactions [13, 17]. However, little has been reported on the construction of multiple heavy metal-resistant biodegradative bacteria through conjugation or on the effect of heavy metals on phenol biodegradation, especially after conjugation.

For this reason, an attempt was made to create efficient phenol-degrading pseudomonads strains in the presence of toxic heavy metals such as $AsO_4^{3-}$, $AsO_2^-$, $SbO_2^-$, $Cd^{2+}$, $Zn^{2+}$, and $Hg^{2+}$. Two different transconjugants (*Pseudomonas* sp. KM12TC and *P. aeruginosa* TC) were constructed by conjugation experiments. In the present report, MIC results, induction results, and growth inhibition results are presented, and the phenol degradation efficiencies of *Pseudomonas* sp. KM12TC and *P. aeruginosa* TC are compared and discussed. These results on the effects of heavy metals on phenol degradation, especially after conjugation, are the first ever reported. The results described in this study strongly suggest that "a designer bacteria" which could degrade certain xenobiotics in the area contaminated with multiple heavy metals could be prepared by conjugation, through horizontal gene transfer.

**Construction of Heavy Metal-Resistant Phenol-Degrading Pseudomonads Strains by Conjugation of Plasmid**

Natural conjugation was exploited to construct a new bacteria strain which is capable of degrading phenol and retains a capability to resist many toxic heavy metals such as $AsO_4^{3-}$, $AsO_2^-$, $SbO_2^-$, $Cd^{2+}$, $Zn^{2+}$, and $Hg^{2+}$. All the bacteria used in this experiment are described in Table 1. *Pseudomonas* sp. KM10 is resistant to both cadmium and mercury, and also capable of using phenol as the sole carbon and energy source and harbors a plasmid named as pKM10 which has genes necessary for phenol degradation [21]. A recently isolated bacteria, *Pseudomonas* sp. KM20, is resistant to arsenate, arsenite, and antimonite and harbors a plasmid named as pKM20 which confers the arsenate-, arsenite-, and antimonite-resistance [22]. The rifampicin-resistant mutant (recipient, $rif^r$) of *Pseudomonas* sp. KM10 was prepared as described elsewhere [11]. Several rifampicin-resistant mutant colonies were selected from *Pseudomonas* sp. KM10 and restreaked onto the basal medium agar [21] containing 25 μg/ml rifampicin, 1 g/l phenol, 1.5 mM $CdCl_2$, and 25 μM $HgCl_2$, and incubated again at 28°C for 2 to 3 days to ensure that both the ability to degrade phenol and the heavy metal-resistant determinants had not been compromised by resistance to rifampicin. A fast-growing colony was obtained, named as *Pseudomonas* sp. KM10R, and used as a rifampicin-resistant recipient of pKM20 in the conjugation experiment.

As pointed out by Hassen *et al.* [14] who selected *P. aeruginosa* as the most important isolate and a bioindicator of toxicity among the isolates from various wastes, in this study *P. aeruginosa*, which belongs to the same genera as *Pseudomonas* sp. KM10 and *Pseudomonas* sp. KM20, was prepared in order to harbor pKM10 and served as a useful control strain which was expected to be heavy metal-sensitive and phenol-degradative, instead of introducing nonsense mutations at cadmium- and mercury-resistant chromosomal determinants of *Pseudomonas* sp. KM10. Cadmium- and mercury-resistant determinants are among the best known heavy metal-resistant determinants [15, 23], therefore, it was of interest to have *P. aeruginosa* harboring pKM10 as a control bioindicator of toxicity. In order to construct *P. aeruginosa* harboring pKM10, the rifampicin-resistant mutant (recipient, $rif^r$) of *P. aeruginosa* (ATCC10145) was first prepared in the same way as described above, except using the *P. aeruginosa* instead of *Pseudomonas* sp. KM10. The rifampicin-resistant mutant was named as *P. aeruginosa* R, and used as a rifampicin-resistant recipient of pKM10 in the conjugation experiment.

The mobilization of pKM20 plasmid from *Pseudomonas* sp. KM20 (donor) into *Pseudomonas* sp. KM10R (recipient) was carried out by conjugation on membrane filters as described elsewhere [11]. Transconjugants were selected by plating dilutions of the mating mixture onto the selective basal medium agar [21] containing 25 μg/ml rifampicin, 1 g/l phenol, 1.5 mM $CdCl_2$, 25 μM $HgCl_2$, and 5 mM arsenate ($Na_2HAsO_4$), and incubated further at 28°C

**Table 1.** Bacterial strains, plasmid, and phenotypes.

| Strains | Plasmid | Phenotype Chromosomal/Plasmid-encoded | Source |
|---|---|---|---|
| *Pseudomonas* sp. KM10 | pKM10 | $rif^s\ Cd^r\ Zn^r\ Hg^r/phenol^+$ | 21 |
| *Pseudomonas* sp. KM20 | pKM20 | $rif^s/As^r$ | 22 |
| *Pseudomonas* sp. KM10R | pKM10 | $rif^r\ Cd^r\ Zn^r\ Hg^r/phenol^+$ | This study |
| *Pseudomonas* sp. KM12TC | pKM10, pKM20 | $rif^r\ Cd^r\ Zn^r\ Hg^r/phenol^+\ As^r$ | This study |
| *Pseudomonas* sp. KM12TCC | –[a] | $rif^r\ Cd^r\ Zn^r\ Hg^r/-$[b] | This study |
| *P. aeruginosa* (ATCC10145) | – | $rif^s/-$ | ATCC |
| *P. aeruginosa* R | – | $rif^r/-$ | This study |
| *P. aeruginosa* TC | pKM10 | $rif^r/phenol^+$ | This study |
| *P. aeruginosa* TCC | – | $rif^r/-$ | This study |

–[a], no plasmid present; –[b], no plasmid-encoded phenotype; $phenol^+$, phenol degradation; $rif^r$, rifampicin resistant; $rif^s$, rifampicin sensitive.

for 2 days. The transconjugants obtained were designated as *Pseudomonas* sp. KM12TC (Table 1). The mobilization of pKM10 plasmid from *Pseudomonas* sp. KM10 (donor) into *P. aeruginosa* R (recipient) was carried out in the same way as described above. Transconjugants were selected by plating dilutions of the mating mixture onto the selective basal medium agar containing 25 μg/ml rifampicin, 1 g/l phenol and incubated further at 28°C for 2 days. The transconjugants obtained were designated as *P. aeruginosa* TC (Table 1).

Plasmid-curing of the *Pseudomonas* sp. KM12TC was carried out by treatment with mitomycin C at a concentration of 10 μg/ml as described elsewhere [11]. After standard mitomycin treatment, colonies which could grow on LB agar plate but could neither grow on LB agar containing arsenate nor on basal medium agar plates containing 1 g/l phenol were selected and named as *Pseudomonas* sp. KM12TCC (The last "C" stood for a cured strain, Table 1). Plasmid-curing of the *P. aeruginosa* TC was carried out in the same way as described above except using basal medium agar plates containing 1 g/l phenol for negative selection of a cured strain, and the selected strain was named as *P. aeruginosa* TCC (Table 1).

The electrophoresis on 0.8% agarose gel revealed that *Pseudomonas* sp. KM12TC carried two kinds of plasmid: The larger and the smaller plasmids were identified as pKM10 and pKM20, respectively, since the size of the larger plasmid was identical to that of pKM10 and the size of the smaller plasmid was identical to that of pKM20 (data not shown). The presence or the absence of plasmids in *Pseudomonas* sp. KM12TCC, *P. aeruginosa* TC, and *P. aeruginosa* TCC were confirmed in the same manner.

### Determinations of the Minimal Inhibitory Concentrations (MICs)

To examine successful phenotype expression of the mobilized plasmid pKM20, the minimal inhibitory concentrations were determined as follows. The overnight cultures were diluted 100-fold in LB media and the diluted cultures were grown for 3 h, then the cultures were diluted 100-fold again. Three μl of newly diluted cultures were placed on the LB plates containing $CdCl_2$ (0, 0.5, 1, 2.5, or 5 mM), $ZnCl_2$ (0, 0.5, 1, 2.5, or 5 mM), $HgCl_2$ (0, 0.01, 0.025, 0.05, 0.1, or 0.25 mM), $Na_2HAsO_4$ (arsenate; 0, 1.0, 2.5, 5, 10, or 25 mM), $NaAsO_2$ (arsenite; 0, 1.0, 2.5, 5, 10, or 25 mM), or $C_4H_4KO_7Sb$ (antimonite; 0, 0.05, 0.1, 0.25, 0.5, or 1 mM). After incubation for 20 h at 28°C, the lowest concentration of heavy metal that completely inhibited growth on LB agar plates was determined.

Colonies of *Pseudomonas* sp. KM10 could be seen clearly on LB agar plate containing 2.5 mM arsenate, 1 mM arsenite, or 0.1 mM antimonite, but could not be observed on the agar plates containing 5 mM arsenate, 2.5 mM arsenite, or 0.25 mM antimonite (Table 2). Colonies of *Pseudomonas* sp. KM12TC harboring additional pKM20 could grow on LB agar plate containing as high as 12.5 mM arsenate, 12.5 mM arsenite, or 0.5 mM antimonite, but were not visible on LB plates containing 25 mM arsenate, 25 mM arsenite, or 1 mM antimonite (Table 2). This result indicated that *Pseudomonas* sp. KM12TC became highly resistant to arsenate, arsenite, and antimonite as compared with *Pseudomonas* sp. KM10. When *Pseudomonas* sp. KM12TCC was examined, arsenic resistance of the microorganism was lost and its MICs were the same as that of arsenic sensitive *Pseudomonas* sp. KM10 (Table 2). These results strongly suggested that the presence of the mobilized pKM20 resulted in arsenic resistance of *Pseudomonas* sp. KM12TC and the phenotype of pKM20 was well expressed. In addition, these results showed that both pKM10 and pKM20 could be self-mobilized and maintained in a single host (i.e. *Pseudomonas* sp. KM12TC).

**Table 2.** Minimal inhibitory concentrations (MIC) on plates.

| Strains | MIC (mM) of heavy metals | | | | | |
|---|---|---|---|---|---|---|
| | $Cd^{2+}$ | $Zn^{2+}$ | $Hg^{2+}$ | $As^{5+}$ | $As^{3+}$ | $Sb^{3+}$ |
| *Pseudomonas* sp. KM10 | 5 | 5 | 0.25 | 5 | 2.5 | 0.25 |
| *Pseudomonas* sp. KM12TC | 5 | 5 | 0.25 | 25 | 25 | 1 |
| *Pseudomonas* sp. KM12TCC | 5 | 5 | 0.25 | 5 | 2.5 | 0.25 |

An additional heavy metal resistance (zinc resistance) was revealed in this experiment. *Staphylococcus aureus* plasmid pI258 cadmium resistance operon (*cadA*) showed zinc resistance [23], although it is not unusual for a cadmium resistance determinant to also confer zinc resistance. The cadmium-resistant chromosomal determinant of *Pseudomonas* sp. KM10 was confirmed to have zinc resistance as well. Hassen *et al.* [14] have reported that the MICs of the isolated *P. aeruginosa* in nutrient broth were 1.5 mM $Cd^{2+}$, 1.5 mM $Zn^{2+}$, 0.08 mM $Hg^{2+}$, 1.2 mM $Cu^{2+}$, 1.2 mM $Cr^{3+}$, and 0.4 mM $Co^{2+}$. In this study, the MICs of *P. aeruginosa* TC were 1.5 mM $Cd^{2+}$, 1.5 mM $Zn^{2+}$, 0.1 mM $Hg^{2+}$, 5 mM $As^{5+}$, 2.5 mM $As^{3+}$, and 0.25 mM $Sb^{3+}$. The MICs of $Cd^{2+}$, $Zn^{2+}$, and $Hg^{2+}$ presented in this study were in good agreement with those reported by Hassen *et al.* [14], although there was a slight difference in the level of inhibition, which would be affected by multiple factors, such as concentration and nature of the heavy metals, media, and microbial species [5]. Both studies also confirmed that mercury is the most toxic heavy metal.

All these MICs results suggested that it might be possible to construct by plasmid conjugation "a designer bacteria" which could degrade certain xenobiotics in areas contaminated with multiple heavy metals.

### Induction of Arsenic, Cadmium (and Zinc), and Mercury Resistances of *Pseudomonas* sp. KM12TC

Since other arsenic, cadmium (and zinc), and mercury resistance systems are inducible [15, 23], it was interesting

to examine the inducibility of heavy metal resistances of *Pseudomonas* sp. KM12TC. For the induction of arsenate, arsenite, and antimonite resistance, the overnight culture of *Pseudomonas* sp. KM12TC was diluted 100-fold into fresh LB medium, pre-incubated at 28°C for 2 h, and the pre-incubated cells were then induced with a subinhibitory concentration of 0.1 mM arsenate for 1 h, and subsequently challenged with 5 mM arsenate, 5 mM arsenite, or 0.3 mM of antimonite, respectively. For the induction of cadmium (and zinc) resistance [23], the pre-incubated cells were induced for 1 h with 30 μM $CdCl_2$ and subsequently challenged with 2.5 mM $ZnCl_2$. For the induction of mercury resistance, the pre-incubated cells were induced with 3 μM $HgCl_2$ for 1 h and subsequently challenged with 70 μM $HgCl_2$. Growth was measured by absorbance at 600 nm using a UV-vis spectrophotometer (Biochrome 4060, Pharmacia Biotech, U.S.A.). For the uninduced cells, cells were grown for 1 h in the absence of any heavy metal ions and subsequently used for challenge in the same procedures as described above.

Figure 1 shows that uninduced cells failed to grow immediately after the addition of an inhibitory concentration of heavy metal ions, and began to grow slowly after 4 to 5 h, because of slow accumulation of enough resistance to the corresponding heavy metals. Such lag period was not observed in the growth of the induced cells (Fig. 1). Cells induced with subinhibitory concentrations of corresponding heavy metal cations grew almost as well in the presence of the inhibitory concentration as in its absence. One-tenth mM arsenate was a strong inducer of the arsenic resistance determinant of pKM20 which conferred resistances primarily to arsenate (Fig. 1C), arsenite (Fig. 1D), and antimonite (Fig. 1E) in *Pseudomonas* sp. KM12TC. Since *Pseudomonas* sp. KM12TC acquired pKM20 by transconjugation, this result suggested that the newly acquired phenotype of pKM20 was not only expressed but also well regulated.

### Growth Inhibition of *Pseudomonas* sp. KM12TC, KM12TCC, and *P. aeruginosa* TC by Heavy Metal Cations

Growth inhibition experiments were carried out in liquid LB media containing increasing amounts of $As^{5+}$, $As^{3+}$, $Sb^{3+}$, $Cd^{2+}$, $Zn^{2+}$, or $Hg^{2+}$ to compare the heavy metal resistances of *Pseudomonas* sp. KM12TC, KM12TCC, and *P. aeruginosa* TC. Thus, the overnight cultures in LB were diluted 100-fold in 5 ml LB medium supplemented with 0–5 mM ($CdCl_2$, $ZnCl_2$, arsenate, or arsenite), or 0–100 μM ($HgCl_2$ or antimonite) of heavy metals, respectively. The diluted cultures were subsequently incubated in a rotary shaker (150 rpm) for 13 h at 28°C. Turbidity ($A_{600}$)

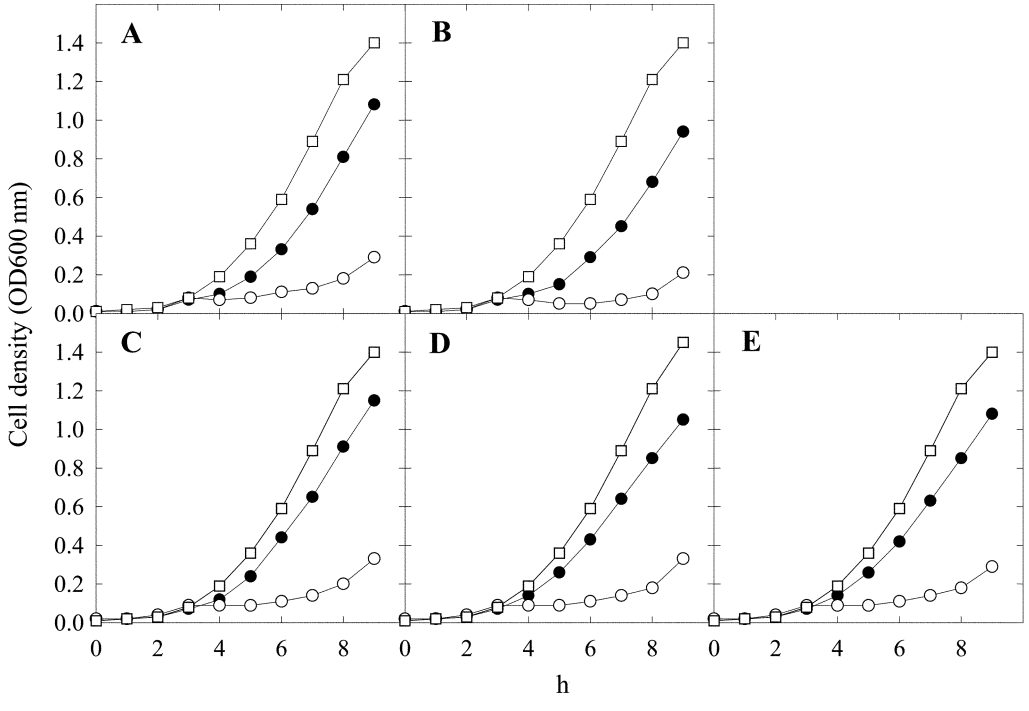


**Fig. 1.** Induction of heavy metal resistances of *Pseudomonas* sp. KM12TC.
For induction of each heavy metal resistance, the overnight culture of each bacteria was diluted 100-fold into fresh LB medium and incubated at 28°C for 2 h and then induced for 1 h with (A, ●), 30 μM $CdCl_2$; (B, ●), 3 μM $HgCl_2$; (C, D, and E, ●), 0.1 mM arsenate and subsequently challenged with (A, and ●), 2.5 mM $ZnCl_2$; (B, ● and ○), 70 μM $HgCl_2$; (C, ● and ○), 5 mM arsenate; (D, ● and ○), 5 mM arsenite; or (E, ● and ○), 0.3 mM antimonite. Growth was measured by the absorbance at 600 nm using a UV-vis spectrophotometer (Biochrome 4060, Pharmacia Biotech, USA). Symbols used in common: ○, uninduced; □, uninduced and unchallenged control.

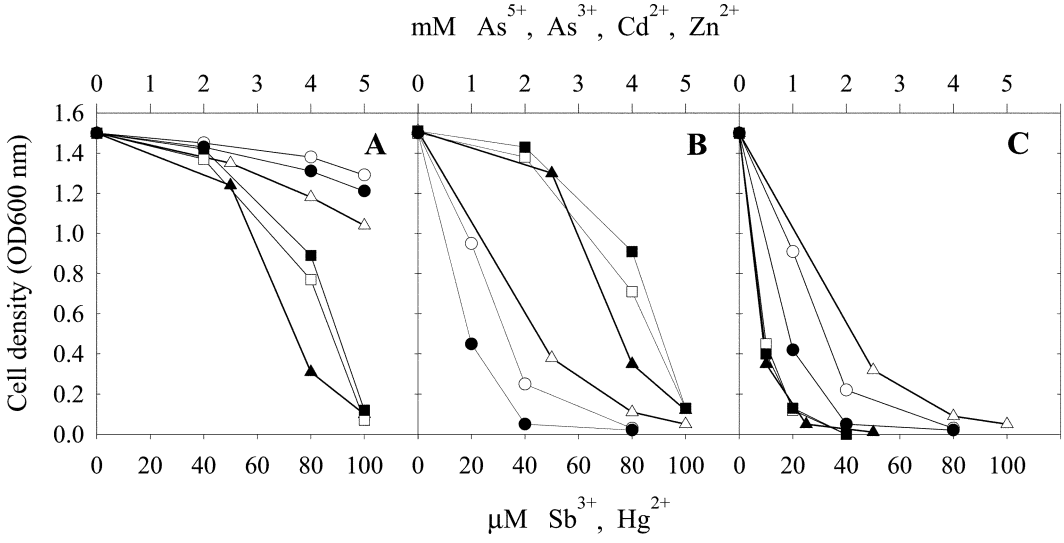


**Fig. 2.** Growth inhibition of *Pseudomonas* sp. KM12TC, KM12TCC, and *P. aeruginosa* TC by heavy metal cations.
Overnight cultures of each *Pseudomonas* strain were diluted 100-fold into fresh LB broth containing increasing amounts of heavy metals and grown at 28°C for 13 h. $A_{600}$ was measured by UV-vis spectrophotometer. Symbols: ○, arsenate ($As^{5+}$); ●, arsenite ($As^{3+}$); □, $CdCl_2$; ■, $ZnCl_2$; △, antimonite ($Sb^{3+}$); ▲, $HgCl_2$. Other symbols: A, *Pseudomonas* sp. KM12TC; B, KM12TCC; C, *P. aeruginosa* TC.

was measured using a UV-vis spectrophotometer (Biochrome 4060, Pharmacia Biotech, U.S.A.).

*Pseudomonas* sp. KM12TC cells were grown well in 4 mM $As^{5+}$, 4 mM $As^{3+}$, 80 μM $Sb^{3+}$, 2 mM $Cd^{2+}$, 2 mM $Zn^{2+}$, or 50 μM $Hg^{2+}$ (Fig. 2A). Cadmium, zinc, and mercury resistance levels of *Pseudomonas* sp. KM12TCC were similar to these of *Pseudomonas* sp. KM12TC (Figs. 2B and 2A), because of the presence of these resistance genes on chromosome [21]. But, in 1 mM $As^{5+}$, 1 mM $As^{3+}$, or 50 μM $Sb^{3+}$, the growth of *Pseudomonas* sp. KM12TCC cells were significantly retarded (Fig. 2B). The loss of arsenic resistance of *Pseudomonas* sp. KM12TCC was due to the loss of pKM20 by the plasmid-curing experiment. The presence of pKM20 in *Pseudomonas* sp. KM12TC was enough to confer full arsenical and antimonial resistance. On the other hand, heavy metal-sensitive *P. aeruginosa* TC showed very poor growth even in a low concentration of heavy metal ions, as was expected (Fig. 2C). The inhibited growth patterns of *P. aeruginosa* TC in Fig. 2C were in good accordance with both the MIC results of *P. aeruginosa* and 26, 3, or 0% growth of the same bacteria in 10, 70, or 100 μM $Hg^{2+}$, respectively, as described by Hassen *et al.* [14], although slightly higher growth was obviously achieved due to longer incubation (48 h).

Results on MIC induction and growth inhibition strongly indicated that pKM20 could be mobilized and expressed in *Pseudomonas* sp. KM10, resulting in newly acquired resistances to $As^{5+}$, $As^{3+}$, and $Sb^{3+}$ in addition to $Cd^{2+}$, $Zn^{2+}$, and $Hg^{2+}$. The ability of both arsenic resistance (pKM20) and phenol utilization (pKM10) to coexist in a single cell provides evidence that the two plasmids might belong to different incompatibility groups.

**Effect of Heavy Metal Toxicity on Phenol Degradation**

The inhibitory effects of metals on the aerobic microbial degradation of the organic chemical, phenol, was examined under a wide range of various metal ion concentrations. Thus, the overnight cultures of heavy metal-resistant *Pseudomonas* sp. KM12TC and heavy metal-sensitive *P. aeruginosa* TC were grown in the basal medium containing 1.0 g/l phenol and increasing amounts of various heavy metal ions such as $As^{5+}$, $As^{3+}$, $Sb^{3+}$, $Cd^{2+}$, $Zn^{2+}$, or $Hg^{2+}$. The phenol degradation efficiency was determined as described elsewhere [21]. In the absence of toxic heavy metals, both bacteria efficiently removed over 90% of 1 g/l phenol within 30 h (Figs. 3A and 3B). The phenol degradation efficiencies of *P. aeruginosa* TC were severely inhibited to 16% in 1 mM $Cd^{2+}$ and to 28% in 1 mM $Zn^{2+}$, and completely inhibited in 2.5 mM $Cd^{2+}$ or $Zn^{2+}$ (Fig. 3B). Similar inhibition patterns of biodegradation by heavy metals have been reported. Amor *et al.* [5] also observed a 55% reduction in the rate of toluene biodegradation for *Bacillus* sp. in either 0.43 mM $Zn^{2+}$ or 0.43 mM $Cd^{2+}$, and almost 100% reduction in 2.5 mM $Cd^{2+}$. In contrast, the phenol-degradation efficiencies of *Pseudomonas* sp. KM12TC were maintained up to 33% in 2.5 mM $Cd^{2+}$ and 43% in 2.5 mM $Zn^{2+}$ (Fig. 3A) due to the cadmium and zinc resistance. The *Pseudomonas* sp. KM12TC could also tolerate a mercury concentration of 0.025 mM with 82% degradation efficiency (Fig. 3A), but the efficiency of *P. aeruginosa* TC was completely inhibited in as low as 0.01 mM mercury concentration (Fig. 3B). Phenol degradation efficiency was most sensitive to mercury. Hassen *et al.* [14] also showed that mercury was the most toxic component for all isolated bacteria, and in the range of

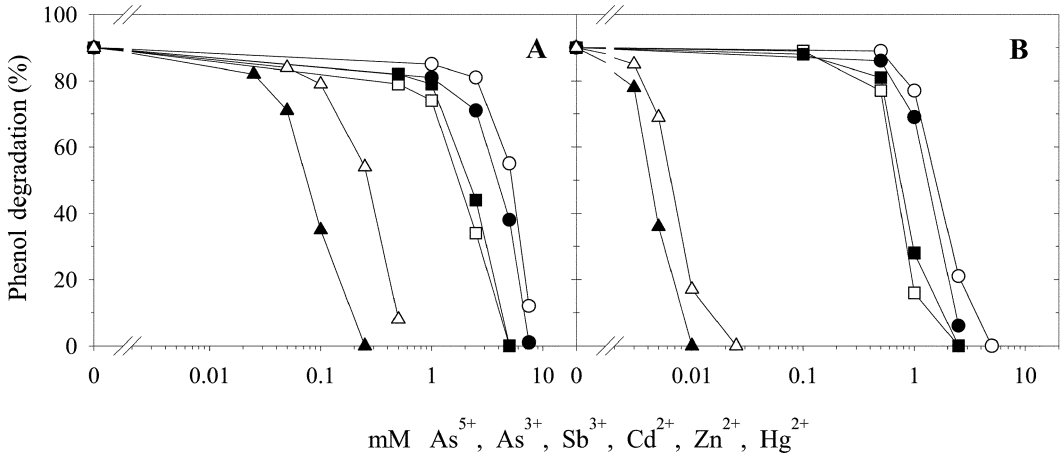


**Fig. 3.** Effect of heavy metals on degradation of phenol by *Pseudomonas* sp. KM12TC and *P. aeruginosa* TC.
Overnight cultures of each *Pseudomonas* strain were diluted 100-fold into the basal medium containing phenol (1.0 g/l) and increased amounts of various heavy metal ions and grown at 28°C for 30 h. Phenol-degradation efficiency was determined as described elsewhere [19]. Symbols: ○, arsenate ($As^{5+}$); ●, arsenite ($As^{3+}$); □, $CdCl_2$; ■, $ZnCl_2$; △, antimonite ($Sb^{3+}$); ▲, $HgCl_2$. Other symbols: A, *Pseudomonas* sp. KM12TC; B, *P. aeruginosa* TC.

0.01 to 0.1 mM of mercury, bacterial growth inhibition varied between 50 and 100% in nutrient broth after incubation at 37°C for 40 h. The *Pseudomonas* sp. KM12TC still retained about 55% efficiency in 5 mM arsenate and 38% in 5 mM arsenite, and 84% in 0.05 mM antimonite (Fig. 3A). However, *P. aeruginosa* TC failed to degrade phenol at the same concentration (Fig. 3B).

Figure 3B clearly shows that even very low concentrations of some metals can severely retard biodegradation rates of phenol. Leighton and Foster [16] reported that all four heavy metals (Pb, Zn, Cu, Ni) at 3 mM caused more than 50% reduction in the methane production of a methanogenic sludge reactor. Among the four heavy metals, lead was shown to have the greatest effect on biogas production. This information will help to understand environmental situations in which biodegradation is unexpectedly low, such as at land disposal sites where metals and metal-containing chemicals are disposed of together with organic matter [8, 12]. Toxic heavy metals exert their toxicity by binding to thiol-containing groups and oxygen sites, which cause improper conformation of nucleic acids and proteins, resulting in impaired oxidative phosphorylation and osmotic balance [20].

Figure 3A suggests a possibility that horizontal gene transfer in indigenous microbial populations would provide a potential for resistance to toxic compounds in bioremediation systems. But, MacDonald *et al.* [17] reported that the rates of plasmid transfer in ecosystems of environmental interest (for example, activated sludge) are low, due to minimal cellular energy levels resulted from operation under substrate-limited conditions. Recently, Hallier-Soulier *et al.* [13] isolated *Pseudomonas putida* harboring a large plasmid (pMX) containing *xyl* genes, which are involved in toluene or meta-xylene degradation pathways via an alkyl monooxygenase and a catechol 2,3-dioxygenase, and indeed showed that, when the pMX was transferred by conjugation to *Pseudomonas putida* F1, the catabolic potential of transconjugant *Pseudomonas putida* F1 acquired a more relaxed substrate specificity than those of parental cells, resulting in growth on benzene, toluene, meta-xylene, para-xylene, and ethylbenzene compounds as the sole carbon sources.

Both Hallier-Soulier *et al*'s study on a self-transferable plasmid (pMX) of *Pseudomonas putida* and this current study on a self-transferable plasmid pKM20 suggest that a low transfer rate could be circumvented by constructing microorganisms, which could degrade phenol in the much more hostile conditions created by toxic heavy metals, in a controlled environment (e.g. conjugation experiments carried out in a research laboratory). All the results described in this study mark encouraging progress towards the goal of making "designer biocatalysts". To accomplish this goal, it would be necessary to bring together new heavy metal-resistant determinants (e.g. Pb, Cu) and desirable biodegradation pathways (e.g. PCBs, TPH, PAHs) in a single cell and to perform field tests with the cell. Such work is currently underway.

## REFERENCES

1. Agency for Toxic Substances and Disease Registry (ATSDR). 1994. *Toxicology Profile for Zinc*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service, U.S.A.
2. Agency for Toxic Substances and Disease Registry (ATSDR). 1997. *Toxicology Profile for Nickel* (updated). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service, U.S.A.

3. Agency for Toxic Substances and Disease Registry (ATSDR). 1999. *Toxicology Profile for Cadmium, Mercury, Lead*. Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service, U.S.A.
4. Agency for Toxic Substances and Disease Registry (ATSDR). 2000. *Toxicology Profile for Arsenic* (updated). Atlanta, GA: U.S. Department of Health and Human Services, Public Health Service, U.S.A.
5. Amor, L., C. Kennes, and M. C. Veiga. 2001. Kinetics of inhibition in the biodegradation of monoaromatic hydrocarbons in presence of heavy metals. *Bioresour. Technol.* **78:** 181–185.
6. Bandhyopadhyay, K., D. Das, and B. R. Maiti. 1999. Solid matrix characterization of immobilized *Pseudomonas putida* MTCC 1194 used for phenol degradation. *Appl. Microbiol. Biotechnol.* **51:** 891–895.
7. Bruins, M. R., S. Kapil, and F. W. Oehme. 2000. *Pseudomonas pickettii*: A common soil and groundwater aerobic bacteria with pathogenic and biodegradation properties. *Ecotoxicol. Environ. Safety* **47:** 105–111.
8. Dua, M., A. Singh, N. Sethunathan, and A. K. Johri. 2002. Biotechnology and bioremediation: Successes and limitations. *Appl. Microbiol. Biotechnol.* **59:** 143–152.
9. El-deeb, B. A. 2001. Catabolic plasmid-mediated heavy metal resistance in herbicide diuron-degrading *Pseudomonas* species. *J. Microbiol. Biotechnol*. **11:** 7–12.
10. Falih, A. M. 1997. Influence of heavy-metals toxicity on the growth of *Phanerochaete chrysosporium*. *Bioresour. Technol.* **60:** 87–90.
11. Gerhardt, P., R. G. E. Murray, W. A. Wood, and N. R. Krieg. 1994. *Methods for General and Molecular Bacteriology*, American Society for Microbiology, Washington, DC, U.S.A.
12. Groudeva, V. I., S. N. Groudev, and A. S. Doycheva. 2001. Bioremediation of waters contaminated with crude oil and toxic heavy metals. *Int. J. Mineral Processing* **62:** 293–299.
13. Hallier-Soulier, S., V. Ducrocq, and N. Truffaut. 1999. Conjugal transfer of a TOL-like plasmid and extension of the catabolic potential of *Pseudomonas putida* F1. *Can. J. Microbiol.* **45:** 898–904.
14. Hassen, A., N. Saidi, M. Cherif, and A. Boudabous. 1998. Resistance of environmental bacteria heavy metals. *Bioresour. Technol*. **64:** 7–15.
15. Ji, G. and S. Silver. 1995. Bacterial resistance mechanism for heavy metals of environmental concern. *J. Ind. Microbiol*. **14:** 61–75.
16. Leighton, I. R. and C. F. Foster. 1998. The effect of heavy metals on a thermophilic methanogenic upflow sludge blanket reactor. *Bioresour. Technol.* **63:** 131–137.
17. MacDonald, J. A., B. F. Smets, and B. E. Rittmann. 1992. The effects of energy availability on the conjugative-transfer kinetics of plasmid RP4. *Water Res.* **26:** 461–466.
18. Ren, S. and P. D. Frymier. 2003. Kinetics of the toxicity of metals to luminescent bacteria. *Adv. Environ. Res.* **7:** 537–547.
19. Shim, H. and S.-T. Yang. 1999. Biodegradation of benzene, toluene, ethylbenzene, and o-xylene by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* immobilized in a fibrous-bed bioreactor. *J. Biotechnol.* **67:** 99–112.
20. Webster, E. A., A. J. Murphy, J. A. Chudek, and G. M. Gadd. 1997. Metabolism-independent binding of toxic metals by *Ulva lactuca*: Cadmium binds to oxygen-containing groups, as determined by NMR. *Biometals* **10:** 105–118.
21. Yoon, K. P. 1998. Isolation and characterization of *Pseudomonas* sp. KM10, a cadmium and mercury-resistant and phenol-degrading bacterium. *J. Microbiol. Biotechnol.* **8:** 388–398.
22. Yoon, K. P. 2002. Plasmid-mediated arsenical and antimonial resistance determinants (*ars*) of *Pseudomonas* sp. KM20. *J. Microbiol. Biotechnol*. **12:** 31–38.
23. Yoon, K. P., T. K. Misra, and S. Silver. 1991. Regulation of the *cadA* cadmium resistance determinant of *Staphylococcus aureus*. *J. Bacteriol*. **173:** 7643–7649.