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Isolation and Characterization of *Pseudomonas* sp. KM10, a Cadmium- and Mercury-resistant, and Phenol-degrading Bacterium

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Abstract A bacterium which is resistant to both mercury and cadmium, and also capable of utilizing phenol as a carbon and energy source, was isolated from the Kumho River sediments near Kangchang Bridge, Taegu, Korea. The isolate was labeled *Pseudomonas* sp. KM10 and characterized. The bacteria grew in 4 mM $CdCl_2$ and in 70 μM $HgCl_2$. The bacteria efficiently removed over 90% of 1 g/l phenol within 30 h. In the presence of 1.250 g/l phenol, the growth of the microorganism was slightly retarded and the microorganism could not tolerate 1.5 g/l phenol. Curing of plasmid from the bacteria was carried out to generate a plasmidless strain. Subsequent experiments localized the genes for phenol degradation in plasmid and the genes for mercury resistance and cadmium resistance on the chromosome. Dot hybridization and Southern hybridization under low stringent conditions were performed to identify the DNA homology. These results showed significant homologies between the some sequence of the chromosome of *Pseudomonas* sp. KM10 and *merR* of *Shigella flexneri* R 100, and between the some sequence of the chromosome of *Pseudomonas* sp. KM10 and *cadA* of *Staphylococcus aureus* pI258. The mechanism of cadmium resistance was efflux, similar to that of *S. aureus* pI258 *cadA*, and the mechanism of mercury resistance was volatilization, similar to that of *S. flexneri* R100 *mer*.

Key words: *Pseudomonas* sp. KM10, cadmium and mercury resistance, phenol degradation, efflux, volatilization

Much environmental pollution has been caused by the release of many aromatic compounds from agricultural (pesticides, herbicides, livestock waste), industrial (solvents, preservatives, detergents, oils), and domestic (household chemicals, sewage sludge) activities. Such compounds are produced in large quantities, are toxic, and accumulate persistently in the environment. The ability of microorganisms to use aromatic compounds as the sole source of carbon and energy has been frequently observed [2, 13, 19, 46]. In general, these microorganisms use a common pathway. Oxygenases prepare the substrates for benzene ring fission by adding hydroxyl substituents to the aromatic nucleus. Subsequently, other oxygenases catalyze the cleavage of the hydroxylated benzene ring to aliphatic and more assimilable compounds [16, 46].

In the activated-sludge process, bacteria play major roles in digestion of organic and inorganic pollutants in wastewater [2, 17, 34], and it is generally thought that fluctuations of bacterial population in an activated-sludge ecosystem exert significant effects on the overall process. Toxic heavy-metal ions in the waste water are one of the important factors capable of causing such fluctuations. Several studies have found that metals influence microorganisms by adversely affecting their growth, morphology, and biochemical activities, resulting in decreased biomass and diversity [3, 5, 26]. 37% of the organic compounds-polluted sites in the United States have also been found to be polluted with metals such as cadmium, mercury, arsenic, lead, and zinc [22]. The presence of heavy metals can impair bioremediation of such sites and make cleanup of contaminated soils and water more challenging. Therefore, more attention should be focused on the treatment of metals although treatment of industrial effluents to remove organic contamination has received the greatest emphasis [6, 42].

Cadmium, mercury, and phenol are important among the many pollutants of our environment. Cadmium and mercury are the two most common toxic heavy metals. Their increased accumulation in the environment, in both plants and animals, has been widely reported. Cadmium is used extensively in industry, such as in electroplating, and protection against corrosion. Mercury compounds are used in industry as catalysts, in the extraction of gold and silver, in dried cell batteries, in dental restorations as a silver-mercury amalgam, in households as mercurochrome,

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etc. The single largest source of mercury is from burning coal and petroleum products. Cadmium and mercury are toxic to microbial and other life forms [5, 10, 27, 38, 40, 42] and are known to block several metabolic processes in bacteria, including respiration by the binding of the ions to thiol groups (-SH) in essential proteins [45]. Phenol and related phenolic derivatives, which are known to be common toxic constituents in industrial aquatic wastes, have been found in drinking water, wells, rivers, lakes, industrial effluents, and sewage treatment plant effluents [2, 13, 48], and a number of studies have also been reported on their bacterial degradation [14, 16, 18, 19, 30, 38].

For this reason, bacteria which are resistant to both cadmium and mercury, and capable of degrading phenol, have been extensively researched. In this paper, results on the isolation and preliminary characterization of a *Pseudomonas* strain, which is resistant to both cadmium and mercury, and capable of utilizing phenol as a single carbon and energy source, are presented.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Escherichia coli JM109 [*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17 *sup*E44 *rel*A1 λ⁻ Δ(*lac-pro*AB)/F': *pro*AB, *lacI*^{q}ZΔM15 *traD*36] was used as a host for the construction of new plasmids. pKPY21 containing an intact 3-kb *cadA* gene [52] was used as a parental plasmid for construction of pKPY26 containing a *cadA*-specific DNA fragment. pTM001 containing an intact *merR* gene [7] was used for construction of pKPY27 containing the *merR*-specific DNA fragment. Bacteria were preserved by freezing at -70°C in LB containing 25% glycerol.

Materials

$CdCl_2$ and other common chemicals were purchased from Sigma (St. Louis, U.S.A.) and culture media were purchased from Difco Laboratories (Detroit, U.S.A.). Southern-Light™ chemiluminescent detection system (Tropix Inc., U.S.A.) was used to prepare biotin labeled *cadA*- and *merR*-specific probes. $^{109}CdCl_2$ and $^{203}HgCl_2$ were obtained from New England Nuclear (Boston, U.S.A.). Restriction nuclease enzymes, calf intestine phosphate, and T4 DNA ligase came from Boehringer Mannheim Biochemicals (Indianapolis, U.S.A.).

Strain Isolation and Characterization

Several sediments were collected from the Kumho River near Kangchang Bridge, Taegu, Korea. The sediments (10 ml slurry of 1 g sediment) were passed through membrane filters (47 mm in diameter, 0.45 μm pore size, Nitrocellulose, Millipore), and the filters were then immersed in 250 ml Erlenmeyer flasks with a basal medium (100 ml) containing the following (per liter of deionized water): 2.13 g of Na_2HPO_4, 2.04 g of KH_2PO_4, 1 g of $(NH_4)_2SO_4$, 0.067 g of $CaCl_2 \cdot 2H_2O$, 0.248 g of $MgCl_2 \cdot 6H_2O$, 0.5 mg of $FeSO_4 \cdot 7H_2O$, 0.4 mg of $ZnSO_4 \cdot 7H_2O$, 0.002 mg of $MnCl_2 \cdot 4H_2O$, 0.05 mg of $CoCl_2 \cdot 6H_2O$, 0.01 mg of $NiCl_2 \cdot 6H_2O$, 0.015 mg of H_3BO_3, and 0.25 mg of EDTA. The pH of the medium was 7.0. Phenol was added as the sole carbon and energy source to the basal medium to a final concentration of 500 mg/l. Cells were grown in flasks on a rotary shaker at 28°C for 5 to 7 days and diluted 100-fold into the same medium and incubated as before. After several transfers, both $CdCl_2$ and $HgCl_2$ were added up to 1 mM and 30 μM, respectively, and the cells were incubated and transferred as before. Fast-growing colonies were obtained on a solid medium of the same composition as the enrichment medium after incubation at 28°C for 5 to 7 days. One fast-growing bacterial isolate, termed KM10, was selected for further study and characterized for morphological and biochemical properties by standard microbiological methods. Results were compared with characteristics cited in *Bergey's Manual of Systematic Bacteriology* [23] and *Methods for General and Molecular Bacteriology* [15].

Heavy Metal Resistance Analysis

The overnight cultures in LB were diluted 100-fold in 5 ml LB medium supplemented with 0, 1, 2, 3, 4, 4.5, and 5 mM $CdCl_2$ for the cadmium resistance test or 0, 5, 10, 25, 50, 70, 80, and 100 μM $HgCl_2$ for the mercury resistance test. The diluted cultures were incubated in a rotary shaker (150 rpm) at 28°C for 12 h. Turbidity (A_{600}) was measured using a UV-vis spectrophotometer (Biochrome 4060, Pharmacia Biotech, U.S.A.).

Phenol-degradation Analysis

Cells in the late log phase were harvested by centrifugation at 10,000 × g for 10 min at 4°C, washed twice with an equal volume of 15 mM phosphate buffer (pH 7.0), and resuspended in the same volume of the buffer. 1 ml of suspended cells was inoculated into 250-ml Erlenmeyer flasks containing the basal medium (100 ml) with phenol (0, 0.5, 1.0, 1.25, 1.5, or 2.0 g/l for tolerance test; 1.0 g/l for degradation test) as the sole carbon and energy source. The cultures were shaken (150 rpm) at 28°C for 30~35 h. Growth was followed by optical determination at 600 nm using a UV-vis spectrophotometer. The modified colorimetric method [13] was used to determine the degradation of phenol. Bacterial culture (1 ml) was added to an Effendorf tube containing 50 μl of 2 N NH_4OH and 25 μl of 2% 4-aminoantipyrine. Then, 25 μl of 8% $K_3Fe(CN)_6$ was added. After mixing, the tube containing the whole mixture was centrifuged to remove cells. A_{510} of the supernatant was measured.

Phenol concentrations were calculated by reference to a standard curve.

Curing

The *Pseudomonas* sp. KM10 was cured by treatment with mitomycin C at a concentration of 10 μg/ml [8]. The strains were grown in LB containing the reagents to the mid-log phase at 28°C and then spread on LB agar plates. Plasmidless colonies were selected after electrophoresis on 0.7% agarose gel.

Construction of pKPY26

pKPY21 [52] was cut by *Xmn*I and the whole mixture was separated in a 1% agarose gel. A 1.35-kb fragment was purified from the 1% agarose gel with a Gene Clean kit and digested again by *Nla*IV. The digested products were electrophoresed on 1% agarose gel and the purified 757-bp fragment was subcloned into the *Sma*I site of pUC19. The resulting construct was named pKPY26 (Fig. 5, B) which has a 757-bp fragment of *cadA* (nucleotides 1117-1874 from published sequence) [31].

Construction of pKPY27

A 620-bp *Eco*RI-*Hin*dIII fragment containing *S. flexneri* R100 *merR* was isolated from pTM001 [7] and cloned into the *Eco*RI and *Hin*dIII sites of pUC19. The resulting plasmid was termed pKPY27 (Fig. 5, D).

Preparation of Biotin Labeled Probe DNA

After digestion of pKPY26 or pKPY27 with *Eco*RI and *Hin*dIII, the *cadA* 757-bp DNA fragments and the *merR* 620-bp DNA fragments to be labeled were obtained by the Gene Clean kit from a 1% agarose gel. Then, the purified fragments were biotinylated with a biotin labeling kit (Southern-Light™, Tropix Inc., U.S.A.) consisting of a dNTP mixture, Biotin-14-dNTP, random octamer primers, and Klenow fragments. The reaction was carried out at 30°C for 30 min according to the manufacturer's instruction.

Preparation of DNA

Total DNA was prepared from the KM10 and plasmidless KM10 with some modification based on the procedure of Beji *et al.* [4]. Late-log-phase cells were harvested and resuspended in saline-EDTA (150 mM NaCl, 100 mM EDTA [pH 8.0]). They were centrifuged and weighed. The cells were lysed with 10 ml of 0.03 M NaOH and 1.5 ml of 25% (wt/vol) SDS and 35 ml of saline-EDTA (pH 7.0) per gram (wet weight) of cells. Then, the cells were treated with 2.5 mg RNase at 60°C for 30 min and 0.6 mg of proteinase K at 37°C for 30 min. The mixture was extracted twice with buffer-saturated phenol, twice with chloroform-isoamyl alcohol (24:1), precipitated with ethanol, and washed in 70% ethanol. The samples were dried and dissolved in 10 mM Tris, 1 mM EDTA (pH 8.0). When plasmid was needed, it was isolated by an alkaline lysis procedure described by Sambrook *et al.* [35].

Dot Hybridization

After the DNA samples were dotted on Hybond N^+ (Amersham, U.S.A.), the membrane was denatured and neutralized by the procedure of Southern [35]. After UV-cross linking (Fluo-link, U.S.A.), hybridization was carried out under high or low stringent conditions. In high stringent condition, prehybridization was performed at 68°C for 4 h and hybridization in a standard hybridization buffer (6× SSC, 0.01 M EDTA, 5× Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon sperm DNA). For hybridization, the biotin labeled *cadA*-specific or *merR*-specific probe (5 ng/cm^2 each) was added to the buffer. The filter was washed twice in 2× SSC, 0.5% SDS for 15 min at room temperature, and washed twice in 0.1× SSC, 0.5% SDS for 1 h at 68°C. In the low stringent condition [25], membranes were prehybridized in 4× SET buffer (0.6 M NaCl, 0.12 M Tris hydrochloride [pH 8.0], 4 mM EDTA), containing 10× Denhardt's solution, 0.2% SDS, 100 μg/ml denatured salmon sperm DNA. Hybridizations were carried out in fresh prehybridization buffer containing 50% deionized formamide (Sigma Chemical Co. U.S.A.) and the biotin labeled probe (5 ng/cm^2) at 40°C overnight. The membranes were washed with 2× SSC, 0.5% SDS at room temperature twice followed by washing in the same buffer at 45°C and treated as described in the Southern-Light™ chemiluminescent detection system for detection and exposed to X-ray films (X-Omat AR, Eastman Kodak Co.) overnight at room temperature.

Southern Hybridization

5 μg of the total DNAs was digested by *Eco*RI, and the digested DNAs were fractionated on a 0.8% agarose gel, and the DNA fragments were transferred to Hybond N^+ (Amersham, U.S.A.) according to the procedure of Southern [35]. After UV-cross linking (Fluo-link, U.S.A.), hybridization was carried out under low stringent conditions with the same probe as described above.

Efflux Assay

The overnight culture of cells was diluted 100-fold in fresh LB medium and the diluted cultures were incubated for 3 h and induced with 2 μM $CdCl_2$ for an additional 45 min at 28°C. For uninduced cells, all the procedures were the same except for the addition of $CdCl_2$. Cultures (15 ml) were centrifuged at room temperature and washed with LB. The cells were suspended in 3 ml LB and the suspended cells (0.3 ml) were incubated with 2 μmol $^{109}CdCl_2$ (sp. act. 773 mCi/mg; New England Nuclear Corp., Boston, U.S.A.) for at

28°C for 5 min, and then the cells were kept at 4°C for 40 min to equilibrate the cells with $^{109}CdCl_2$. Loaded cells were diluted 20-fold in the prewarmed LB medium containing 0.15 M sodium acetate (pH 5.5) at 28°C. The first sample was taken after 10 sec and subsequent samples were taken every 5 min. The samples (0.3 ml each) were filtered (0.45 μm, nitrocellulose; Millipore) and rinsed twice with 5 ml of 20 mM $CdCl_2$ in TE buffer (10 mM Tris plus 1 mM EDTA, pH 7.5). Washed filters were counted in a liquid scintillation spectrophotometer.

Mercury Volatilization Assay

Cells were grown in LB medium to the mid-logarithmic phase of growth, diluted 100-fold, and then the ^{203}Hg-volatilization assays were performed as follows. The diluted cells were grown for 3 h and induced in the presence of 3 μM $HgCl_2$ for 1 h. For uninduced cells, the cells were grown for an additional hour in the absence of Hg^{2+}. Cultures (15 ml) were centrifuged at room temperature and washed with LB broth. The cells were suspended in 3 ml LB broth. 0.3 ml of the suspension was added to 2.7 ml of prewarmed LB broth and the mixture was incubated in a shaker at 28°C for 2 min. Then, 5 μM $^{203}HgCl_2$ (sp. act. 82 mCi/g; New England Nuclear Corp., Boston, U.S.A.) was added. 0.3 ml of aliquots was withdrawn after 0, 5, 10, and 15 min. The cells were centrifuged and the supernatant was collected. Individually collected samples were counted in a liquid scintillation spectrophotometer.

RESULTS AND DISCUSSION

Isolation and Identification of Cadmium- and Mercury-resistant, and Phenol-degrading Bacterium

After enrichment in media containing cadmium and mercury, with phenol as the sole carbon source as described in Materials and Methods, a microorganism which was cadmium- and mercury-resistant, and phenol-degrading was isolated from the sediments from the Kumho River where many industries are located. From sediments of 10 different places, a sample which was taken near Kangchang Bridge contained a bacterium which was resistant to both heavy metals and capable of utilizing phenol as the sole carbon and energy source and was termed KM10. The KM10 strain was further characterized according to *Bergey's Manual of Systematic Bacteriology* [23] and *Methods for General and Molecular Bacteriology* [15]. The strain was determined to be a gram-negative, motile, nonspore-forming, and aerobic rod-shaped bacteria which generated a mucoid colony on nutrient agar. In addition, it was positive for oxidase, denitrification, gelatin hydrolysis, starch hydrolysis, and growth at 41°C and was negative for β-galactosidase, glucose acidification, Indole production, V-P test, and growth at 4°C (Table 1). Based on these characteristics, the isolate was identified as a *Pseudomonas* sp.

Table 1. Morphological and physiological characteristics of the isolated *Pseudomonas* sp. KM10.

Characteristics	KM10
Morphological	
Gram-staining	-[a]
Shape	rod
Motility	+
Spore	-
Colony on nutrient agar	Mucoid
Physiological	
β-galactosidase (hydrolysis of ONPG[b])	-
Glucose acidification	-
Oxidase	+
Denitrification	+
Indole production	-
V-P test	-
Hydrolysis of:	
Gelatin	+
Starch	+
Growth at 4°C	-
Growth at 41°C	+

[a]+, positive; -, negative.
[b]ONPG, *o*-nitrophenylgalactoside

The ability to utilize aromatic growth substrate is widely distributed among soil bacteria. The genus *Pseudomonas* is a group of gram-negative motile rods known for their large metabolic versatility as well as pathogenicity to plants, animals, and humans [32]. Phenol metabolism in various *Pseudomonas* strains has been examined [14, 16, 18, 19, 30, 38]. The isolation of microorganisms resistant to toxic heavy-metal ions and capable to degrade toxic organic compounds would certainly aid in the genetic manipulation of bacteria to create or improve their ability to degrade other pollutants. Such new bacteria (GEM, genetically engineered microorganisms) would be useful in the biodegradation of chemical contaminants at heavy metal-contaminated sites.

Growth of *Pseudomonas* sp. KM10 in Cadmium, Mercury, and Phenol

Growth studies were conducted with *Pseudomonas* sp. KM10 in liquid culture to determine the lowest concentration of mercury, cadmium, and phenol inhibiting the growth of the bacteria. To determine the MIC (minimal inhibitory concentration) of cadmium, the growth of the bacteria was monitored in LB containing an increased amount of $CdCl_2$ as described in Materials and Methods. Figure 1 (○) shows that bacteria could grow in 4 mM Cd^{2+}, but not in 5 mM Cd^{2+}. *S. aureus* and *Bacillus subtilis* could not grow in 50 and 20 μM Cd^{2+}, respectively [52, 53]. When compared with these

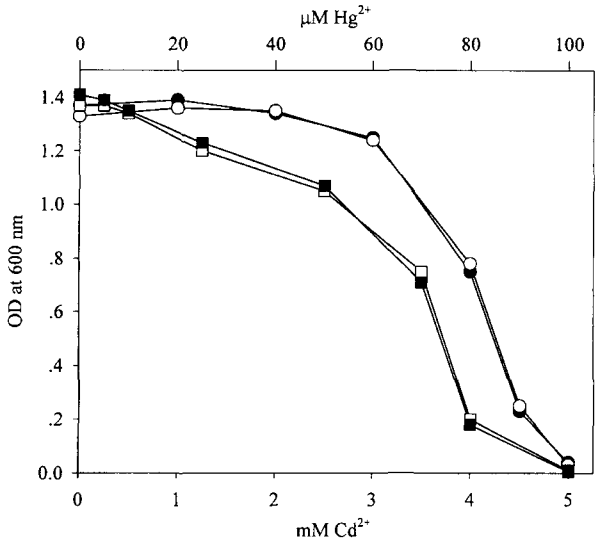

Fig. 1. Growth inhibition of *Pseudomonas* sp. KM10 and plasmidless KM10 by cadmium or mercury.

Overnight cultures of *Pseudomonas* sp. KM10 and plasmidless KM10 were diluted 100-fold into fresh LB broth containing increased amounts of $CdCl_2$ or $HgCl_2$ and grown as described in Materials and Methods. A_{600} was measured by a UV-vis spectrophotometer. Symbols: ○, KM10 in Cd^{2+}; ●, plasmidless KM10 in Cd^{2+}; □, KM10 in Hg^{2+}; ■, plasmidless KM10 in Hg^{2+}.

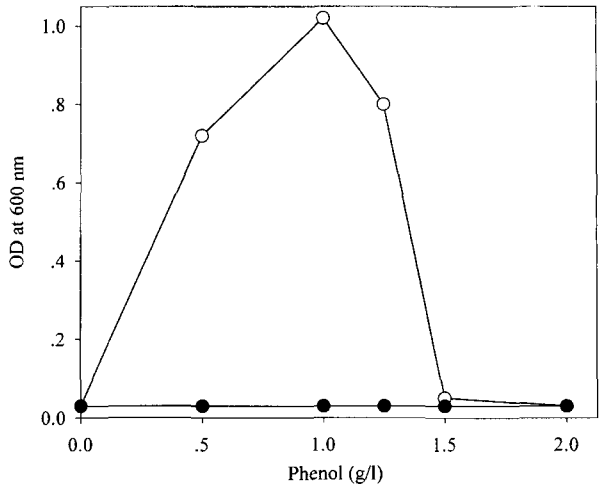

Fig. 2. Growth of *Pseudomonas* sp. KM10 and plasmidless KM10 in phenol.

Overnight cultures of *Pseudomonas* sp. KM10 and plasmidless KM10 were diluted 100-fold into the basal medium containing increased amounts of phenol and grown in a shaker at 28°C for 30 h. A_{600} was measured by a UV-vis spectrophotometer. Symbols: ○, KM10; ●, plasmidless KM10.

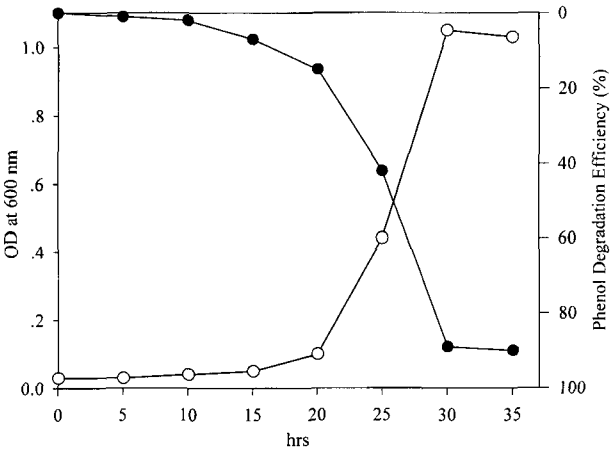

Fig. 3. Phenol degradation by *Pseudomonas* sp. KM10.

Overnight cultures of *Pseudomonas* sp. KM10 were diluted 100-fold into the basal medium containing phenol (1.0 g/l) and grown in a shaker at 28°C for a given time. The amount of phenol was determined as described in Materials and Methods. Symbols: ○, OD at 600 nm; ●, phenol degradation efficiency (%).

results, the newly isolated microorganism was highly resistant to cadmium. To determine the MIC of mercury, the growth of the bacteria was monitored in the same way. Figure 1 (□) shows that the bacteria grew well in 70 μM Hg^{2+}. But, the bacteria could not grow well in 80 μM Hg^{2+}, and not at all in 100 μM Hg^{2+}. When compared with 10 μM Hg^{2+} at which both *P. aeruginosa* and *E. coli* could not grow at all [7], this microorganism was indeed highly resistant to mercury. To determine the highest phenol concentration which *Pseudomonas* sp. KM10 could tolerate, the bacteria in the late log phase were washed and diluted 100-fold into fresh basal media containing increased amounts of phenol and the growth was monitored as described in Materials and Methods. Figure 2 (○) shows that the maximum growth was achieved in a culture containing 1 g/l phenol. The growth was significantly impaired in 1.25 g/l phenol. At higher phenol concentrations (1.5~2.0 g/l), the bacteria could not grow at all due to the toxicity of the phenol. These results were in good accordance with previous reports [1, 20, 23]. *Pseudomonas putida* [23], *Fusarium* sp. [1], and *Candida tropicalis* [20] were shown to be able to degrade 1, 1, and 2 g/l phenol respectively. *Pseudomonas* sp. KM10 seemed to be able to grow and degrade a slightly higher concentration of phenol (1.25 g/l) than *P. putida* [23]. Figure 3 shows the time course study of relationship between growth of the cells and degradation of phenol. The study showed the significant degradation of phenol and the corresponding increase in cell growth in the medium containing 1 g/l phenol. After 30 h, this strain could efficiently degrade about 90% of the phenol.

Phenol and related phenolic derivatives are frequently found in industrial effluents, drinking water, wells, rivers, and sewage treatment plant effluent. Industrial waste water often contains a high concentration of phenol (one of the widest spread environmental pollutants), as well as heavy metal ions such as cadmium and mercury, resulting

in reduction of treatment efficiency by the normal activated sludge system due to the toxicity [2, 11, 17]. These results suggested that *Pseudomonas* sp. KM10 could be developed as a degrader of contaminating phenol in the heavy metal-contaminated wastewater treatment process.

Curing of Plasmid and Localization of the Genes

Since many heavy metal resistances and phenol metabolism in bacteria are known to be conferred by plasmids [7, 18, 19, 24, 29, 37-39], this isolate was suspected to harbor the necessary genes in the plasmid. To determine if this plasmid encoded the genes for cadmium resistance, mercury resistance, and phenol-degradation, an attempt was made to cure plasmids from the strain with mitomycin C (10 μg/ml) as described in Materials and Methods. Agarose gel electrophoresis of the total DNA isolated from the original and the cured strain revealed a single species of plasmid band from the original strain (Fig. 4, lanes 1 and 2). Cured (plasmidless) strain derived in this way was unable to grow in basal medium containing various concentrations of phenol (Fig. 2. ●), whereas the resistance to both cadmium and mercury was not impaired at all (Fig. 1. ●, ■). Several phenol-nondegrading colonies derived by mitomycin C treatment were checked for spontaneous reversion to the phenol-degrading phenotype, but the frequency in every case was below the limits of detection.

The following results indicated that both cadmium and mercury resistance could be due to chromosomally mediated resistance. First, curing with mitomycin C resulted in the loss of plasmid but the resistant level to both cadmium and mercury was not changed at all. Second, plasmid DNA was successfully isolated from the original strain but not from its phenol-nondegrading variant. The role of the plasmid in phenol-degradation was deduced from the observations from that (i) curing with mitomycin C resulted in the loss of both the plasmid and the ability to utilize phenol as a carbon and energy source, (ii) repeated attempts to isolated plasmid DNA from phenol-nondegrading variant were unsuccessful, and (iii) when this apparently plasmidless strain was transformed with plasmid DNA from the original strain, *Pseudomonas* sp. KM10, the transformants not only regained the ability to degrade phenol-degrading characteristics identical to those of the original strain but also had a single species of plasmid band corresponding in size to that of the original strain (data not shown). Further investigations are under way to more fully elucidate the nature of this phenol degradation and its effect on the host.

It is evident from this curing experiment that resistant genes for cadmium and mercury are located on the chromosome and genes for phenol-degradation at least partially on the plasmid. Further characterization of the *Pseudomonas* sp. KM10 in this study was expected to yield insight into the bacterial mechanisms of heavy metal resistance and to provide the basis for possible exploitation in the remediation of metal-contaminated soils.

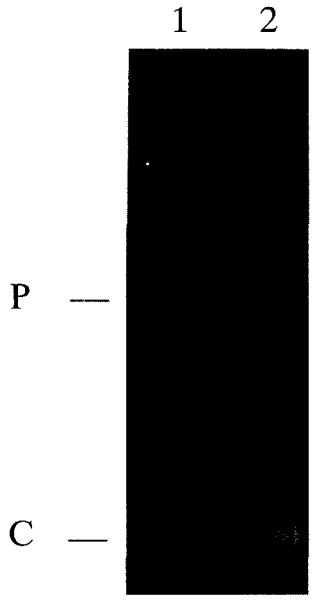

Fig. 4. Electrophoresis of *Pseudomonas* sp. KM10 and plasmidless KM10. Total DNA was isolated and electrophoresed in 0.7% agarose gel as described in Materials and Methods.
Lanes: 1, KM10; 2, plasmidless (cured) KM10. Symbols: P, plasmid DNA; C, chromosomal DNA.

DNA-DNA Hybridization

DNA-DNA hybridizations were carried out at high and low stringencies. The subcloned *cadA*-encoding gene fragment (Fig. 5, pKPY26) and the subcloned *merR*-encoding gene fragment (Fig. 5, pKPY27) were used as probes in both dot hybridization and Southern hybridization to detect similarities. Dot hybridization showed that the *cadA*-specific probes and the *merR*-specific probes were well hybridized with the *cadA*-specific control (Fig. 6A, lane 1, C and lane 3, C) and *merR*-specific control (Fig. 6A, lane 2, C and lane 4, C), respectively, under both the high and the low stringent conditions. However when these specific probes were hybridized with total DNA under the high stringent condition, there was no signal detectable (Fig. 6A, lane 1, T and lane 2, T). The weak hybridization signals could be detected only when the stringency was lowered (Fig. 6A, lane 3, T and lane 4, T). The results of dot hybridization were further investigated by Southern blot with the same *Eco*RI digested total DNA under the same low stringent conditions. When hybridized with the *cadA*-specific probe, there were two bands detectable (Fig. 6B, lane 1). The sizes of the two bands were 2.6 kb and 2.0 kb

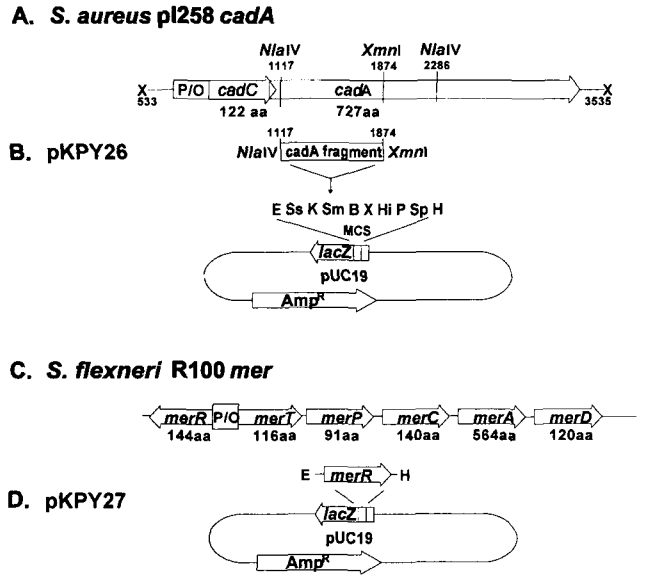

Fig. 5. Structure of pKPY26 and pKPY27. pKPY26 and pKPY27 were constructed as described in Materials and Methods.

Operator/Promoter region (P/O), and genes are marked by open bars, with arrows inside indicating the direction of transcription. Thin lines flanking *cadA* and *cadC* indicate additionally cloned DNA outside of the genes. Numbering of base pair positions came from the published 3535 bp sequence [31]. E, Ss, K, Sm, B, X, Hi, P, Sp, and H represent restriction nuclease sites for *Eco*RI, *Sst*I, *Kpn*I, *Sma*I, *Bam*HI, *Xba*I, *Hinc*II, *Pst*I, *Ssp*I, and *Hin*dIII, respectively, within the plasmid multicloning site. See Materials and Methods for the detailed construction of pKPY26 and pKPY27. A, Structure of *S. aureus* pI258 cadmium-resistant gene [31]; B, pKPY26; C, *S. flexneri* R100 mercury-resistant gene [7]; D, pKPY27.

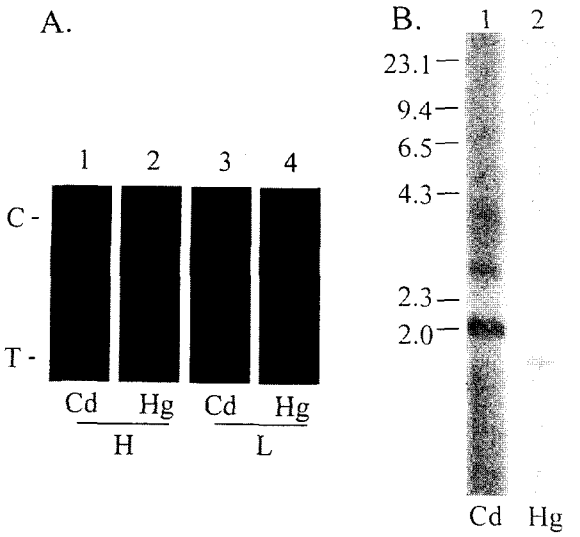

Fig. 6. DNA-DNA hybridization. Hybridizations were performed as described in Materials and Methods.

A. Dot blot: *cadA*-specific probe was hybridized with 0.1 μg of the *cadA* unlabeled DNA fragments (lane 1, C and 3, C), and *merR*-specific probe with 0.1 μg of *merR* unlabeled DNA fragments (lane 2, C and lane 4, C) for control; 5 μg of total DNA of *Pseudomonas* sp. KM10 was hybridized with a *cadA*-specific probe (lane 1, T and 3, T) and with a *merR*-specific probe (lane 2, T and 4, T). Symbols: C, control; T, total DNA; H, high stringent condition; L, low stringent condition. B. Southern blot: Total DNAs (5 μg/lane) of *Pseudomonas* sp. KM10 were digested with *Eco*RI restriction enzymes and hybridized with a *cadA*-specific probe (lane 1) and a *merR*-specific probe (lane 2). DNA molecular weight markers (Lambda DNA - *Hin*dIII digest) are indicated on the left.

respectively. When hybridized with the *merR*-specific probe, there was only one band observable, whose size was estimated as 1.5 kb (Fig. 6B, lane 2). Since the hybridization signal only showed up in the low stringent condition, the homologies among them might not be quite high enough. Nevertheless, there must be some homologies and the results strongly suggested that the probable mechanisms of cadmium- and mercury-resistance could be efflux and volatilization, respectively.

Cadmium efflux is energized only by ATP in everted membrane vesicles of *B. subtilis* harboring *S. aureus* pI258 *cadA*, proving that the mechanism of cadmium resistance is mediated by a cadmium-transporting ATPase [41, 43]. Reagents that affect the proton-motive force only partially inhibited transport, whereas the Ca^{2+}/H^{+} antiporter was completely inhibited [43]. This was the first report proving that CadA is P-ATPase. Lebrun *et al.* [25] showed the DNA-DNA hybridization between the cadmium-resistant gene of *Listeria monocytogenes* and *cadA* of *S. aureus* pI258, and mentioned from the results of sequence analysis that there are 65.8% amino acid sequence similarity between those two investigated genes. They subsequently suggested that *L. monocytogenes* CadA could be considered a P-type ATPase since it shared many basic structural elements and regions found in different organisms, such as CadA *S. aureus*, CadA *B. firmus*, CopA *Enterococcus hirae*, KdpB *E. coli*, and Mcl Human Menkes. The *cadA*-specific probe used in this report encompasses the N-terminal half of the CadA. This portion was thought to have a well conserved metal binding locus and phosphatase domain of the ATPase domain found among many different organisms mentioned above [12, 25, 33, 36]. Since the high similarities of cadmium-resistant genes are present among widely different organisms, the DNA-DNA hybridization results suggested that it was quite feasible for the cadmium-resistant gene of *Pseudomonas* sp. KM10 to share some similarity with *cadA* of *S. aureus* pI258.

Mercury resistance results from enzymatic reduction by a reductase enzyme such as mercuric reductase which reduces Hg^{2+} to volatile Hg^{0} [40, 50, 51]. Nakamura *et al.* [28] used low-stringent Southern blot to analyze 78 Minamata Bay *Bacillus* strains and reported that restriction nuclease site maps of the *mer* operon for the new isolates closely resembled the *mer* determinant of *Bacillus* strain RC607. *S. aureus* pI258 *merR* was also found to share

amino acids sequence homology with pDU1358 *merR* (35%) from *Serratia*, Tn*501* *merR* (36%) from *P. aeruginosa*, and R100 *merR* (35%) from *S. flexneri* [39, 40, 50].

Choi *et al.* [9] screened 42 *Bacillus thuringiensis* strains by Southern hybridization with a *cryl*-specific probe and identified two strains generating weak signals under low-stringency hybridization conditions. Lebrun *et al.* [25] identified restriction fragments of *L. monocytogenes* plasmids hybridizing with an *S. aureus cadAC*-specific probe under low-stringency conditions. Since the total DNA of *Pseudomonas* sp. KM10 also showed hybridizing signals with *S. aureus cadA*-specific probe and with *S. flexneri* R100 *merR*-specific probe under low-stringency conditions, a similar strategy could be employed to clone and sequence both cadmium-resistant and mercury-resistant genes of the bacteria. The exact DNA nucleotide sequence data of those genes would be necessary to understand heavy metal resistance mechanisms.

Efflux Assay

All the results shown above suggested that the cadmium resistance might be due to the efflux mechanism. Lowered cadmium uptake by cells containing the *cadA* cadmium resistance determinant had initially been shown to be the basis for resistance [44, 45]. But there are many other ways to be resistant to heavy metals such as biosorption and enzymatic reduction [39, 40, 42]. Efflux experiments were carried out to further examine the resistance mechanism of *Pseudomonas* sp. KM10.

To demonstrate the inducible efflux nature of cadmium transport, both the original and plasmidless KM10 were induced and filtered after 10 sec and every 5 min as described in Materials and Methods. When the filters were counted in a liquid scintillation spectrophotometer, the radioactivity reflected the remaining $^{109}CdCl_2$ amount still inside cells after efflux during a given time. Figure 7 shows that when the cells were uninduced, neither the original nor plasmidless KM10 could efflux $^{109}Cd^{2+}$ out of cells even after 15 min. There was no significant reduction in the amount of $^{109}Cd^{2+}$ inside the cells. But when the cells were induced with 2 μM $CdCl_2$, both showed that decreased amounts of $^{109}Cd^{2+}$ remained inside the cells. Both could efflux about half of the $^{109}Cd^{2+}$ in 5 min. There was no significant difference between them. These results confirmed that the cadmium-resistant gene was located on the chromosome. Based on these efflux data, the cadmium resistance mechanism of the *Pseudomonas* sp. KM10 could be explained as being the same inducible efflux mechanism as that of *S. aureus* pI258 *cadA*.

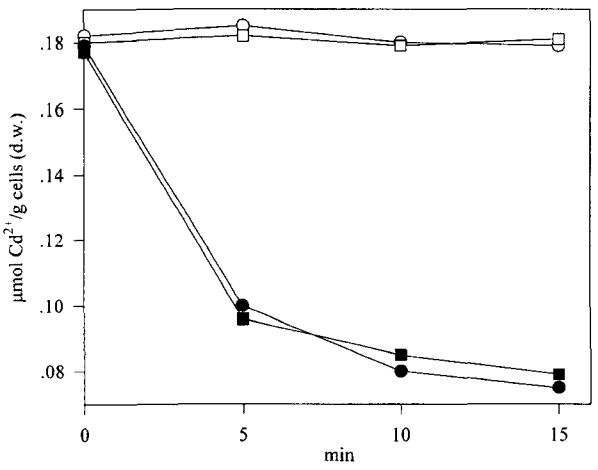

Fig. 7. Cadmium resistance of the *Pseudomonas* sp. KM10 is due to inducible cadmium efflux. Induced and uninduced cultures were prepared and loaded with $^{109}CdCl_2$ and filtered. The filters were counted in a liquid scintillation spectrophotometer as described in Materials and Methods. Symbols: ○, uninduced *Pseudomonas* sp. KM10; ●, induced KM10; □, uninduced plasmidless KM10; ■, induced plasmidless KM10.

Mercury Volatilization Assay

Mercury resistance results from the mercuric reductase activity responsible for the reduction of Hg^{2+} to Hg^0 which volatilizes subsequently [7, 40, 42]. Plasmid-mediated bacterial Hg^{2+} reduction to Hg^0 is the mechanism of the resistance of *S. flexneri* to Hg^{2+} [51]. Overnight cultures of *Pseudomonas* sp. KM10 and plasmidless KM10 were diluted 100-fold in LB and grown for 3 h and induced for 1 h in the presence of 3 μM $HgCl_2$. The cells were then harvested and the remaining $^{203}Hg^{2+}$ was counted as described in Materials and Methods. Figure 8 shows that when uninduced, neither the original nor the plasmidless KM10 could volatilize $^{203}Hg^{2+}$ even after 15 min. There was no significant reduction in the amount of $^{203}Hg^{2+}$ inside the cells. But when induced with 3 μM $HgCl_2$, both showed the same rapidly decreased amount of $^{203}Hg^{2+}$ remaining inside the cells. Both could remove about 70% of the $^{203}Hg^{2+}$ in 5 min and almost 90% after 15 min. There was no difference between them. These results proved that the mercury-resistant gene was also located on the chromosome and the mercury resistance mechanism of the *Pseudomonas* sp. KM10 was volatilization, as is that of *S. flexneri* R100 *mer*.

Many metals are essential for microbial growth and metabolism at low concentrations (e.g., Cu, Fe, Zn, Co, Mn), whereas cadmium and mercury are toxic and biologically inessential heavy metals. For many recent decades, its extensive use in industry resulted in cadmium and mercury contamination of the environment, including various kinds of life forms and foods. Cadmium and mercury ions exert their toxicity by binding to thiol groups (-SH) of proteins, which leads to the blockage of several metabolic processes in bacteria, including respiration [27, 39, 42, 44, 45]. Despite this, the ability of microorganisms

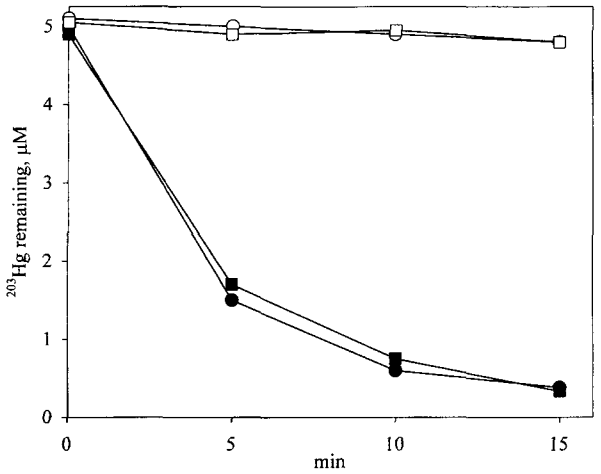

Fig. 8. Mercury resistance of the *Pseudomonas* sp. KM10 is due to inducible volatilization of mercury.

Overnight cultures were diluted and prepared and the rates of volatilization of 5 μM $^{203}Hg^{2+}$ were determined as described in Materials and Methods. Symbols: ○, uninduced *Pseudomonas* sp. KM 10; ●, induced KM10; □, uninduced plasmidless KM10; ■, induced plasmidless KM10.

to survive and grow in the presence of heavy metals is a frequent phenomenon [39, 42]. Microorganisms can be resistant to heavy metals in many ways [39, 42]. First, metal-binding proteins such as metallothionein isolated from animals, yeast, algae, and fungi [21] were shown to bind and sequester cadmium ions. Second, biosorption by bacterial cell walls can also sequester heavy-metal ions, resulting in heavy-metal resistance [47]. Third, reduction in uptake rate by blocking the cellular uptake pathway was demonstrated in *B. subtilis* 168 [24]. Fourth, enzymatic reduction by a reductase enzyme such as mercuric reductase which reduces Hg^{2+} to volatile Hg^{o} was demonstrated [40, 50, 51]. Fifth, highly specific efflux systems found in *S. aureus* [31, 43, 49] and *Alcaligenes eutrophus* [29] were shown to rapidly pump out toxic heavy metals. From the above results, *Pseudomonas* sp. KM10 could be explained by the fourth and fifth mechanisms.

In conclusion, it was not until the modern era of industrialization that there was widespread environmental contamination by toxic elements such as toxic heavy metals and a diverse group of toxic chemicals. Microorganisms play an important role in the degradation of these xenobiotics. But it was soon realized that the microorganisms have to be heavy-metal resistant in order to maximize their ability to attack xenobiotics because most environments contaminated by modern industrial activity contain substantial amounts of heavy metals. Therefore, it has become necessary to find microorganisms which can degrade a wide variety of organic chemicals and also are resistant to toxic heavy metals to accomplish expected bioremediation. Once such microorganisms are found, we may direct the genetic manipulation of bacteria to create or improve their ability to degrade various pollutants and such bacteria would be useful in the biodegradation of chemical pollutants in areas contaminated with several toxic heavy-metal ions and organic xenobiotics. In this laboratory, a *Pseudomonas* sp. KM10 was isolated from the Kumho River sediments near Kangchang Bridge, Taegu, Korea. The isolated bacteria was characterized and named as *Pseudomonas* sp. KM10. The isolate was found to be resistant to both cadmium and mercury since the bacteria could grow in 4 mM Cd^{2+} and grow well in 70 μM Hg^{2+}. The isolate was also capable of degrading phenol and using it as a carbon and energy source, efficiently removing over 90% of 1 g/l phenol within 30 h. After curing, a plasmidless variant was obtained and it was concluded that genes for phenol degradation were located on the plasmid and the genes for mercury resistance and cadmium resistance on chromosomes. Dot blot and Southern blot showed hybridizing signals between the chromosome of *Pseudomonas* sp. KM10 and *cadA* of *S. aureus* pI258, and between the chromosome of *Pseudomonas* sp. KM10 and *merR* of *S. flexneri* R100. The mechanism of cadmium resistance was efflux, similar to that of *S. aureus* pI258 *cadA*, and the mechanism of mercury resistance was volatilization, similar to that of *S. flexneri* R100 *mer*.

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