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Silver accumulation in *Pseudomonas stutzeri* AG259

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Summary. Silver toxicity to *Pseudomonas stutzeri* AG259 was strongly dependent on the NaCl concentration in the medium, which reduced the availability of Ag^+ by precipitation as AgCl. Accumulation of Ag by growing cultures was low being $\leqslant 7.5$ nmol (mg dry mass)$^{-1}$ over all treatments examined. The presence of NaCl in the growth medium did not markedly affect the amounts of Ag accumulated by the cells but influenced toxicity as manifest by a lag period which was greatest at low NaCl concentrations ($\leqslant 0.1\%$ mass/vol.). In NaCl-free medium, *P. stutzeri* did not grow in the presence of 0.5 mM $AgNO_3$ in contrast to Ag-free controls. The majority of Ag accumulation by resting cells of *P. stutzeri* occurred within 1 min of incubation and there was little difference in uptake capacities between cells previously grown in the absence or presence of $AgNO_3$. Lowest amounts of Ag uptake by resting cells occurred when suspended in 1 mM Mes pH 6.5, containing 1% (mass/vol.) NaCl. Prior exposure of *P. stutzeri* to $Cu(NO_3)_2$ resulted in a marked reduction in Ag uptake when suspended in 1 mM Mes pH 6.5, containing 0.5 mM $AgNO_3$.

Key words: Silver accumulation — Silver toxicity — *Pseudomonas stutzeri* AG259

Introduction

The toxicity of Ag and other metals towards bacteria is well documented (Bragg and Rainne 1974; Gadd and Griffiths 1978; Sterritt and Lester 1980; Schreurs and Rosenberg 1982; Silver 1983; Trevors et al. 1985). Bacterial strains that are sensitive to Ag compounds accumulate Ag and become non-viable (Trevors 1987). However, several Ag-resistant bacterial strains have been isolated from a variety of environments that are able to grow in the presence of silver concentrations that are normally considered to be toxic to most bacterial strains (Annear et al. 1976; Bridges et al. 1979; Charley and Bull 1979; Hendry and Stewart 1979; Pooley 1982; Belly and Kydd 1982; Trevors 1987).

Some Ag-resistant bacteria, such as *Escherichia coli* R1 (Starodub and Trevors 1989) and *Pseudomonas stutzeri* AG259 are able to grow in the presence of Ag concentrations >0.5 mM (Haefeli et al. 1984). In contrast, Ghandour et al. (1988) found that batch growth of *E. coli* K12 was completely inhibited at $AgNO_3$ concentrations above 2.5 μM in a chloride-free medium. It was also reported that growth yields in chemostat cultures were diminished in the presence of added Ag^+ and this effect was moderated by Cu^{2+}, possibly by means of competition for binding sites on the cells (Ghandour et al. 1988). Other studies have shown that Ag-resistance in *E. coli* was dependent on chloride concentration (Silver 1983). Relatively few studies have been carried out on silver accumulation in bacteria. Ghandour et al. (1988) found that total Ag accumulated by *E. coli* K12 was about 67 mg (g dry mass)$^{-1}$, a higher value than those reported for a range of Ag-resistant bacterial strains isolated from polluted soils. Pümpel and Schinner (1986) found accumulation ranged over 7.3–44 mg (g dry mass)$^{-1}$. There are other examples of bacterial accumulation up to 3000 mg (g dry mass)$^{-1}$ (Charley and Bull 1979). In contrast, Ag-resistant *P. stutzeri* AG259 accumulated approximately 2 mg (g dry mass)$^{-1}$ when grown on agar supplemented with 5 mM $AgNO_3$

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(Haefeli et al. 1984). This organism was originally isolated from the soil of a silver mine and is known to contain a non-conjugative 49.4-MDa plasmid (PKK1) that is responsible for Ag-resistance (Haefeli et al. 1984). However, no information is available that identified the resistance mechanism(s).

This paper examines Ag uptake and toxicity by both growing and resting cells of *P. stutzeri* AG259 and the influence of NaCl and Cu^{2+} in an attempt to further understand possible mechanism(s) of Ag-resistance in this organism.

Materials and methods

Organism, and growth conditions. A silver-resistant strain of *Pseudomonas stutzeri* AG259, originally isolated from the soil of a silver mine (Haefeli et al. 1984), was maintained at 25° C on agar of composition ($g\,l^{-1}$): D-glucose, 10; yeast extract (Lab M), 5; tryptone (Oxoid), 10; agar (Oxoid, Technical Grade 3), 15; $AgNO_3$, 0.085 (=0.5 mM). Cultures were grown at 25° C on an orbital shaker (100 rev min^{-1}) in liquid medium of the above composition, but omitting the agar, and adjusted to pH 6.5 using 6 M HNO_3. Medium was supplemented with NaCl and/or $AgNO_3$ from concentrated stock solutions as appropriate.

Silver uptake. An overnight culture of *P. stutzeri* was harvested by centrifugation (70000 *g*, 10 min) washed twice with and finally resuspended in either (a) 1 mM 2(N-morpholino)ethanesulphonic acid (Mes) adjusted to pH 6.5 using solid tetramethylammonium hydroxide, (b) 1 mM Mes pH 6.5 containing 1% (mass/vol.) NaCl or (c) liquid growth medium, pH 6.5, to an absorbance at 550 nm, of approximately 0.5. Cell suspensions were incubated on an orbital shaker (120 rev min^{-1}) at 25° C for 10 min prior to the addition of $AgNO_3$ from a 0.1 M stock solution. At time intervals, 1.5-ml aliquots were centrifuged (8000 *g*, 20 s). Cells were washed twice with 1 mM Mes pH 6.5 and finally resuspended in 1 ml 6 M HNO_3 and incubated for 1 h at 100° C. Silver concentrations in acid digests of cells or supernatant buffer were determined using a Pye Unicam SP9 atomic absorption spectrophotomter, after dilution with distilled deionised water, with reference to appropriate $AgNO_3$ standards. For analysis of silver uptake during growth, 1-ml aliquots were taken at intervals and treated as described above.

Agar diffusion assays for toxicity assesment. Single-well agar diffusion assays employed the agar medium described above (10 cm^3 per 90-mm-diameter Petri dish) adjusted to pH 6.5 using 6 M HNO_3. NaCl was also incorporated in this medium at concentrations ranging over 0–20 $g\,l^{-1}$. Plates were spread inoculated with 0.1 ml of a cell suspension in sterile distilled water ($A_{550} \approx 0.5$) and 20-µl aliquots of $AgNO_3$ or $Cu(NO_3)_2$ solutions were added to 6-mm-diameter wells in the plate centre. Plates were incubated at 25° C in the dark for 48 h prior to measuring the inhibition zone diameters. Two-dimensional gradient plates (Wimpenny 1981; Gadd 1986) were used to estimate the effect of NaCl on silver toxicity. Each square plate (10 × 10 cm) received 60 ml agar with or without 1% (mass/vol.) NaCl. Wells (1 × 7 cm) were cut aseptically in the agar on two adjacent sides and 2 ml 0.1 M $AgNO_3$ was placed in one well and 2 ml 2% (mass/vol.) NaCl placed in the other well. After a 24-h incubation at 25° C in the dark, diffusion gradients were stopped by inserting a stainless steel grid that divided the agar into compartments of 49 × 1 cm^2 (Wimpenny 1981). Each compartment was inoculated with approximately 1 µl of a cell suspension of *P. stutzeri* in sterile distilled deionised water ($A_{550} \approx 0.5$). Plates were incubated for 48 h at 25° C in the dark.

Results

The toxicity of Ag to *P. stutzeri*, as assessed using a single-well diffusion assay, was strongly dependent on the NaCl content of the assay medium. The largest zone of inhibition way obtained in NaCl-free medium whereas little or no toxicity was evident at NaCl concentrations >0.5% (mass/vol.) (Fig. 1a). In contrast, the concentration of NaCl had little influence on the toxicity of Cu except at 1% and 2% (mass/vol.) NaCl where some increase in inhibition zone diameters was recorded (Fig. 1b). Confirmation of the effect of NaCl can be seen from the results of the two-dimensional diffusion assay. *P. stutzeri* was able to grow at all the Ag concentrations along a linear Ag gradient in medium containing 1% (mass/vol.) NaCl whereas a toxic effect was clearly apparent in NaCl-free medium (Fig. 2A, B). The creation of

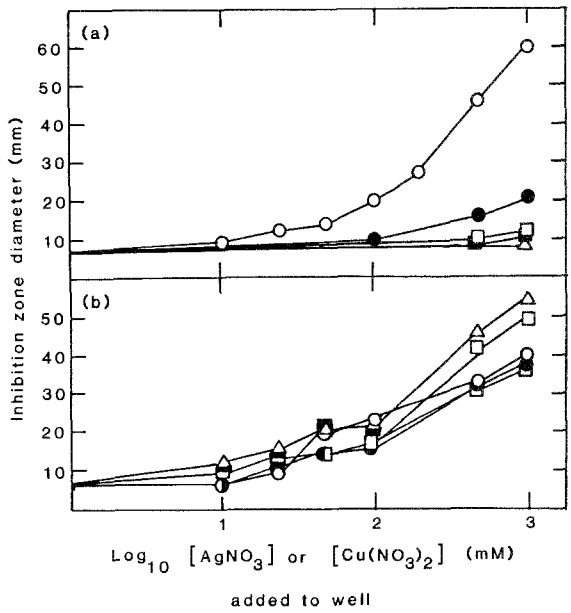

Fig. 1. Toxicity of **(a)** $AgNO_3$ and **(b)** $Cu(NO_3)_2$ towards *P. stutzeri* AG259 assessed using a single-well diffusion assay. The diameter of the diffusion well was 6 mm. Average values are shown from three separate determinations. Concentrations of NaCl in the agar were (% mass/vol.): (○) zero; (●) 0.1; (□) 0.5; (■) 1.0; (△) 2.0

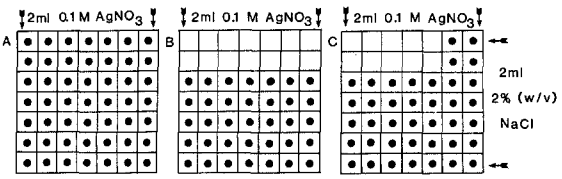

Fig. 2. Influence of NaCl on the toxicity of $AgNO_3$ to *P. stutzeri* AG259 assessed using single and two-dimensional gradient plates. **(A)** Single-dimension diffusion gradient of $AgNO_3$ in agar containing 1% (mass/vol.) NaCl; **(B)** single-dimension diffusion gradient of $AgNO_3$ in NaCl-free agar; **(C)** two-dimensional diffusion gradient of $AgNO_3$ *versus* NaCl in NaCl-free agar; (●) indicates growth. Typical results are shown from one of three experiments

an NaCl gradient at right-angles to the Ag gradient resulted in precipitation of AgCl at the interface between the two gradients and a reduction in Ag toxicity (Fig. 2C).

Ag accumulation by growing cultures of *P. stutzeri* was extremely low, being $\leqslant 7.5$ nmol (mg dry mass)$^{-1}$ over all treatments examined (Table 1). Furthermore, for a given culture, the specific amount of Ag accumulated decreased with increasing time of incubation. The presence of NaCl in the growth medium did not markedly affect the amounts of Ag accumulated by the cells but did influence toxicity as manifest by a lag period (Fig. 3a, b). The lag period in the presence of Ag was greatest at an NaCl concentration of 0.1% (mass/vol.; Fig. 3b) while in NaCl-free medium, no growth of *P. stutzeri* occurred in the presence of Ag over the duration of the experiment (Fig. 3a, b). The presence or absence of NaCl had little effect on the growth of Ag-free cultures (Fig. 3a).

When the uptake of Ag by resting cells of *P. stutzeri* was examined, it was found that the majority of uptake occurred within 1 min of incubation (Figs. 4–7). For cells pre-grown with 0.1 mM $AgNO_3$, uptake was maximal at a Ag^+ concentration of 1 mM with lower amounts of Ag^+ taken up at concentrations of 0.5 and 2.0 mM Ag^+ (Fig. 4). Uptake values for cells not previously exposed to Ag^+ were higher (Fig. 4) although it should be noted that values for Ag^+-exposed cells were corrected for their initial Ag content (approximately 25 nmol (mg dry mass)$^{-1}$). This means that there was little difference between Ag^+-exposed cells and non-exposed cells in respect of Ag accumulation (Fig. 4). With cells not previously grown with $AgNO_3$, Ag^+ uptake was again maximal at 1 mM $AgNO_3$ and lowest at 0.5 mM $AgNO_3$, values for 2 mM being intermediate between these two concentrations (Fig. 4).

When cells were suspended in liquid growth medium, the uptake of Ag^+ was lower than that recorded in Mes buffer, and was similar in both Ag-grown and control cells (Fig. 5). For Ag-grown cells, the highest values of uptake occurred in 0.5 and 1.0 mM $AgNO_3$ (Fig. 5). In control cells, uptake values were similar in both 1 and 2 mM

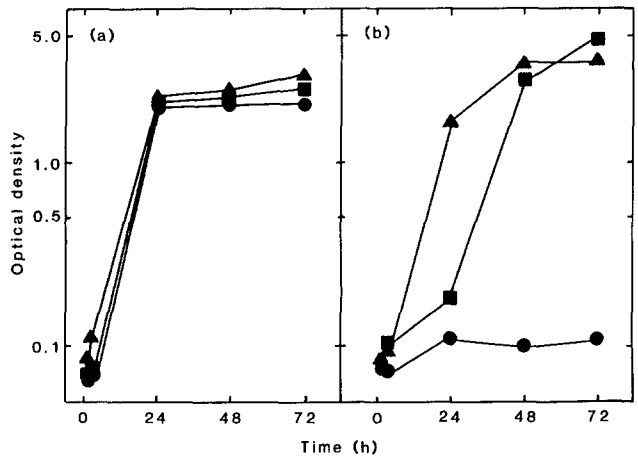

Fig. 3. Effect of $AgNO_3$ on the growth of *P. stutzeri* AG259 in liquid medium containing varying concentrations of NaCl. **(a)** $AgNO_3$-free controls; **(b)** 0.5 mM $AgNO_3$. NaCl concentrations (% mass/vol.) were (●) zero; (■) 0.1; (▲) 1.0. Average values are shown from three separate experiments

Table 1. Silver accumulation by *P. stutzeri* AG259 during growth in liquid medium containing 0.5 mM $AgNO_3$ and varying concentrations of NaCl

Time (h)	Silver accumulated [nmol (mg dry mass)$^{-1}$] with NaCl conc (% mass/vol.)					
	0.1%	0.25%	0.5%	1.0%	1.5%	2.0%
24	n.d.	7.5±1.1	5.6±0.4	5.4±0.3	n.d.	n.d.
48	4.4±0.1	4.3±1.1	3.7±0.6	3.4±0.2	4.3±0.2	6.7±0.3
72	3.3±0.1	3.6±0.2	3.3±0.6	3.5±0.4	3.3±0.1	1.7±0.5
96	2.5±0.1	3.2±1.2	2.5±0.1	2.1±0.2	1.2±0.5	1.3±0.1

Samples were taken from experimental flasks at biomass concentrations ranging over 1.5–4.0 mg dry mass (ml)$^{-1}$ (see also Fig. 3). Values shown are ±SEM; n.d. = not determined

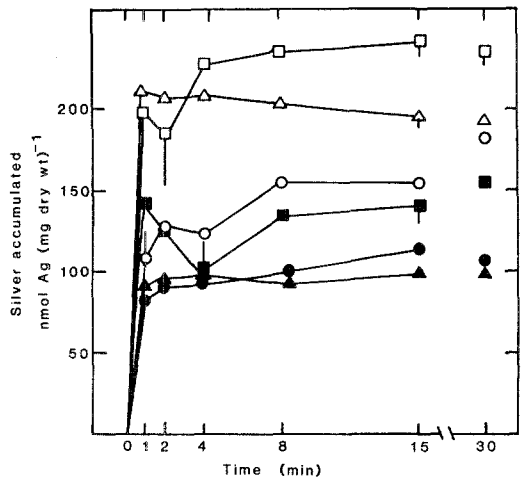

Fig. 4. Silver accumulation by resting cells of *P. stutzeri* AG259 suspended in 1 mM Mes pH 6.5 at 25° C. Open symbols refer to cells previously grown in 0.5 mM $AgNO_3$. $AgNO_3$ concentrations in uptake experiments were (○, ●) 0.5 mM; (□, ■) 1.0 mM; (△, ▲) 2.0 mM. Average values are shown, bars indicate ±SEM

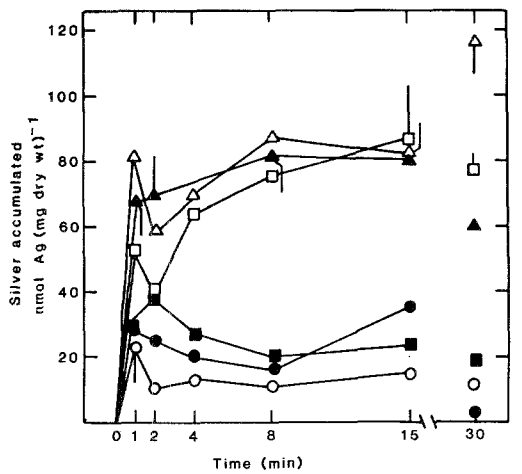

Fig. 5. Silver accumulation by resting cells of *P. stutzeri* AG259 suspended in liquid medium, pH 6.5, at 25° C. Open symbols refer to cells previously grown in the absence of $AgNO_3$; closed symbols to cells previously grown with 0.5 mM $AgNO_3$. $AgNO_3$ concentrations in uptake experiments were (○, ●) 0.5 mM; (□, ■) 1.0 mM; (△, ▲) 2.0 mM. Average values are shown bars indicate ±SEM

$AgNO_3$ with the lowest values occurring in 0.5 mM $AgNO_3$ (Fig. 5). Control and Ag-grown cells of *P. stutzeri* exhibited the lowest values of Ag^+ accumulation when suspended in 1 mM Mes pH 6.5 containing 1% (mass/vol.) NaCl and there was little difference between the $AgNO_3$ concentrations tested (Fig. 6).

The presence of glucose and/or Cu^{2+} had a significant effect on Ag^+ uptake. Control cells

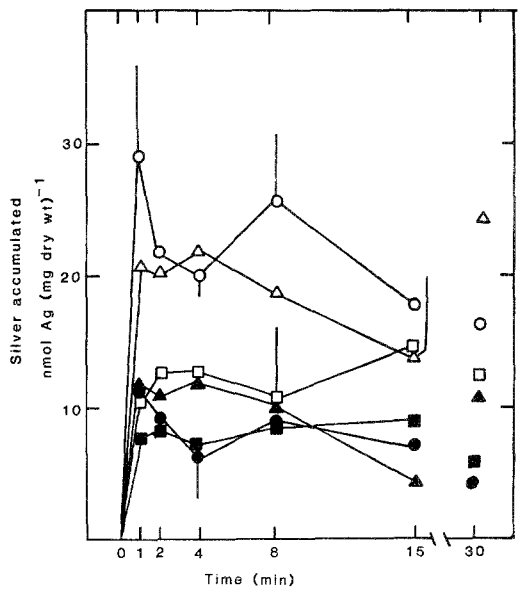

Fig. 6. Silver accumulation by resting cells of *P. stutzeri* AG259 suspended in 1 mM Mes pH 6.5 containing 1% (mass/vol.) NaCl at 25° C. Open symbols refer to cells previously grown in the absence of $AgNO_3$; closed symbols to cells previously grown with 0.5 mM $AgNO_3$. $AgNO_3$ concentrations in uptake experiments were (○, ●) 0.5 mM; (□, ■) 1.0 mM; (△, ▲) 2.0 mM. Average values are shown, bars indicate ±SEM

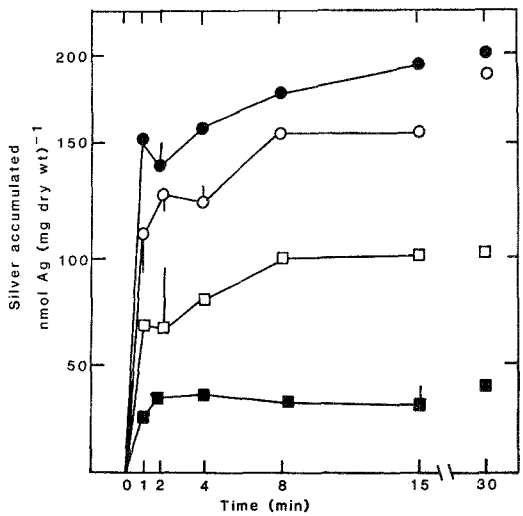

Fig. 7. Silver accumulation by resting cells of *P. stutzeri* AG259 suspended in 1 mM Mes pH 6.5 at 25° C in the absence (open symbols) or presence of 50 mM glucose (closed symbols). The $AgNO_3$ concentration used was 0.5 mM. (○, ●) Cells previously grown in the absence of $AgNO_3$; (□, ■) cells previously grown in liquid medium containing 0.5 mM $Cu(NO_3)_2$

took up slightly more Ag^+ in the presence of 50 mM glucose than in its absence (Fig. 7). However, cells previously grown in the presence of

0.5 mM $Cu(NO_3)_2$ took up approximately half the amount of Ag accumulated by the control cells in the absence of glucose. In the presence of glucose, Cu-exposed cells accumulated very little Ag^+ (Fig. 7).

Discussion

Some Ag-resistant strains of bacteria accumulate low amounts of Ag (Kaur and Vadehra 1986; Starodub and Trevors 1989). It seems likely that Ag-resistant cells must exclude Ag unless a mechanism for intracellular detoxification is possessed, such as synthesis of a metal-binding protein (see Gadd 1988). Decreased Ag accumulation may also be advantageous in preventing interference with the uptake of essential metal ions, e.g. Mg^{2+}, Mn^{2+}, Cu^{2+}, and phosphate (Schreurs and Rosenberg 1972).

While *P. stutzeri* AG259 appears to be markedly more Ag-resistant than most other bacteria, this study demonstrates that Ag toxicity, and therefore apparent resistant behaviour, is markedly influenced by the composition of the growth or suspending medium and that this also influences levels of Ag uptake. Maximal Ag toxicity in solid or liquid growth media occurred at zero or low concentrations of NaCl since, at high NaCl concentrations, toxic Ag^+ is removed from solution by the formation of insoluble AgCl. This can be contrasted with the influence of NaCl on copper toxicity to *P. stutzeri* where little effect was observed because of the solubility of $CuCl_2$. Similar features were observed in *Citrobacter* spp. where in the absence of Cl^-, cells with or without a silver-resistance plasmid were sensitive to Ag^+ (Silver 1981). It was proposed that sensitive *Citrobacter* strains strongly bound Ag^+ and removed it from the AgCl whereas cells possessing the resistance plasmid did not bind Ag^+ to the same extent (Silver 1981). NaCl is often a major component of bacterial culture media and the use of such media for the isolation of so-called resistant strains, or other experimentation, should be tempered by the realisation that any resistance observed may be indirect and due to the low biological availability of Ag^+. Not surprisingly, Ag accumulation by *P. stutzeri* was lowest in cells suspended in medium containing NaCl. Other binding interactions may also contribute to a lower availabilty of Ag^+ (Tilton and Rosenberg 1978). It should be noted, however, that in Ag-containing low-NaCl medium, *P. stutzeri* was able to grow after a lag period and reach similar biomass densities as Ag-free controls. It is not known whether this indicates the expression of an inducible resistance mechanism, the selection of resistant variants, reflects the time scale of Ag detoxification by medium components, or is a combination of these factors.

Plasmid-encoded Hg^{2+} resistance results in a reduction of Hg^{2+} to Hg^0 by mercuric reductase (Williams and Silver 1985; Foster 1987). The capacity to reduce Ag^+ to Ag^0 has been observed in a silver-resistant *Pseudomonas* strain (Belly and Kydd 1982). It is not clear whether Ag^+ reduction is related to silver resistance in *P. stutzeri* since the reductive capacities of silver-sensitive strains were identical to those of silver-resistant strains like AG259 (Haefeli et al. 1984).

The majority of Ag uptake by resting cells of *P. stutzeri* occurred during the first minute of incubation which suggests that Ag accumulation largely comprised non-specific binding to negatively charged groups and other ligands on and/or within the cells. That this is a relatively unspecific process, not relying on metabolism, is further borne out by the fact that the extent of Ag accumulation achieved in uptake experiments was similar in cells previously grown in the absence or presence of $AgNO_3$ (when Ag-exposed cells were corrected for their initial Ag content). In *E. coli*, Ag^+ uptake occurred at the cell surface and also inside the cells, both processes being independent of metabolism (Ghandour et al. 1988). Amounts of Ag^+ bound to cell surfaces and intracellularly in *E. coli* were determined as being 460 and 160 nmol (mg dry mass)$^{-1}$ respectively (Ghandour et al. 1988). For a series of bacterial strains tolerant to >0.1 mM Ag^+, accumulation ranged over 7.3–44 mg (g dry mass)$^{-1}$ with a mean value of 23 mg (g dry mass)$^{-1} \equiv 213$ nmol (mg dry mass)$^{-1}$ (Pumpel and Schinner 1986). *Thiobacillus ferrooxidans* accumulated approximately 50 nmol (mg dry mass)$^{-1}$ (Norris and Kelly 1978) while a multispecies bacterial community was capable of accumulating >3000 mg (g dry mass)$^{-1} \equiv 28$ μmol (mg dry mass)$^{-1}$ (Charley and Bull 1979). Levels of Ag accumulation by *P. stutzeri* were lower than most of these values, especially in growing cells, and this is clearly an important determinant of resistant behaviour.

Work with *E. coli* has demonstrated moderation of silver toxicity by added copper (Ghandour et al. 1988). Prior growth of *P. stutzeri* in the presence of $Cu(NO_3)_2$ resulted in approximately half the amount of Ag^+ being taken up by Cu^{2+}-grown cells compared to control cells. In the presence of glucose, Ag^+ accumulation was further

decreased in Cu^{2+}-grown cells. The mechanism for this remains unclear but may involve glucose affecting the energetic status of the cell membrane and/or the expression of an inducible resistance mechanism. Control cells took up slightly more Ag^+ in the presence of glucose but the overall pattern of uptake was similar in the absence and presence of glucose.

This study has demonstrated that the silver resistance of *P. stutzeri* is linked with low accumulation of Ag and this is highly dependent on the nature of the growth or suspension medium. Ag^+ toxicity and accumulation were greatly reduced in the presence of NaCl, due to precipitation of AgCl, though there were also indications that cellular attributes may have contributed to resistance. It is confirmed that Ag uptake by growing cultures markedly differs from Ag uptake by resting cells and these observations, together with the dramatic influence of medium composition on Ag uptake and toxicity, serve to stress the importance of cautious interpretation in studies concerned with bacterial interactions with heavy metals.

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