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Silver accumulation and resistance in *Pseudomonas stutzeri*

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Abstract. Silver (Ag) resistance and accumulation were investigated in Ag-resistant *Pseudomonas stutzeri* strain AG259 and Ag-sensitive *P. stutzeri* strain JM303. Both strains exhibited a similar pattern of silver accumulation although to different final concentrations. Energy-dispersive X-ray analyses revealed the association of dense silver deposits with the Ag-resistant strain, but not the Ag-sensitive strain. Toluene permeabilization or incubation of cells at 2 °C resulted in decreased Ag accumulation in both strains. This suggests that Ag accumulation may be energy dependent. A decrease in Ag accumulation was observed when cells were pretreated with 2,4-dinitrophenol (2,4-DNP). No decrease was observed using carbonyl cyanide m-chlorphenyl-hydrazone (CCCP). However, it was observed that both 2,4-DNP and CCCP complexed to Ag, making interpretation of accumulation results difficult. Washing of cells incubated in the presence of Ag with ethylenediaminetetraacetic acid (EDTA) or hydrochloric acid did not result in decreased Ag accumulation.

Key words: Accumulation – Complexation – Energy-dispersive X-ray – Metabolic inhibitors – *Pseudomonas stutzeri* – Ag-resistance – Ag-sensitivity

Silver (atomic weight 107.868) is a biologically non-essential metal used in electroplating and photographic industries as well as in jewelry manufacture and medicine. The toxicity of silver has generated interest in the isolation and study of metal-resistant microorganisms (Gadd et al. 1989; Haefeli et al. 1984; Slawson et al. 1990, 1992; Starodub and Trevors 1989, 1990). *Pseudomonas stutzeri* AG259 is able to grow in the presence of 25 mM $AgNO_3$ (Haefeli et al. 1984). Gadd et al. (1989) showed this resistance was dependent on the concentration of NaCl in the medium. Although this microorganism contains a 49.4 Mdal plasmid (pKK1) that confers Ag-resistance (Haefeli et al. 1984), the mechanism of resistance is not known (Gadd et al. 1989). Gadd et al. (1989) also determined that prior exposure of *P. stutzeri* AG259 cells to $Cu(NO_3)_2$ resulted in decreased Ag accumulation. *Pseudomonas stutzeri* JM303 is an Ag-sensitive strain that is unable to grow at or above Ag concentrations of about 0.05 mM. The transfer of the resistance plasmid pKK1 from *P. stutzeri* AG259 to *P. stutzeri* JM303 using high voltage electrotransformation was unsuccessful (Trevors and Starodub 1990). However, transfer into *P. putida* CYM318 was successful, and the transformant was resistant to silver (Trevors and Starodub 1990). The Ag-resistance plasmid pJT1 has also been mobilized between two strains of *Escherichia coli* using the Tn5-Mob transposon and an RP4-4 as the mobilizing plasmid (Starodub and Trevors 1990).

In the present study, characteristics of Ag accumulation in Ag-sensitive and Ag-resistant *P. stutzeri* strains were compared. Cells were treated with metabolic inhibitors (2,4-DNP, CCCP), permeabilized with toluene or incubated at 2 °C to study the mechanism(s) of Ag accumulation.

Materials and methods

Microorganisms

Pseudomonas stutzeri AG259 was kindly obtained from K. Hardy, Biogen S.A. Switzerland. *Pseudomonas stutzeri* JM303 was provided by J. Ingraham, University of California, Davis, U S.A. Ag-resistant *P. stutzeri* AG259, was maintained in Lennox L (LB) medium containing 0.5 mM silver nitrate. The Ag-sensitive strain, *P. stutzeri* JM303, was maintained in LB medium only, as no growth occurred on LB agar or in LB broth containing silver nitrate at a concentration as low as 0.05 mM. For storage, cells were grown to late exponential phase (18 h) and kept in 10% (v/v) sterile glycerol at −80 °C.

Chemicals

Silver was added during accumulation experiments as a sterile solution of $AgNO_3$. All media and solutions were prepared using ultrapure type I water from a Sybron/Barnstead Nanopure II Water System. Silver nitrate and other chemicals were reagent grade.

Inocula preparation

Cultures of both strains were prepared by adding one loopful of cells from an LB agar slant to 10 ml of LB broth (pH 6.8). The

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culture was incubated in 50-ml Erlenmeyer flasks at 28 °C, in the dark with gyratory shaking at 200 rpm. Inocula consisted of adding 1% (v/v) (0.5 ml) of each culture into 250-ml Nalgene polypropylene flasks containing 50-ml LB broth. Cultures were grown for 18 h at 28 °C with shaking at 200 rpm in a controlled environment incubator shaker (New Brunswick Scientific Co., New Brunswick, N.J., USA).

Silver accumulation by whole resting cells

Cells from an 18-h culture were harvested by centrifugation at 7700 × *g* for 15 min at 4 °C. Cells were washed twice in 20 ml of 5 mM piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES) buffer (Sigma Chemical Co., St. Louis, USA) (pH 6.8), and harvested as before. PIPES buffer exhibits negligible binding to ions such as magnesium, calcium, manganese and copper (Gueffroy 1990), and was chosen in these studies as it binds less silver than other buffers. Cell pellets were resuspended in 20 ml PIPES buffer and incubated at 28 °C at 120 strokes/min for 15 min in a Yamato (Model BT-25) constant temperature shaking water bath. One-ml samples were periodically removed from duplicate flasks immediately after addition of 0.5 mM $AgNO_3$. Samples were centrifuged in an Eppendorf centrifuge at 15000 × *g* for 20 s. The supernatant was removed, cell pellets washed and resuspended in 1 ml PIPES. Cells were pelleted for 20 s, the supernatant discarded, and 1 ml 6 M ultrapure HNO_3 added to digest cells for atomic absorption analysis. Suspensions in acid were heated at 80 °C for 1 h in a Tempblock heater. All metal analyses were performed using a Buck Scientific (Model 200) Atomic Absorption/Emission Spectrophotometer operated in the acetylene flame mode. The Ag resolution was 0.06 µg/ml for 1% absorption at 328.1 nm. Cell dry weights were determined by vacuum filtration of a 2-ml suspension through 0.22-µm nylon filters, which were then dried overnight at 80 °C in a vacuum oven. In some experiments the Ag-resistant AG259 strain exhibited variable accumulation of Ag (Table 1) even though it remained resistant to this metal.

Effect of temperature and toluene treatment on silver accumulation

The effect of temperature on Ag accumulation was studied by performing accumulation experiments at 2, 12 and 28 °C. Lower temperatures were maintained by placing crushed ice in the water-bath. Cells were permeabilized with toluene (Van Dyke et al. 1989) in a ratio of 1 ml toluene to 100 ml washed cells for 15 min at 28 °C prior to Ag accumulation studies.

Effect of washing treatments and glucose on silver accumulation

Cells were removed from the accumulation buffer and washed with EDTA or HCl to remove surface-bound Ag. A mixture of 1 mM EDTA : 5 mM PIPES (1 : 1 v/v) replaced washing with 5 mM PIPES buffer. Similarly, 0.1 or 10 mM HCl replaced PIPES in the washing of whole cells. Two acid concentrations were used to determine the effect on surface-bound silver. The influence of D-glucose on Ag accumulation was investigated by growing a culture for 18 h in LB broth amended with 50 mM D-glucose. Cells were harvested, washed as before, and D-glucose-starved in PIPES buffer for 2 h at 28 °C with shaking at 120 rpm. During the accumulation assay, cells were exposed to 50 mM D-glucose and 0.5 mM $AgNO_3$.

Effect of chemical inhibitors on silver accumulation

The influence of 2,4-DNP and CCCP on Ag accumulation was investigated. Each strain was grown in LB broth for 18 h, harvested and washed in PIPES buffer as described previously. Cells were exposed to 0.5 mM concentrations of the inhibitor for 10 min prior to the addition of 0.5 mM $AgNO_3$. Samples were removed and Ag accumulation measured as before.

Determination of Ag^+ complexation to metabolic inhibitors

The capacity of 2,4-DNP and CCCP to complex silver was studied using an Orion Research (Cambridge, Mass., USA) Ionalyzer/model 407A ion meter with an Orion Research Ag^+-specific electrode. Each inhibitor was titrated into a solution of 0.5 mM $AgNO_3$ dissolved in ultrapure water to determine its complexation to Ag^+.

Energy-dispersive X-ray analyses

Whole cells from an 18-h culture were harvested, washed, resuspended in PIPES buffer, and collected on copper grids. Energy dispersive X-ray analysis was performed using a Philips EM400T scanning transmission electron microscope, operated in the TEM mode and equipped with an X-ray analyser/image processor and Link Analytical LZ-5 light element detector, operated at 100 kV (−180 °C, liquid N_2) with an anticontaminator in place. Electron micrographs were prepared using a Philips EM300, operated at 60 kV with anticontaminator in place (Mullen et al. 1989). Beam spot size was 10 nm and current settings were 50 to 60 nA.

Table 1. Ag accumulation by whole resting cells of *Pseudomonas stutzeri* AG259 and JM303 after washing with EDTA or dilute HCl, or exposure to metabolic inhibitors

Strain	Treatment	Ag accumulation (µg/mg dry wt. cells)[a] Time (min)		
		1	8	30
AG259	Control	11.46 (4.68)	21.39 (3.94)	23.74 (5.30)
	EDTA	5.57 (0.11)	23.71 (1.27)	25.80 (3.38)
JM303	Control	7.94 (0.10)	19.10 (0.84)	22.72 (1.36)
	EDTA	7.37 (1.37)	20.19 (0.10)	23.79 (0.49)
AG259	Control	4.42 (4.01)	16.58 (0.38)	18.47 (1.34)
	HCl	12.09 (1.41)	25.31 (0.59)	29.63 (1.76)
JM303	Control	7.99 (0.95)	20.14 (0.11)	23.67 (0.21)
	HCl	11.62 (2.16)	21.74 (1.48)	21.10 (0.57)
AG259	Control	5.74 (0.33)	12.93 (0.66)	21.38 (1.47)
	2,4-DNP	3.84 (0.18)	11.62 (0.18)	12.87 (0.53)
JM303	Control	3.91 (0.77)	8.44 (0.11)	14.67 (0.33)
	2,4-DNP	4.63 (0)	6.64 (0.12)	7.86 (0.34)
AG259	Control	3.57 (0.35)	4.79 (0.35)	8.22 (0.35)
	CCCP	8.15 (1.76)	7.92 (0.80)	9.62 (0.96)
JM303	Control	5.57 (0.30)	10.92 (1.04)	18.36 (1.19)
	CCCP	8.91 (2.44)	14.26 (2.20)	14.52 (0.12)

[a] Values are means (SD) of a minimum of 2 independent determinations

Results

Pseudomonas stutzeri AG259 and JM303 were exposed to 0.5 mM $AgNO_3$ during accumulation experiments. Viable cells of both strains were recovered on LB agar after 30 min exposure to $AgNO_3$. This revealed that not all cells were killed, as they were by toluene treatment (Fig. 1). Both strains exhibited a similar pattern of Ag accumulation at 28 °C whereby the majority of Ag was accumulated in the first 8 min (Table 1). After this initial rapid phase, further increase was gradual up to 30 min. Under these control conditions, the Ag-resistant strain accumulated similar concentrations of Ag as the Ag-sensitive strain (Table 1).

Silver accumulation was conducted using cells incubated at 2, 12 and 28 °C or pretreated with toluene, 2,4-DNP or CCCP. These treatments were intended to inhibit energy-dependent Ag accumulation. The Ag-resistant AG259 strain exhibited lower Ag accumulation in toluene-treated than in untreated cells (Fig. 1A). Toluene-treated Ag-sensitive cells also accumulated lower levels of Ag than cells not incubated with toluene (Fig. 1B). Viable cells were not detected after incubation with toluene and subsequent exposure to Ag during the accumulation experiment.

At 2 °C, both strains accumulated 70 to 80% lower amounts of Ag than at 28 °C (Fig. 2). Ag accumulation at 12 °C and 28 °C was similar (Fig. 2). D-glucose did not affect Ag accumulation in either strain (data not shown).

Cells were washed with HCl or EDTA to determine the extent of surface binding. Washing *P. stutzeri* AG259 cells with EDTA and HCl did not consistently change

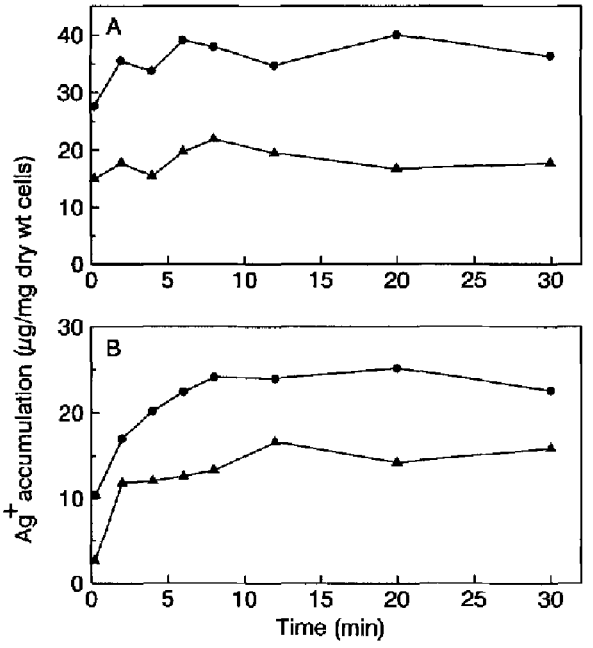

Fig. 1. Effect of toluene on Ag accumulation in **(A)** *Pseudomonas stutzeri* AG259 and **(B)** *P. stutzeri* JM303. Control, 0.5 mM $AgNO_3$ (●); toluene and 0.5 mM $AgNO_3$ (▲). Each point is the mean of two determinations and typical results are shown from a minimum of two independent experiments

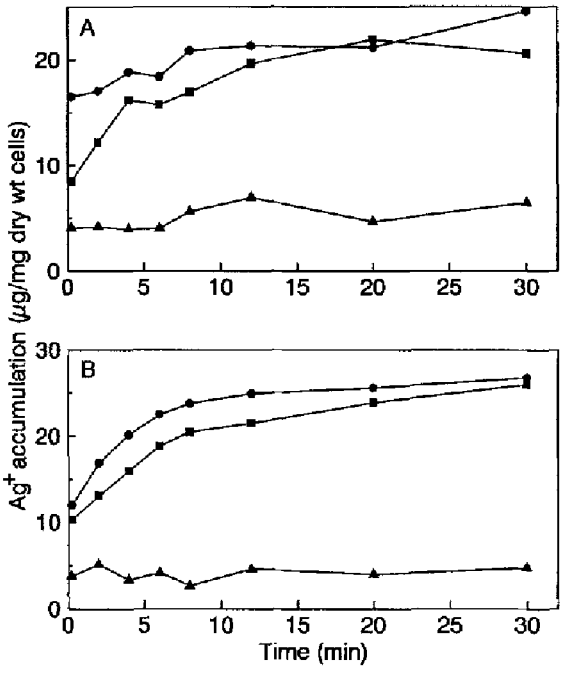

Fig. 2. Effect of temperature on Ag accumulation in **(A)** *P. stutzeri* AG259 and **(B)** *P. stutzeri* JM303. Control, 28 °C (●); 12 °C (■); 2 °C (▲). Each point is the mean of two determinations and typical results are shown from a minimum of two independent experiments

Ag accumulation. Similar results were obtained with the Ag-sensitive strain (Table 1). Silver accumulation by both Ag-sensitive and Ag-resistant cells in the presence of 2,4-DNP was lower than without the inhibitor (Table 1). The presence of CCCP did not decrease Ag accumulation in either strain (Table 1). However, both 2,4-DNP (from 0.001 to 0.5 mM) and CCCP (from 0.001 to 1 mM) readily complexed to Ag^+ as determined by an Ag^+-specific electrode (Table 2). For example, all Ag^+ was completely complexed in the presence of 0.2 and 0.5 mM

Table 2. Complexation of 2,4-DNP and CCCP to 0.5 mM $AgNO_3$ as determined using an Ag^+-specific electrode

Metabolic inhibitor concentration added (mM)	Ag^+ concentration (mM)
2,4-DNP	
0	0.54
0.001	0.53
0.01	0.50
0.05	0.39
0.10	0.26
0.15	0.11
0.20	0
0.50	0
CCCP	
0	0.58
0.001	0.58
0.01	0.57
0.05	0.52
0.10	0.47
0.25	0.33
0.50	0.08
1.00	0.02

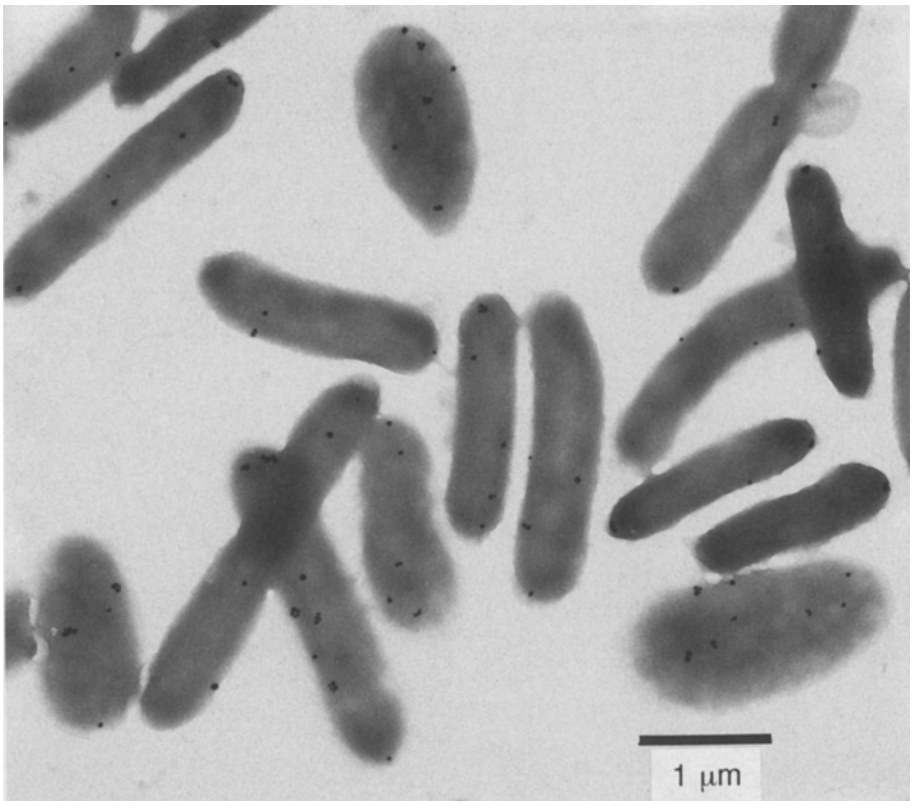

Fig. 3. Electron micrograph of whole cells of *P. stutzeri* AG259 after exposure to 0.5 mM $AgNO_3$

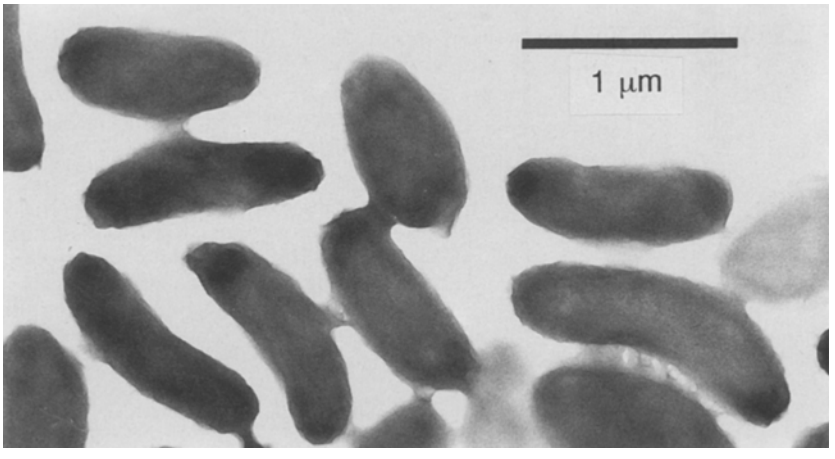

Fig. 4. Electron micrograph of whole cells of *P. stutzeri* JM303 after exposure to 0.5 mM $AgNO_3$

2,4-DNP. In the presence of 0.5 mM CCCP (the concentration used in the accumulation assay), only 0.08 mM Ag^+ was detected. This makes interpretation of Ag accumulation data in the presence of either 2,4-DNP or CCCP difficult.

Energy-dispersive X-ray analysis revealed dense Ag deposits associated with the Ag-resistant AG259 strain (Figs. 3 and 5), whereas no deposits were observed with the sensitive strain (Fig. 4). The corresponding element profiles revealed relatively low Ag levels in both *P. stutzeri* AG259 and JM303 (Fig. 5). A high sulfur signal was detected in the resistant strain (Fig. 5A) as compared to the sensitive strain (Fig. 5B). A high Ag signal was associated with the dense deposits observed in the Ag-resistant strain (Fig. 6). A frequency histogram of these deposits in the resistant strain revealed that Ag-resistant cells contained from 1 to 11 Ag deposits per cell (Fig. 7). The median number was 4 deposits per cell. Deposit sizes ranged from 35 to 46 nm in Ag-resistant *P. stutzeri* cells.

Discussion

The mechanism of Ag-resistance in bacteria has not been fully determined and information on resistant and sensitive strains is limited (Hughes and Poole 1991; Slawson et al. 1990, 1992). Microbial resistance to metals and metal accumulation has been of interest in metal recovery and from a fundamental perspective. The nature of this

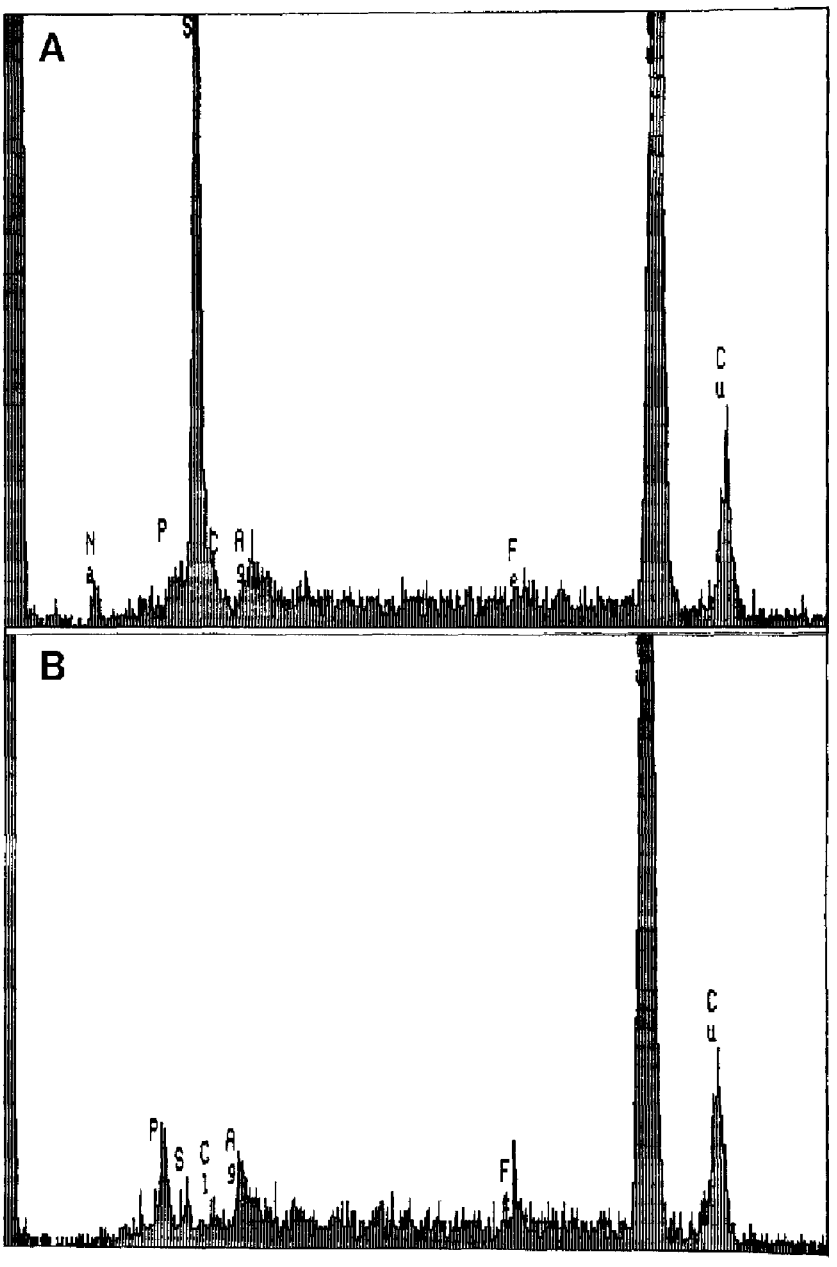

Fig. 5. Representative energy dispersive X-ray analysis of whole cell preparations of *P. stutzeri* AG259 **(A)** and JM303 **(B)** after exposure to $AgNO_3$. The high Cu signals (last two peaks) were caused by the copper grids. Energy on the X axis is 0 to 20 keV. Vertical full scale (VFS) on Y axis is 0 to 127 peaks units

accumulation depends on the type of metal and speciation (Hughes and Poole 1991), the particular organism involved (de Rome and Gadd 1991; Mullen et al. 1989; Starodub and Trevors 1989; Trevors 1989; Van Dyke et al. 1989; Williams and Silver 1984) as well as numerous interacting physical, chemical and biological factors.

Previous studies have shown that Ag resistance in *Pseudomonas stutzeri* AG259 is associated with metal accumulation that is dependent on the composition of the culture medium (Gadd et al. 1989). In this study, rapid accumulation of Ag in the first 8 min was consistent with earlier reports (Gadd et al. 1989). However, our studies also showed that Ag-sensitive and -resistant strains accumulated comparable amounts of Ag. Moreover, the Ag-resistance mechanism was not an uptake/efflux system as Ag was rapidly accumulated and immobilized in dense deposits, most likely as Ag-sulfide.

Toluene treatment of cells decreased Ag accumulation, suggesting that it may in part be an energy-dependent mechanism. Toluene disturbs biological membranes, disrupting membrane structure and function (Serrano et al. 1973). It is also noteworthy that while cells were rendered non-viable by toluene treatment, no leakage of K^+ from cell suspensions was detected using a K^+-specific ion electrode. Low levels of Ag were accumulated at 2 °C by both the Ag-sensitive and resistant strains, also suggesting Ag accumulation may be energy dependent. Alternatively, an enzyme-linked Ag-resistance mechanism may be inhibited at low temperatures.

Ag was not removed by washing sensitive or resistant cells with EDTA or HCl. This suggests Ag was bound to the cell surface, and/or accumulated intracellularly. Currently, we are investigating the cellular location of

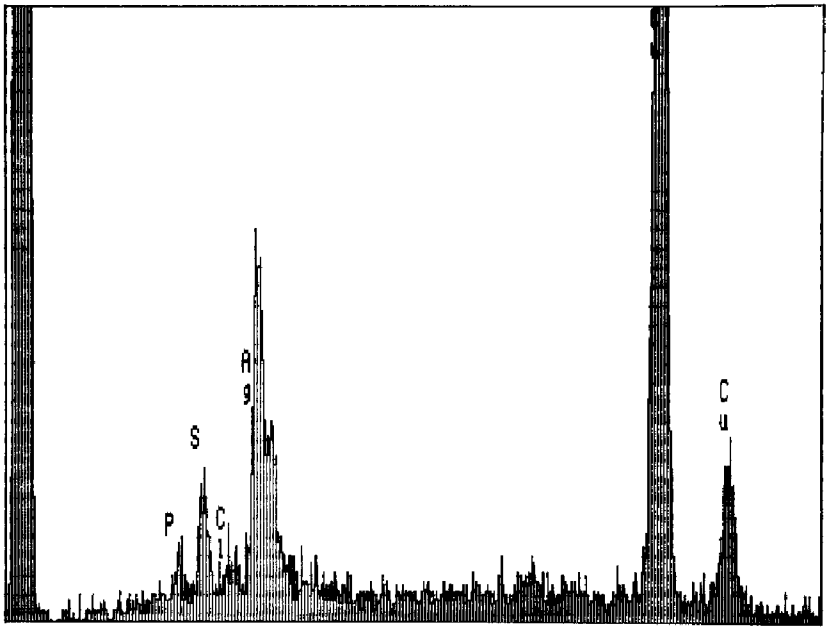

Fig. 6. Representative energy dispersive X-ray analysis of whole cell preparations of *P. stutzeri* AG259 after exposure to 0.5 mM $AgNO_3$, with high resolution of deposits. Scales are as in Fig. 5

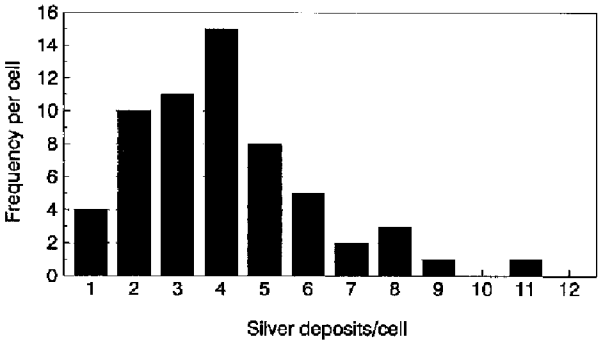

Fig. 7. Frequency histogram of Ag deposits in whole cells of *P. stutzeri* AG259. Deposits were tabulated as one deposit or cluster(s) of indistinguishable deposits

the accumulated Ag. Goddard and Bull (1989 a, b) reported that Ag accumulation by *Citrobacter intermedius* B6 was associated with the cell envelope. Adsorption of Ag to the surface of cells was discounted as the accumulation process. In another study, actively growing cells of *Escherichia coli* R1 did not accumulate Ag, yet remained resistant to the toxic metal (Starodub and Trevors 1989).

Providing a metabolizable C-source such as D-glucose to starved cells did not increase Ag accumulation. This supports the view that accumulation may not be an active uptake process. Common methods to inhibit active transport or energy-dependent efflux of metals in bacterial cells include use of chemical agents such as 2,4-DNP (uncoupler, proton-motive force inhibitor) (Bauda et al. 1987; Tynecka et al. 1981; Van Dyke et al. 1990), CCCP (uncoupler of oxidative phosphorylation), DCCD (N,N'dicyclohexylcarbodiimide, specific inhibitor of membrane-bound ATPase) (Bauda et al. 1987; Harold et al. 1969; Tynecka et al. 1981), valinomycin and nigercin (ionophores) (Tynecka et al. 1981), or incubation at 4 °C (Tynecka et al. 1981; Van Dyke et al. 1990). However, caution is necessary when interpreting decreased accumulation of a metal in the presence of metabolic inhibitors. For example, Bauda et al. (1987) reported decreased accumulation of Cd by *P. fluorescens* cells was not due to inhibitory effects of DNP and DCCD on metabolism. Rather, these inhibitors complexed Cd, making the metal less available to cells. Such complexation may explain the effect of 2,4 DNP on Ag accumulation. CCCP, which exhibited less complexation than 2,4 DNP to Ag^+ (Table 2) did not affect Ag accumulation by either strain. The research by Bauda et al. (1987) and this study suggest the use of metabolic inhibitors that complex metals may lead to incorrect conclusions regarding energy requirements for metal accumulation. It is necessary to first determine the complexation of each metal to each inhibitor. In addition, buffers that complex metals should not be used in accumulation assays (Hughes and Poole 1991). In situations where metals complex chemical inhibitors, alternative methods should be used to metabolically inhibit bacterial cells. Useful treatments may include incubation at 4 °C (Bauda et al. 1987; Tynecka et al. 1981; Van Dyke et al. 1990), toluene permeabilization of cell membranes or ultraviolet irradiation to kill cells prior to the accumulation assay (Van Dyke et al. 1990).

This study showed the Ag-resistant strain sequestered Ag as dense deposits. Electron dense deposits have been reported by Belly and Kydd (1982) in a Ag-resistant *Pseudomonas* sp. Both the Ag-resistant AG259 strain and the Ag-sensitive JM303 strain, produced H_2S as determined by the lead acetate test of MacFaddin (1976). It is possible the Ag-resistant strain produces higher levels of H_2S and /or intracellular sulfide that complex to Ag and form insoluble Ag-sulfide deposits as observed in electron micrographs. An Ag-sensitive derivative of *E. coli* R1 (designated S1) contained dense silver deposits as determined by electron microscopy and energy dispersive X-ray analysis (Starodub and Trevors 1989). Belliveau et al. (1991) reported on decreased H_2S production and

intracellular SH levels in mercury-resistant *Bacillus* spp. grown in the presence of $HgCl_2$. It was suggested that cellular sulfides complexed Hg^{2+} in these strains. In a similar manner, intracellular immobilization of Ag by sulfide may account in part for resistance of *P. stutzeri* AG259 to this metal. Aiking et al. (1982) reported that adaptation to Cd by *Klebsiella aerogenes* proceeded *via* formation of Cd sulfide. Pan-Hou and Imura (1981) observed that H_2S played a role in Hg resistance in *Clostridium cochlearium* T-2. In addition, H_2S production was plasmid-encoded in this strain.

Research is in progress to quantify production of H_2S in both the Ag-resistant and Ag-sensitive strains. Incubation at 2 °C during the assay may decrease H_2S production. It is possible that increased production of sulfide in the Ag-resistant strain plays a role in the resistance mechanism. A decrease in H_2S production may lower H_2S-Ag complexation, and this in turn can reduce Ag accumulation. Further studies and careful interpretation must be undertaken to elucidate mechanism(s) of Ag uptake and resistance.

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