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Plasmid-Mediated Arsenical and Antimonial Resistance Determinants (*ars*) of *Pseudomonas* sp. KM20

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Abstract Bacteria have evolved various types of resistance mechanism to toxic heavy metals, such as arsenic and antimony. An arsenical and antimonial resistant bacterium was isolated from a shallow creek draining a coal-mining area near Taebaek City, in Kangwon-Do, Korea. The isolated bacterium was identified and named as *Pseudomonas* sp. KM20 after biochemical and physiological studies were conducted. A plasmid was identified and its function was studied. Original cells harboring the plasmid were able to grow in the presence of 15 mM sodium arsenite, while the plasmid-cured (plasmidless) strain was sensitive to as little as 0.5 mM sodium arsenate. These results indicated that the plasmid of *Pseudomonas* sp. KM20 does indeed encode the arsenic resistance determinant. In growth experiments, prior exposure to 0.1 mM arsenate allowed immediate growth when they were challenged with 5 mM arsenate, 5 mM arsenite, or 0.1 mM antimonite. These results suggested that the arsenate, arsenite, and antimonite resistance determinants of *Pseudomonas* sp. KM20 plasmid were indeed inducible. When induced, plasmid-bearing resistance cells showed a decreased accumulation of ^{73}As and showed an enhanced efflux of ^{73}As. These results suggested that plasmid encoded a transport system that extruded the toxic metalloids, resulting in the lowering of the intracellular concentration of toxic oxyanion. In a Southern blot study, hybridization with an *E. coli* R773 *arsA*-specific probe strongly suggested the absence of an *arsA* cistron in the plasmid-associated arsenical and antimonial resistance determinant of *Pseudomonas* sp. KM20.

Key words: *Pseudomonas* sp. KM20, arsenical and antimonial resistance, uptake, efflux

Heavy metals such as Ag^{+}, AsO_2^{-}, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2+}, Cu^{2+}, Hg^{2+}, Ni^{2+}, SbO_2^{-}, TeO_3^{2-}, and Zn^{2+} are very toxic to microorganisms [11, 13, 14]. The continual use of herbicidal, insecticidal, and medical arsenicals, as well as release from natural sources, results in the widely disseminated presence of arsenic in the environment [9, 16, 30]. Arsenic in the environment commonly exists in pentavalent [arsenate (As^{5+})] and trivalent [arsenite (As^{3+})] ionic forms [16]. Arsenate is a toxic analog of phosphate that is taken up by the bacterial transport systems [4, 13, 18].

Many microorganisms show resistance to metals in water, soil, and industrial waste. Genes located on chromosomes, plasmids, or transposons mediate specific resistance to a variety of metal ions [8, 11, 13, 18, 24, 27, 34]. Closely related bacterial arsenic and antimonite resistance systems are found on plasmids, the chromosome of *Escherichia coli*, and plasmids of *Staphylococcus* [2, 3, 6, 20, 25, 33] (Fig. 1). Decreased intracellular concentration of arsenic is the result of an active efflux system [17, 23]. These arsenic resistance determinants have been cloned and sequenced. The *ars* operon of the *E. coli* plasmid R773 and R46 consists of five genes (*arsR*, *arsD*, *arsA*, *arsB*, and *arsC*) [2, 6, 31]. *Staphylococcus* plasmid pI258 and pSX267, and the *E. coli* chromosome also carry *ars* operons [3, 7, 20, 33], but those operons only have three genes (*arsR*, *arsB*, and *arsC)* without *arsD* and *arsA* genes (Fig. 1). The *arsR*, *arsB*, and *arsC* gene products of both microorganisms are similar in sequence and function. The ArsR proteins of R773 (*E. coli* plasmid) and pI258 (*S. aureus* plasmid) are repressors and 30% identical in amino acid residues [33]. The *E. coli* ArsB protein is a membrane protein with 12 transmembrane α-helices [32] and pumps arsenite across the inner membrane [32]. The ArsC is an arsenate reductase enzyme that reduces intracellular arsenate to arsenite, which is extruded from the cells by the pump [12]. The ArsA protein functions as an arsenite- or antimonite-stimulated soluble ATPase [19, 26]. Under normal physiological conditions, the ArsA protein is a part of the ArsB protein in the inner membrane of *E. coli* [19, 28]. The ArsD protein is a secondary down-regulatory protein which

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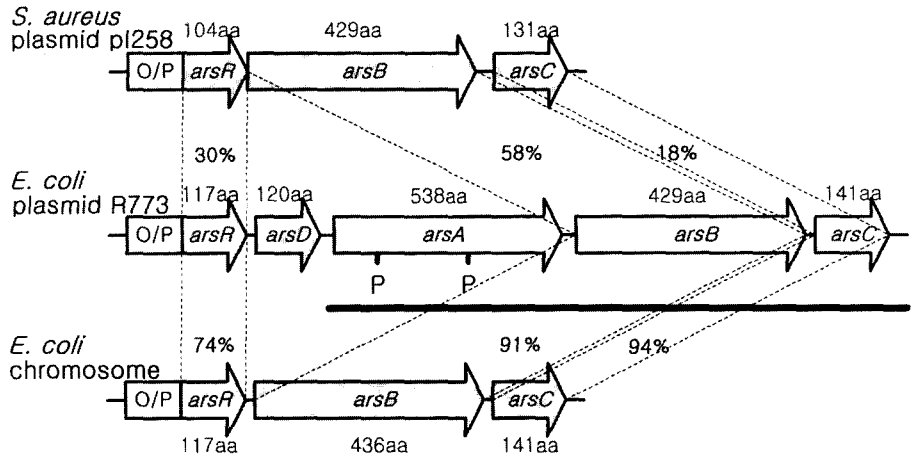

Fig. 1. Amino acids homologies among the arsenic resistance determinants of *Staphylococcus aureus* pI258 [12 13], *E. coli* plasmid R773 [6], and the chromosomal *ars* operon of *E. coli* [3, 7, 33].
Operator/Promoter region (P/O), and genes are marked by open bars, with arrows inside indicating the direction of transcription. The sizes (in amino acids [aa]) of each protein are shown above or below genes. The numbers between operons are percentages of similarity among ArsR, ArsB, and ArsC proteins. P represents restriction nuclease sites for *Pst*I. A thick line represents subcloned *ars* structural genes (*arsA*, *ardB*, and *arsC* in a *Eco*RI and *Hin*dIII fragment of pKPYa3).

functions separately from the repressor ArsR, and its absence has a little effect on the level of resistance [31]. The newly reported *ars* operon in the *Mycobacterium tuberculosis* chromosome consists of three ORFs [22]. The *ars* operon in the skin element of *Bacillus subtilis* is inducible by arsenate, arsenite, and antimonite and the *B. subtilis* ArsB protein is strongly homologous (51%) to that of the putative ArsB of *M. tuberculosis* [22].

In this study, the isolation and preliminary characterization of *Pseudomonas* sp. KM20 harboring a plasmid, which confers arsenical and antimonial resistance, are presented. The arsenical and antimonial resistance determinant in the plasmid was inducible by arsenate, arsenite, and antimonite that endows the cells with resistance to arsenic (as both arsenate and arsenite). The mechanism consisted of expelling arsenite or antimonite, which might be mediated through a specific anion pump. The *Pseudomonas* sp. KM20 *ars* determinant resembles that of the *E. coli* chromosomal *ars* operon organization, which lacks *arsD* and *arsA*.

Materials and Methods

Bacterial Strains, Plasmids, Chemicals, and Growth Conditions

For comparison of resistance level, *Pseudomonas* sp. KM20, *Escherichia coli*, and *Staphylococcus aureus* were used as follows: *Pseudomonas* sp. KM20 (plasmid-cured); *Pseudomonas* sp. KM20 (wild-type) harboring arsenate, arsenite, and antimonite resistance plasmid; *E. coli* J53 (*pro*, *met*, plasmid-free); *E. coli* J53 (*pro*, *met*, R773) harboring arsenate, arsenite, and antimonite resistance R773 plasmid [6, 25]; *S. aureus* RN1 (plasmid-free); *S. aureus* RN23 (RN1 harboring arsenate, arsenite, antimonite cadmium, and mercury resistance plasmid pI258 [12, 25, 29, 35]). Standard methods were used for plasmid construction and DNA manipulation [21]. Arsenate (As^{5+}; Na_2HAsO_4, $7H_2O$, di-sodium hydrogen arsenate heptahydrate), arsenite (As^{3+}; $NaAsO_2$, sodium arsenite), and antimonite (Sb^{3+}; $C_4H_4KO_7Sb$, potassium antimonial tartrate) were purchased from Sigma-Aldrich (St. Louis, U.S.A.). Other chemicals were of the analytical grade. $Na_3{}^{73}AsO_4$ was purchased from New England Nuclear (Boston, U.S.A.). *Pseudomonas* sp. KM20 was grown at 28°C, but *E. coli* and *S. aureus* were grown at 37°C.

Sample Collection and Isolation of Bacteria

Ten sediment samples were collected from a shallow creek draining a coal-mining area near Taebaek City, Kangwon-Do, Korea. The samples were pooled and thoroughly mixed and an enrichment culture was established as follows. Three g of the mixed sediments was placed in 300 ml of minimal basal salts (MBS) medium (pH 7.0) [21] supplemented with yeast extract, peptone, and soluble starch at a final concentration of 0.5 g/l each and 0.5 mM arsenate (Na_2HAsO_4). The culture was incubated in a rotary shaker at 150 rpm (28°C). At 1-week intervals, subenrichment cultures were made with 5% inoculum of the previous enrichment culture and transferred into a fresh MBS medium supplemented as described above. In addition to establishing a stable consortium by following the above methods, arsenate-resistant bacteria were selected and isolated by diluting 100-fold in 250-ml Erlenmeyer flasks with LB medium containing 3 mM arsenate. Cells were grown in the flasks on a rotary shaker at 28°C for 3–4 days and diluted 100-fold into the same medium containing an

increased amount of arsenate (up to 5 mM), and incubated as before. An individual arsenate-resistant bacterium was isolated by plating subenrichment cultures onto the LB agar containing 5 mM arsenate. Fast-growing colonies were obtained on the solid LB medium after incubation at 28°C for 3 to 4 days. One fast-growing bacterial isolate, designated as KM20, was selected for further study, and morphological and biochemical properties were characterized by applying the standard microbiological methods. Results were compared with characteristics cited in *Bergey's Manual of Systematic Bacteriology* [15] and *Methods for General and Molecular Bacteriology* [10].

Plasmid-Curing

Plasmid-curing of the *Pseudomonas* sp. KM20 was carried out by treating with mitomycin C at a concentration of 10 μg/ml, as described elsewhere [5].

Resistance of Arsenate, Arsenite, and Antimonite in *Pseudomonas* sp. KM20, *E. coli*, and *S. aureus*

The resistance of three different strains was compared. Arsenate-, arsenite-, and antimonite-resistant bacteria (*Pseudomonas* sp. KM20, *E. coli* J53 [R773], *S. aureus* RN23 [pI258]) and arsenate-, arsenite-, and antimonite-sensitive bacteria (*Pseudomonas* sp. KM20 [plasmid-cured], *E. coli* J53 [plasmid-free], *S. aureus* RN1 [plasmid-free]) were grown overnight. Each overnight culture was diluted 100-fold into a fresh LB broth containing an increasing amounts of arsenate, arsenite, or antimonite. Growth was measured by the absorbance at 600 nm after 10 h.

Induction of Arsenate, Arsenite, and Antimonite Resistance

Overnight cultures of *Pseudomonas* sp. KM20 were diluted into a fresh LB medium, and grown at 28°C for 2 h. For expression of the *ars* genes, the cultures were induced with a subinhibitory concentration of arsenate at 0.1 mM for 1 h and challenged with 5 mM arsenate, 5 mM arsenite, or 0.1 mM antimonite. The growth was measured by the absorbance level at 600 nm.

Arsenic Uptake in Whole Cells

Cells were grown to an optical density (600 nm) of 0.35 at 28°C with aeration in LB medium, and induced with 50 μM arsenite at 28°C for 1 h, harvested, washed twice at room temperature with TEA phosphate-free buffer [25], and then suspended in the same buffer at a cell density of approximately 70 mg (dry weight) per ml. To initiate the uptake assay, the cells were diluted 35-fold into a prewarmed buffer containing 0.2% glucose and 5 μM (1.25 μCi) $Na_3{}^{73}AsO_4$. Samples (0.7 ml each) were withdrawn at intervals, filtered through nitrocellulose filters (0.45-μm pore diameter, Whatman), and washed with 5 ml of TEA buffer. The filters were counted in a liquid scintillation counter (Packard Instrument Co. Inc., U.S.A.).

Arsenic Efflux in Whole Cells

Arsenic efflux was measured after loading cells with radioactive $Na_3{}^{73}AsO_4$ [12, 23]. Cells were grown and induced as described above. Cells were harvested, washed twice with TEA buffer, and suspended at a density of 200 mg (dry weight) per ml in TEA buffer. Cells were loaded with 50 μM $Na_3{}^{73}AsO_4$ for 30 min at 28°C and stored on ice. Efflux was initiated by 100-fold dilution into TEA buffer (pH 7.5) plus 0.2% glucose and 5 mM sodium phosphate. Samples (0.7 ml) were filtered and washed, and scintillation counting (Packard Instrument Co. Inc., U.S.A.) was carried out.

Construction of pKPYa3

Standard procedures were used for plasmid DNA manipulations and agarose gel electrophoresis [21]. The sequencing phage, M13mCMC6 [3, 6], containing *ars* structural gene (*arsA*, *ardB*, and *arsC* in a 4.3 kb fragment) was digested with *Eco*RI and *Hin*dIII. The digested products were ligated into the *Eco*RI and *Hin*dIII sites of pUC19. The resulting construct was named pKPYa3, which has a 4,347 bp fragment of *arsABC* (nucleotides 1-4,347 from published sequence [6]).

Southern Hybridization

For preparation of biotin-labeled *arsA*-specific probe, a 687 bp *Pst*I fragment containing an internal part of *arsA* (nucleotides 639–1,326 from published sequence [6]) was purified from the agarose gel and biotinylated with a biotin labeling kit (Southern-Light™, Tropix Inc., U.S.A.) according to the manufacturer's instruction. For Southern hybridization, the total DNA of *Pseudomonas* sp. KM20 and plasmid-cured *Pseudomonas* sp. KM20 were prepared by the procedure of Beji *et al.* [1]. The total DNA samples (10 μg per lane) were digested by *Pst*I, and the digested DNAs were fractionated on a 0.8% agarose gel, followed by standard methods described by Sambrook *et al.* [21].

RESULTS AND DISCUSSION

Isolation and Characterization of the Bacteria, *Pseudomonas* sp. KM20

The simple enrichment culture techniques yielded an arsenate-resistant bacterium on the LB agar containing 5 mM arsenate. The standard microbiological methods described in *Bergey's Manual of Systematic Bacteriology* [15] and *Methods for General and Molecular Bacteriology* [10] were used for morphological and biochemical studies. The bacterium was aerobic, Gram-negative, rod-shape, motile in semisolid nutrient medium, and produced a mucoid

Table 1. Taxonomical characteristics of the isolated strain KM20.

Characteristics	KM20
Morphological	
Gram-staining	-[a]
Shape	rod
Colony on nutrient agar	Mucoid
Motility	+
Spore	-
Physiological	
β-Galactosidase (hydrolysis of ONPG[b])	-
Glucose acidification	-
Catalase	+
Oxidase	+
Nitrate reduction	-
Indole production	-
V-P test	-
Hydrolysis of:	
Gelatin	+
Starch	+
Casein	+
Growth at 4°C	-
Growth at 41°C	+
Fluorescent pigment	-

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

colony on the nutrient agar (Table 1). In addition, the bacterium was found to be negative for β-galactosidase, glucose acidification, nitrate reduction, indole production, V-P test, growth at 4°C, fluorescent pigment, and positive for oxidase, catalase, growth at 41°C, hydrolysis of gelatin, starch, and casein (Table 1). The strain showed no significant differences in the growth rate in the presence of NaCl concentration up to 10 g/l. On the basis of these characteristics, the strain was tentatively identified as *Pseudomonas* sp. and designated as *Pseudomonas* sp. KM20.

Plasmid-Curing of the *Pseudomonas* sp. KM20

Plasmid-encoded arsenical resistance (*ars*) operons are found on plasmids of both Gram-positive [12, 20, 31] and Gram-negative bacteria [2, 3, 6, 33], however, they have not yet been detected in *Pseudomonas*. Plasmid-curing of the *Pseudomonas* sp. KM20 with mitomycin C was carried out to examine whether arsenate resistance phenotype of the *Pseudomonas* sp. KM20 was caused by the acquisition of the arsenate resistance plasmid. Curing and subsequent agarose gel electrophoresis were performed as described in Materials and Methods.

Original *Pseudomonas* sp. KM20 showed a single species of plasmid (Fig. 2, lane 1). But curing with mitomycin C resulted in the loss of plasmid (Fig. 2, lane 2). The plasmid DNA could be isolated from the original strain but not from the cured strain. When this plasmid-cured strain was transformed with plasmid DNA from the original strain, by applying electrophoresis, the transformants showed a single species of plasmid band corresponding in size to the original strain (Fig. 2, lane 3).

Resistance of Arsenate, Arsenite, and Antimonite in *Pseudomonas* sp. KM20, *E. coli*, and *S. aureus*

The biochemical and molecular basis of arsenate, arsenite, and antimonite resistance are most thoroughly known in *E. coli* and *S. aureus* [2, 3, 6, 12, 20, 31, 33]. In both organisms, arsenate, arsenite, and antimonite resistances are determined by a single gene complex (Fig. 1); therefore, they are coinduced by any of those ions [13]. It is interesting to compare the resistance level of *Pseudomonas* sp. KM20 with those of *E. coli* and *S. aureus*. Therefore, *E. coli* J53 (*pro*, *met*, plasmid-free), *E. coli* J53 (*pro*, *met*, R773) harboring arsenate, arsenite, and antimonite resistance R773 plasmid [5, 25], *S. aureus* RN1 (plasmid-free), *S. aureus* RN23 (RN1 harboring arsenate, arsenite, antimonite cadmium, and mercury resistance plasmid pI258 [12, 24, 29, 35]), *Pseudomonas* sp. KM20 (plasmid-cured), *Pseudomonas* sp. KM20 (original) harboring the plasmid, and *Pseudomonas* sp. KM20 (transformants) transformed with the same plasmid originated from *Pseudomonas* sp. KM20 (original) were grown on LB medium and the cell growth was determined by measuring the absorbance at 600 nm as described in Materials and Methods.

E. coli J53 (R773) could actually grow well in 5 mM arsenate, 7.5 mM arsenite, and 0.5 mM antimonite (Fig. 3,

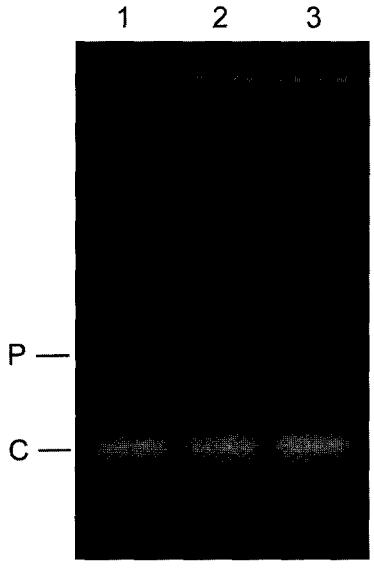

Fig. 2. Electrophoresis of total DNAs isolated from *Pseudomonas* sp. KM20, plasmid-cured KM20, and *Pseudomonas* sp. KM20 (transformant).
Total DNA was isolated and electrophoresed in 0.8% agarose gel as described in Materials and Methods. Lanes: 1, KM20 (original); 2, plasmid-cured KM20 (plasmidless); 3, KM20 (transformant). Symbols: P, plasmid DNA; C, chromosomal DNA.

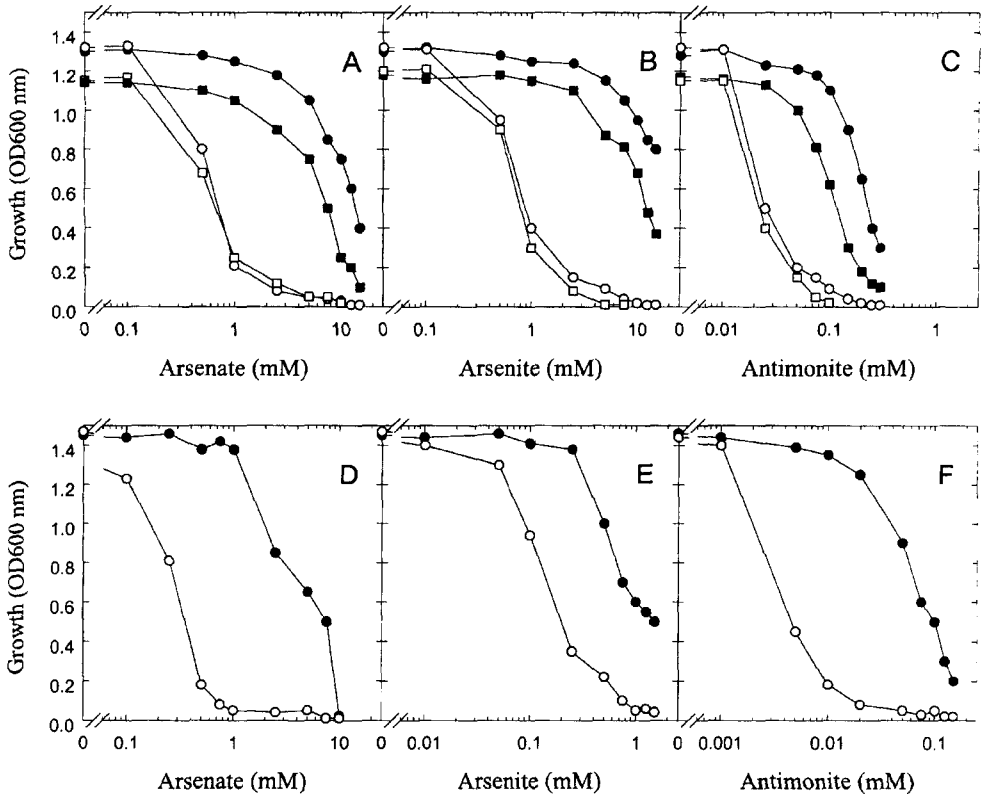

Fig. 3. Arsenate, arsenite, and antimonite resistances of *Pseudomonas* sp. KM20 (A, B, C,), *E. coli* J53 (A, B, C), and *S. aureus* (D, E, F).

Overnight cultures of each bacterium species were diluted 100-fold into fresh LB broth containing increasing amounts of arsenate, arsenite, or antimonite as described in Materials and Methods. Growth was measured by the absorbance at 600 nm after 10 h. Symbols in A, B, C: ○, *E. coli* J53 (plasmid-free); ●, *E. coli* J53 (R773); □, *Pseudomonas* KM20 (plasmid-cured); ■, *Pseudomonas* KM20 (original). Symbols in D, E, F: ○, *S. aureus* RN1 (plasmid-free); ●, *S. aureus* RN23 (pI258).

● in A, B, and C), but *E. coli* J53 (plasmid-free) was not able to grow at all in the same concentration of arsenate, arsenite, and antimonite (Fig. 3, ○ in A, B, and C). *S. aureus* RN23 (pI258) was able to grow well in 1 mM arsenate, 0.75 mM arsenite, and 0.02 mM antimonite (Fig. 3, ● in D, E, and F), but *S. aureus* RN1 (plasmid-free) was not able to grow in the same concentration of arsenate, arsenite, and antimonite (Fig. 3, ○ in D, E, and F). Direct comparisons of the plasmid-carrying and plasmid-free strains demonstrated that the plasmid-carrying strains were resistant to arsenate, arsenite, and antimonite.

The original strain of *Pseudomonas* sp. KM20 could grow well in 5 mM arsenate, 5 mM arsenite, and 0.1 mM antimonite (Fig. 3, ■ in A, B, and C), but the plasmid-cured strain of *Pseudomonas* sp. KM20 could not grow at all in the same concentrations of arsenate, arsenite, and antimonite (Fig. 3, □ in A, B, and C). *Pseudomonas* sp. KM20 (transformants) failed to show any significant difference in growth when compared with the original strain of *Pseudomonas* sp. KM20 (data not shown). *E. coli* J53 gave a significantly higher resistance to each metalloid salt than *Pseudomonas* sp. KM20 (original). The difference in resistance could be due to a different level of transcripts or protein, or different efficiencies of the transport systems. The role of the plasmid in arsenical and antimonial resistance level was deduced from the observations from that (i) curing with mitomycin C resulted in the loss of both the plasmid (Fig. 2, lane 2) and the arsenical and antimonial resistances (Fig. 3, □ in A, B, and C), and (ii) when the cured plasmidless strain was transformed with the plasmid DNA from the original strain, *Pseudomonas* sp. KM20 (transformants), it not only regained the arsenical and antimonial resistances identical to those of the original strain (data not shown), but it also had a single species of plasmid band of size corresponding to that of the original strain (Fig. 2, lane 3). In the *Pseudomonas* sp. KM20, arsenic and antimonial resistances are plasmid-mediated.

Induction of Arsenate, Arsenite, and Antimonite Resistances of *Pseudomonas* sp. KM20

Both *E. coli* and *S. aureus ars* systems are inducible [3, 6, 20, 33]. Only a basal level of expression of the R773 *ars* operon occurs in the absence of inducer (arsenate, arsenite, or antimonite) [33]. To determine whether the expression

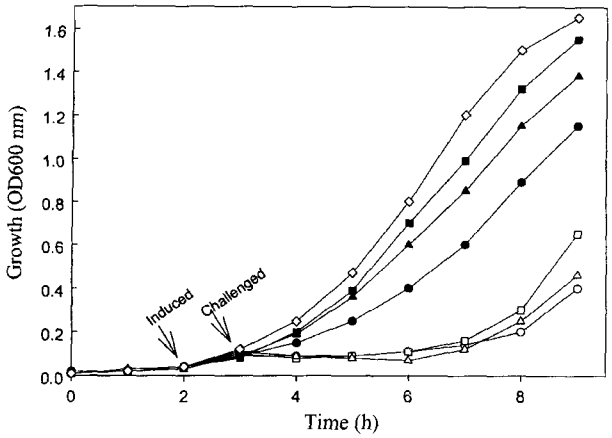

Fig. 4. Induction of arsenate, arsenite, and antimonite resistances of *Pseudomonas* sp. KM20.
Overnight cultures of *Pseudomonas* KM20 (original) were diluted 100-fold. After 2 h of subculture, the cells were induced with a subinhibitory concentration of 0.1 mM arsenate for 1 h and challenged with 5 mM arsenate, 5 mM arsenite, or 0.1 mM antimonite, respectively. For uninduced cells, 0.1 mM arsenate was not added. Growth was measured by the absorbance at 600 nm at 28°C. Symbols: uninduced and challenged by arsenate (○), arsenite (□), or antimonite (△); induced and challenged by arsenate (●), arsenite (■), or antimonite (▲); uninduced and unchallenged (◇, control).

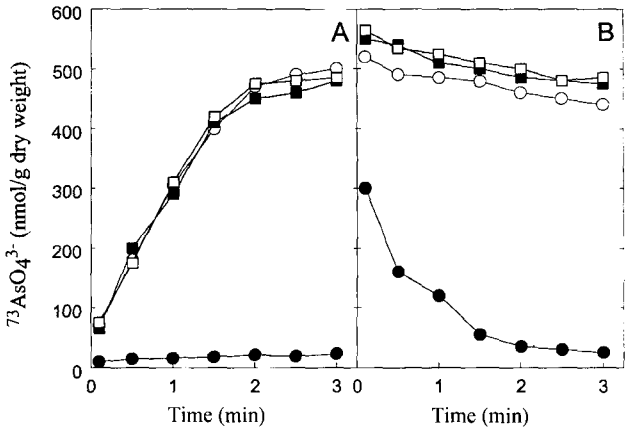

Fig. 5. Uptake and efflux of ^{73}As by *Pseudomonas* sp. KM20 and plasmid-cured *Pseudomonas* sp. KM20. Arsenical resistance of the *Pseudomonas* sp. KM20 is due to inducible arsenic efflux.
(A) Uptake of $^{73}AsO_4^{3-}$. Cells were grown, induced with 50 μM of arsenite, centrifuged, washed, and suspended in TEA buffer with 2% glucose. At time zero, 5 μM of $^{73}AsO_4^{3-}$ was added and uptake was measured by filtering 0.7 ml samples. Symbols: ○, uninduced KM20; ●, induced KM20; □, uninduced plasmid-cured km20; ■, induced plasmid-cured KM20. (B) Efflux of preloaded $^{73}AsO_4^{3-}$. Cells were grown and induced in LB broth, centrifuged, washed, and suspended in TEA buffer. Cells were preloaded with 50 μM $^{73}AsO_4^{3-}$ for 30 min. Efflux was initiated by 100-fold dilution into TEA buffer (pH 7.5). Efflux was measured by filtering 0.7 ml samples. Symbols are as defined for panel A.

of *Pseudomonas* sp. KM20 plasmid *ars* determinant is also inducible, *Pseudomonas* sp. KM20 (original) were induced with a subinhibitory concentration of 0.1 mM arsenate and challenged with 5 mM arsenate, 5 mM arsenite, or 0.1 mM antimonite. Growth was measured by the absorbance at 600 nm at 28°C. Figure 4 shows that, when uninduced cells were challenged with a high concentration of arsenate (○), arsenite (□), or antimonite (△), not only the growth of cells were significantly impaired but they showed a delay of 4–5 h before growth commenced again. Prior exposure of cells to 0.1 mM arsenate allowed for immediate growth when they were challenged with a high concentration of arsenate (●), arsenite (■), or antimonite (▲). The growth of cells was not effected when induced. This growth study confirms that the plasmid-mediated arsenate, arsenite, and antimonite resistances of *Pseudomonas* sp. KM20 is also inducible, and strongly suggests that the resistance level might also be determined by a single gene complex.

Arsenic Uptake and Efflux from *Pseudomonas* sp. KM20

An active efflux of the metal is a frequently utilized method to produce resistance by lowering the intracellular concentration levels [11, 13, 24, 29, 34, 35]. But no reports have been found in arsenic uptake and efflux of *Pseudomonas*. Therefore, it is interesting to characterize the plasmid-mediated arsenical resistance of *Pseudomonas* sp. KM20. Arsenic efflux was measured after loading the cells with radioactive $Na_3{}^{73}AsO_4$ as described in Materials and Methods [12, 23, 35]. When induced, plasmid-bearing resistant cells showed a lowered uptake of ^{73}As (Fig. 5, ● in A) and enhanced efflux of ^{73}As (Fig. 5, ● in B) compared to the uninduced cells (Fig. 5, ○ in A and B). On the other hand, plasmid-cured sensitive cells showed neither decreased accumulation nor efflux of ^{73}As (Fig. 5, □ and ■ in A and B). Lower uptake and rapid efflux were found when the plasmid of *Pseudomonas* sp. KM20 was present and induced. These data were consistent with the resistance data shown in Figs. 3 and 4. The results that (i) the plasmid of *Pseudomonas* sp. KM20 determined the inducible arsenical and antimonial resistances and (ii) the plasmid alone resulted in low accumulation of arsenate or arsenite, strongly suggested that the plasmid encodes an arsenite extrusion system that confers arsenite or antimonite resistances to *Pseudomonas* sp. KM20 by extruding arsenicals and antimonials out of the cell, thus lowering their intracellular concentration.

Southern Blot Analysis

Because of the high degree of homology between the protein products of the *E. coli* chromosomal *ars* operon and those found on plasmids from both Gram-negative and Gram-positive bacteria, it was suggested that these operons may be closely related, which might be progenitors of the common plasmid-borne *ars* operon (Fig. 1) [2-4, 6, 13, 20, 33]. For this reason, it is interesting to determine (i) if the

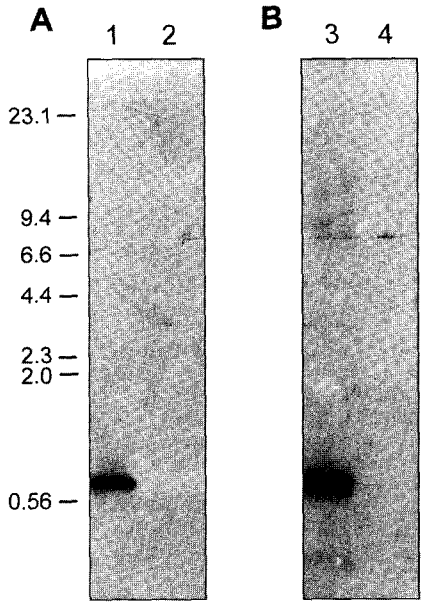

Fig. 6. Southern hybridization analysis of *Pseudomonas* KM20 plasmid to detect sequences homologous to *arsA* of the *E. coli* R773 *ars* gene.

The total DNAs samples (10 μg per lane) of *Pseudomonas* sp. KM10 were digested with *Pst*I restriction enzymes and hybridized with a *arsA*-specific probe (lanes 2 and 4). For a positive control, pKPYa3 (5 μg/lane) were digested with *Pst*I and hybridized with a *arsA*-specific probe (lanes 1 and 3). DNA M.W. markers (Lambda DNA - *Hin*dIII digest) are indicated on the left. A, high stringent condition; B, low stringent condition.

plasmid-associated arsenical and antimonial resistances of *Pseudomonas* sp. KM20 were conserved at the DNA level, (ii) the structure of the *Pseudomonas* KM20 plasmid *ars* determinant, and (iii) its relationship to other *ars* operons. The total DNA was isolated from *Pseudomonas* sp. KM20, hybridized with an *E. coli* plasmid R773 *arsA*-specific probe, and Southern blotted in high (Fig. 6A) and low (Fig. 6B) stringent conditions after agarose gel electrophoresis, as described in Materials and Methods.

However, no signal was detected with the R773 *arsA*-specific probe (Fig. 6, lanes 2, and 4) even after long exposure times, except for a strong single signal received from pKPYa3 that was digested with *Pst*I to be used as control (Fig. 6, lanes 1, and 3). These results implied that the *arsA* cistron was not present on the plasmid of *Pseudomonas* sp. KM20. The overall results that (i) the *Pseudomonas* sp. KM20 *ars* determinant did not contain an *arsA* gene, and (ii) the resistant cells actively extruded arsenite (Fig. 5), strongly suggested that the ArsB protein (an arsenite specific efflux system) might transport arsenite in the absence of an *arsA* gene product. In the well-studied plasmid pI258 from the *S. aureus* and *E. coli* chromosomal *ars* operon, the *arsR*, *arsB*, and *arsC* cistrons are conserved, while the *arsD* and *arsA* cistrons are absent [12, 26, 28]. In this case, the ArsB protein is believed to use the cell's membrane, having a potential to drive the efflux of intracellular arsenite ions [12]. It is likely that the *Pseudomonas* sp. KM20 *ars* determinant more closely resembles that of the *E. coli* chromosomal *ars* operon organization which lacks *arsD* and *arsA*. More detailed analysis is necessary to define the similarities and differences between the functional modes of the *ars* operons in *S. aureus* and *E. coli*, and the probable *ars* operon of *Pseudomonas* sp. KM20.

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