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Plasmid-Determined Silver Resistance in *Pseudomonas stutzeri* Isolated from a Silver Mine

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A silver-resistant strain of *Pseudomonas stutzeri* was isolated from a silver mine. It harbored three plasmids, the largest of which (pKK1; molecular weight, 49.4×10^6) specified silver resistance. Plasmid pKK1 was apparently nonconjugative but could be transferred to *Pseudomonas putida* by mobilization with plasmid R68.45.

Plasmids encoding silver resistance have been found in several clinical isolates of bacteria, predominantly members of the *Enterobacteriaceae*, especially after the topical use of silver compounds, which are frequently used for prophylactic treatment of burns (1, 8, 18, 24, 34). Such plasmids have also been found in bacteria, again primarily enterobacteria, isolated from sewage and from the sludge of industrial plants which reprocess used photographic film (4, 34). We report here the isolation of a strain of *Pseudomonas stutzeri* carrying a plasmid encoding silver resistance.

P. stutzeri AG259 was isolated from the soil of a silver mine in Utah. Approximately 1 g of soil was added to 100 ml of LB-medium (17) containing 1 mM $AgNO_3$ and incubated with gyratory shaking for 48 h at 30°C.

Bacteria in the culture were isolated on LB-agar containing 1 mM $AgNO_3$. The original isolate, AG259 (Table 1), had a rough colony morphology. When it was restreaked, it gave rise to both rough and smooth colony forms which were equally resistant to silver. A similar variation in colony morphology upon subculture of a fresh isolate of *P. stutzeri* was noted by van Niel and Allen (37). Strain AG259 was identified as *P. stutzeri* by the method of Buchanan and Gibbons (9) by using biochemical identification methods described by Cowan and Steele (12). Some of these tests were made at The National Collection of Industrial and Marine Bacteria Ltd., Torry Research Station, Aberdeen, Scotland. Strains AG257 and AG259 formed colonies on LB-agar containing up to at least 25 mM $AgNO_3$.

Plasmids were isolated from strains AG257 (smooth colony forms derived from AG259) and AG259 by the methods of Birnboim and Doly (6) and Hansen and Olsen (15) and were analyzed by agarose gel electrophoresis (Fig. 1) as previously described (5). Both strains had three plasmids, pKK1, pKK2, and pKK3. The size of each plasmid was determined by digestion with the restriction endonuclease *Hin*dIII and agarose gel electrophoresis, with size standards provided by fragments of phage λ *c*I857 generated by *Hin*dIII and *Eco*RI, *Hin*dIII-generated fragments of plasmid pWWO (13, 19), and *Hin*fI-generated fragments of Plasmid pBR322 (32).

To determine the origin of each fragment, *Hin*dIII digestions were made of plasmid DNA isolated from strain AG259, which has pKK1, pKK2, and pKK3; strain AG635, which has pKK1 and pKK3; and strain AG724, which has only pKK1 (Table 1 and Fig. 1). This allowed an unambiguous assignment of the fragments to each plasmid. Plasmid pKK1 (total length, 74.6 kilobases) gave *Hin*dIII fragments of 14.1, 13.0, 10.8, 8.7, 8.1, 7.2, 5.8, 2.6, 2.5, 1.45, and 0.35 kilobases. Plasmid pKK2 (11.3 kilobases) gave *Hin*dIII fragments of 10.4 and 0.93 kilobases. Plasmid pKK3 (9.3 kilobases) gave three *Hin*dIII fragments of 4.6, 3.1, and 1.6 kilobases.

LB-medium containing mitomycin C (1 μg/ml) was inoculated with AG259 and incubated overnight at 30°C. Colonies selected on LB-agar were tested for resistance to silver (1 mM $AgNO_3$ in LB-agar). Of 50 colonies tested, 14 were sensitive to silver. None of the 50 colonies isolated from an overnight broth culture not containing mitomycin C was Ag^+ sensitive. The plasmids present in silver-resistant and silver-sensitive bacteria from the mitomycin C-treated culture were examined again. All 19 silver-resistant isolates of AG259 had the large plasmid pKK1. None of the five silver-sensitive colonies examined had this plasmid. The MIC of $AgNO_3$ for a cured derivative (AG256) was 0.25 mM, compared with greater than 25 mM for AG259. The MIC was determined from the ability of bacteria to form single colonies on LB-agar containing $AgNO_3$.

We examined the resistances of strains AG259 and AG256 (cured of pKK1 and silver sensitive) to the following antibiotics: ampicillin, tetracycline, gentamicin, streptomycin, chloramphenicol, neomycin, sulfathiazole, tobramycin, clindamycin, and erythromycin. The two strains were equally sensitive to these antibiotics and $HgCl_2$, as indicated by the similar sizes of the inhibition zones surrounding disks containing these antimicrobial agents.

To determine whether pKK1 specified silver resistance, we attempted to transfer a determinant for silver resistance from AG259 to AG266. No silver-resistant derivatives of AG266 were found after the strain was mixed with AG259 on Millipore filters on the surface of LB-agar under conditions which permit the transfer of other *Pseudomonas* plasmids (3) (frequency of silver-resistant recipients, $<1 \times 10^{-10}$). The conjugative plasmid R68.45 (33) was transferred from *Pseudomonas aeruginosa* PAO25(R68.45) to AG259, as previously described (3); selection was applied for silver (2 mM $AgNO_3$) and kanamycin resistance (100 μg/ml). The resultant recipient (AG643) was mated with AG266 and with *Pseudomonas putida* BG410; selection was applied for chloramphenicol (200 μg/ml) and silver resistance (5 mM $AgNO_3$) or for rifampin (30 μg/ml) and silver resistance (2 mM $AgNO_3$), respectively. Silver-resistant recipients of strain AG266 were found at a frequency of 5×10^{-6}, and those of *P. putida* BG410 were found at a frequency of 3×10^{-7}.

The plasmids found in two silver-resistant exconjugants of

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TABLE 1. Bacteria and plasmids

Strain	Description[a]	Plasmids	Reference or source
P. stutzeri			
AG257	Ag^r, smooth colonies	pKK1, pKK2, pKK3	This study
AG259	Ag^r, rough colonies	pKK1, pKK2, pKK3	This study
AG256	Ag^s, derived from AG259 after mitomycin C treatment	pKK2, pKK3	This study
AG266[b]	Ag^s Cm^r Sm^r, antibiotic-resistant mutant of AG256	pKK2, pKK3	This study
AG635	Ag^r, spontaneous loss of pKK2 from AG257	pKK1, pKK3	This study
AG643	Ag^r Ap^r Km^r Tc^r	pKK1, pKK2, pKK3, R68.45	This study
AG722	Ag^r, pKK1 transferred from AG643 to AG266	pKK1, pKK2, pKK3	This study
P. aeruginosa PAO25(R68.45)	*argF leu-10* Ap^r Km^r Tc^r	R68.45	25, 33
P. putida			3
BG410	Ag^s Rif^r, rifampin-resistant mutant of strain, mt-2 2440		
AG724	Ag^r	pKK1	This study
E. coli J53	*pro met*		2

[a] Resistance markers are abbreviated as follows: silver ions, Ag^r; ampicillin, Ap^r; chloramphenicol, Cm^r; kanamycin, Km^r; rifampin, Rif^r; tetracycline, Tc^r. Ag^s, Silver ion sensitivity.

[b] Isolated as a mutant of AG256 spontaneously resistant to streptomycin (300 μg/ml in LB agar) which was subsequently used to isolate a mutant spontaneously resistant to chloramphenicol (300 μg/ml in LB agar).

AG266 and *P. putida* BG410 were examined. Silver-resistant derivatives of AG266 were checked for streptomycin resistance, an unselected marker to confirm that they were not simply mutants of the donor strain.

Each strain had a large plasmid corresponding to that seen in AG259. None of the four exconjugants examined had a plasmid corresponding to R68.45, nor did they have antibiotic resistance markers corresponding to those of R68.45. The fragments generated by cleavage of plasmid DNA with *Hin*dIII were identical to those corresponding to pKK1 in the donor strain.

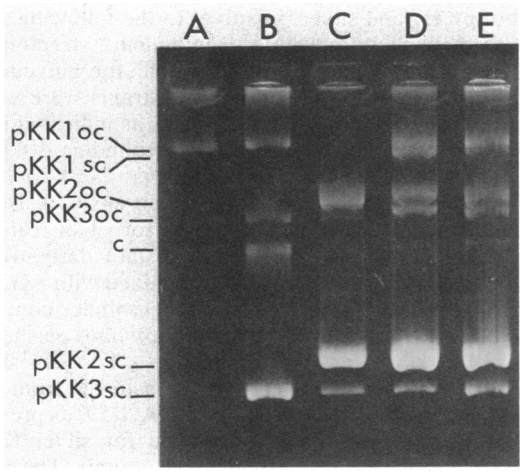

FIG. 1. Agarose gel electrophoresis of plasmid DNA isolated from *P. stutzeri* or *P. putida*. Lane A, Strain AG724(pKK1); lane B, strain AG635(pKK1, pKK3); lane C, strain AG266(pKK2, pKK3); lane D, strain AG722(pKK1, pKK2, pKK3); lane E, strain AG259(pKK1, pKK2, pKK3). The positions of the various forms of each plasmid are shown on the left. oc, Open circles; sc, supercoiled; c, chromosome. Certain preparations of plasmid DNA from each of the five strains contained both open circular and supercoiled forms of pKK1, as shown in lanes D and E. Electrophoresis through 1% agarose was performed as previously described (5).

Strains AG257 and AG259 growing on media containing silver and subsequently exposed to light formed black colonies surrounded by a region of agar which did not become dark (Fig. 2). LB-agar contains 171 mM NaCl; precipitated AgCl darkens on exposure to light because metallic silver is formed. We examined the concentration of silver in bacterial colonies and agar taken from light and dark areas surrounding the bacteria. Table 2 shows that the concentration of silver in agar taken from light and dark areas of the plate was not significantly different. The results do not indicate that the light areas are caused by bacteria which have accumulated silver from the surrounding agar, but rather that the silver in the agar close to the bacteria is in a form which does not darken on exposure to light. The silver concentration in bacteria growing on the agar was 0.5 mg per g (wet weight) of bacteria (2 mg per g (dry weight) of bacteria). In the silver assay used (Table 2, footnote *a*), the material was first treated with concentrated H_2SO_4 to oxidize metallic silver to Ag^+ so that both Ag(0) and Ag^+ were assayed. There have been reports of bacteria which can accumulate substantial amounts of silver, at least 300 mg per g (dry weight) of bacteria (11, 20).

This is the first report of plasmid-determined resistance to silver in *Pseudomonas* spp. However, Bridges et al. (8) have isolated a silver-resistant strain of *P. aeruginosa* from a patient in a burn unit which readily lost resistance when subcultured, which strongly suggests that silver resistance was also plasmid-determined in this strain. Plasmids conferring silver resistance have been found in numerous enterobacteria isolated from hospital or industrial environments in which silver compounds are used (1, 4, 8, 18, 24, 34). The finding reported here of a silver resistance plasmid isolated

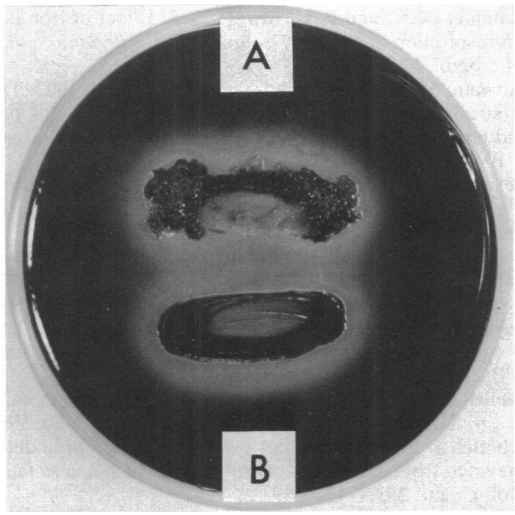

FIG. 2. *P. stutzeri* strains AG259 (A) and AG257 (B) growing on LB-agar containing 5 mM $AgNO_3$. After incubation for 3 days in the dark at 30°C, the plate was incubated for 2 days at 25°C in daylight.

from a region naturally rich in silver compounds suggests that this provides a natural environment for the evolution and selection of silver resistance genes. In contrast to the results of Smith (31), we found that silver resistance genes do not commonly occur on enterobacterial plasmids. Ninety-four conjugative R plasmids were transferred (by E. Meynell) from *Salmonella typhimurium* or *Escherichia coli* to *E. coli* K-12J53, but none increased the MIC of the strain more than twofold toward silver, cobalt, nickel, or copper.

The mechanism of plasmid-determined resistance to silver is unknown. Resistance to mercuric ions is frequently encoded by plasmids found in enterobacteria and in *P. aeruginosa*; the plasmids encode an inducible enzyme (mercuric reductase) which reduces Hg(II) to Hg(0) (27, 28, 35, 36). Additional polypeptides specified by the *mer* operon of plasmid R100 are involved in the uptake of Hg(II) and the regulation of the operon (14). Belly and Kydd (4) have isolated a silver-resistant bacterium, probably a species of *Pseudomonas*, which produced a volatile compound that reduced Ag^+. The strain also reduced resazurin and methylene blue which were incorporated in Sabouraud dextrose broth agar plates at a concentration of 0.005 and 0.002% (wt/vol), respectively. When grown on LB-agar containing the same concentrations of these indicators, strains AG256, AG257, and AG259 all reduced resazurin; methylene blue close to the colonies was also reduced. The reduction caused by strains AG257 and AG259 was not affected by the presence of $AgNO_3$ in the medium. As the reductive capacities of the silver-sensitive strain AG256 were indistinguishable from those of AG257 and AG259, it is not clear whether the reduction is related to silver resistance.

TABLE 2. Silver concentations in bacterial colonies and the surrounding agar

Sample[a]	Silver (μg per g of agar or bacteria)	
	Expt 1	Expt 2
Ag agar, uninoculated[b]	540	514
Ag agar, 2–8 mm from bacteria[c]	570	592
Ag agar, 25–35 mm from bacteria[d]	547	564
Bacteria from Ag agar	428	566

[a] The silver assay method used was essentially that described by Sandell (26). The silver content of agar or bacteria was measured by adding 0.5 ml of concentrated H_2SO_4 to a weighed amount (0.9 to 1.2 g) of agar or bacteria in a glass tube. After 5 min at 100°C, the sample was diluted 1,000-fold in 0.5 N H_2SO_4. To 4 ml of the dilution, 2 ml of a 0.001% solution of copper dithizone in chloroform was added. The tube was blended with a Vortex mixer for 1 min, and the phases were allowed to separate. The absorbance of the chloroform phase was measured at 465 nm (maximum absorbance for silver dithizone). (The absorbance was also measured at 603 nm, maximum absorbance for copper dithizone. Values for Ag obtained from measurements at these two wavelengths were in agreement.) The standard curve in the range 0.04 to 0.9 μg of Ag^+ per ml was made with dilutions of a stock solution of $AgNO_3$ in 0.5 N H_2SO_4.

[b] LB-agar containing 5 mM $AgNO_3$ (540 μg of Ag^+ per ml) exposed to daylight for 2 days at 25°C after incubation for 3 days at 30°C.

[c] The sample was taken from agar which did not darken on exposure to light (see Fig. 1).

[d] The sample was taken from a region which darkened on exposure to light.

Plasmid-determined silver resistance might involve the production of a compound which forms an inert complex with Ag^+. The region surrounding bacterial colonies which does not darken on exposure to light indicates that the bacteria may produce a molecule which binds Ag^+. The region close to the bacteria contains as much silver as the dark area in which metallic silver is formed from Ag^+. S. Silver (30) has found that silver resistance specified by a plasmid isolated from *Citrobacter* spp. was constitutive in *E. coli* and that the level of resistance depended on the availability of halide ions. The difference between sensitive and resistant cells was most apparent in the presence of halide ions, for example 5 mM Cl^-; in the absence of Cl^-, cells with or without the resistance plasmid were highly sensitive to Ag^+. S. Silver has proposed that sensitive bacteria bind Ag^+ tightly so that it is removed from the precipitated AgCl, whereas the surfaces of cells carrying a resistance plasmid do not bind Ag^+. The silver resistance of AG257 and AG259 was also markedly affected by the Cl^- concentration. When tested on LB-agar that did not contain the usual 171 mM NaCl, the MIC of $AgNO_3$ for strains AG257 and AG259 was reduced from more than 25 to 0.8 mM, whereas the MIC for AG256 remained at 0.25 mM.

Ag^+ apparently kills bacteria by acting on a number of cell surface components (23, 24), but the most well-characterized effect of Ag^+ on *E. coli* is the inhibition of respiration (7, 38). Ag^+ also appears to act as an uncoupler (10, 21). Silver ions inhibit the uptake of phosphate and also cause the release of phosphate, mannitol, succinate, proline, and glutamine from *E. coli* cells (22, 23, 29). However, Schreurs and Rosenberg (29) have found that these effects of Ag^+ could not be ascribed to a single inhibitory mechanism of Ag^+ acting as an uncoupler, a respiratory chain inhibitor, or a thiol reagent. The efflux of phosphate caused by silver could be reversed by dithiothreitol or by mercaptoethanol, presumably because the thiol groups of these compounds combine with Ag^+ to form an inactive complex (29). Possibly, the production by *P. stutzeri* AG259 of a molecule analogous to metallothionein (16) which binds and inactivates Ag^+ could protect the bacteria from these ions.

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