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Silver resistance in *Pseudomonas stutzeri*

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Silver resistance was studied in a silver-resistant *Pseudomonas stutzeri* AG259 strain and compared to a silver-sensitive *P. stutzeri* JM303 strain. Silver resistance was not due to silver complexation to intracellular polyphosphate or the presence of low molecular weight metal-binding protein(s). Both the silver-resistant and silver-sensitive *P. stutzeri* strains produced H_2S, with the silver-resistant AG259 strain producing lower amounts of H_2S than the silver-sensitive JM303 strain. However, intracellular acid-labile sulfide levels were generally higher in the silver-resistant *P. stutzeri* AG259 strain. Silver resistance may be due to formation of silver–sulfide complexes in the silver-resistant *P. stutzeri* AG259 strain.

Keywords: bacteria, hydrogen sulfide, *Pseudomonas stutzeri*, resistance, silver accumulation

Introduction

Silver is a biologically non-essential heavy metal used for jewelry, electroplating, photographic and medicinal purposes. Silver occurs in its native form as well as in ores such as argenite (Ag_2S), horn silver (AgCl), lead, lead–zinc, copper, gold and copper–nickel ores (Vernier 1992). Pure silver has a white, metallic lustre, with the highest electrical and thermal conductivity of all metals. The metal tarnishes upon exposure to ozone, H_2S or air containing sulfur (Vernier 1992).

The toxicity of silver to bacteria has been documented, and has led to its use in treatment of burns in patients and disinfection of water systems (Yahya *et al.* 1990). Silver ions at 86 μM inhibited oxidation of glucose, glycerol, fumarate, succinate, and D- and L-lactate by cell suspensions of *Escherichia coli* (Bragg & Rainnie 1974). Silver ions inhibited the respiratory chain at two sites; between *b*-cytochromes and cytochrome *d*; and between the site of substrate entry into the respiratory chain and flavoprotein in the NADH and succinate dehydrogenase regions. Antimicrobial activity of silver results primarily from its complexation to membranes, enzymes, nucleic acids and other cellular components. Silver ions complex to electron donor groups containing nitrogen, oxygen and sulfur (Grier 1977) present in silver-sensitive microbial cells as amines, hydroxyls, phosphates and thiols. As with other heavy metals, silver reacts readily with sulfur-containing compounds (Grier 1977).

Competition with silver for binding sites on bacterial surfaces by other metal ions such as copper has been reported to reduce silver toxicity. For example, 0.66 μM Ag^+ exerted a smaller toxic effect toward *Escherichia coli* K12 when 2.16 μM Cu^{2+} was also present (Ghandour *et al.* 1988). Hence, silver toxicity may depend on the $[Ag^+]/[Cu^{2+}]$ ratio, rather than $[Ag^+]$ alone. It is possible that silver competes for cellular entry by a copper transport system.

Accumulation of silver by various bacteria has been reported, although a specific mechanism has not been established. Intracellular metal accumulation offers a greater uptake capacity than simple silver binding to the cell surface (Hughes & Poole 1989). Modak & Fox (1973) showed that $^{110+}$Ag was detected intracellularly in exposed *Pseudomonas aeruginosa* cells, where most of the silver was complexed to DNA. Binding of $^{110+}$Ag to RNA and the cell wall/membrane fractions was about 1/40 and 1/10, respectively, of the amount bound to DNA. McNeilage & Whittingham (1984) also reported that silver bound to nucleic acids. Phillips & George (1968) reported 1 mol of Ag^+ binds 2 mol of

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adenosine. Further studies demonstrated binding to be reversible (Grier 1977). Silver stains proteins and has been used after gel electrophoresis to visualize protein bands (Merril *et al.* 1984). Clark *et al.* (1990) described an SSB-1 RNA-binding protein in the yeast *Saccharomyces cerevisiae* that was demonstrated to be a nucleolar-specific silver-binding protein in this organism.

Strains of *Thiobacillus* can accumulate excess amounts of silver from sulfide minerals (Pooley 1982). Electron micrographs revealed the bacterial membrane was covered with silver sulfide. As much as 25% of the bacterial biomass recovered after leaching of a silver sulfide mineral consisted of silver (Pooley 1982).

Goddard & Bull (1989) studied the ability of *Citrobacter intermedius* B6 to accumulate silver. They found that growing bacteria accumulated silver in dense deposits on the cell envelope. Cells harvested during mid or late exponential phase showed the highest levels of silver accumulation, about 4.35% (w/w) from an external concentration of 2.11 mmol Ag^+ l^{-1}. Accumulation increased with increasing external silver concentrations, although growing cells exhibited higher levels of silver accumulation below 200 μmol Ag^+ l^{-1}.

The composition of the growth medium can influence silver resistance, uptake and toxicity by affecting the metal's availability. For example, at a high NaCl concentration (1% w/v), Ag^+ ions were removed from solution by forming insoluble AgCl (Gadd *et al.* 1989). Further evidence that the presence of NaCl reduced the toxicity of silver was provided when silver accumulation was found to be the lowest in *Pseudomonas stutzeri* cells grown in a medium supplemented with NaCl (Gadd *et al.* 1989).

Traxler & Wood (1990) reported a coryneform bacterial strain isolated from metal-contaminated sediment could remove lead, cadmium, mercury, nickel, zinc and silver from an aqueous solution. After 10 min of incubation in a solution of 470 μM Ag^+, 18.5 μmol Ag mg^{-1} cell dry weight were accumulated. This value decreased to 15 μmol mg^{-1} dry weight cells after 130 min. This may indicate rapid silver binding followed by release of some metal into the external solution. Alternatively, cell lysis may have occurred followed by metal release.

To prevent accumulation of toxic levels of silver in bacterial cells, some cellular mechanism(s) of decreasing accumulation of free metal may exist. Gadd *et al.* (1989) stated that unless some type of intracellular detoxification mechanism exists, it is likely resistant cells exclude or efflux silver. Some silver-sensitive *E. coli* and *P. stutzeri* strains accumulate higher levels of silver than strains considered resistant (Gadd *et al.* 1989, Slawson *et al.* 1990, Starodub & Trevors 1990).

The mechanism of silver resistance in some bacterial strains may be plasmid-encoded. A silver-resistant *E. coli* R1 strain grew without an extended lag phase in the presence of 1.0 mM $AgNO_3$ and initial evidence indicated that the resistance was encoded on the 83 kb plasmid, pJT1 (Starodub & Trevors 1989). Electron microscopy and energy dispersive X-ray analysis showed the resistant strain did not accumulate silver as compared with a plasmid-cured sensitive strain. Although H_2S production was observed in both the sensitive and resistant strains, it was unclear if *E. coli* R1 could overproduce H_2S and if this was the mechanism of silver detoxification (Starodub & Trevors 1989, 1990).

The extent of silver-resistance may depend on silver-complexing components such as halide ions. Possibly, silver-sensitive cells bind Ag^+ tightly enough to extract it from AgCl and other complexed forms (Silver & Misra 1988). However, cells with silver-resistance plasmids do not successfully compete with other precipitates for silver. Haefeli *et al.* (1984) postulated the existence of a metallothionein in *P. stutzeri* AG259 which may protect cells by binding and inactivating silver.

In a previous study with *P. stutzeri* strains, electron microscopy and energy-dispersive X-ray analysis revealed the presence of dense silver deposits associated with cell surfaces of silver-resistant *P. stutzeri* AG259 cells but not with silver-sensitive *P. stutzeri* JM303 cells (Slawson *et al.* 1992). In this study, possible metal–phosphate interactions in *P. stutzeri* strains AG259 and JM303 were studied using ^{31}P-NMR spectroscopy. The possibility that silver may be complexed to low molecular weight metal binding proteins or bound to sulfide in both strains was also examined to provide additional knowledge on silver-resistance in *P. stutzeri* AG259.

Materials and methods

Microorganisms

P. stutzeri AG259 (Haefeli *et al.* 1984) was provided by Dr K. Hardy (Biogen SA, Switzerland). *P. stutzeri* JM303 was obtained from Dr J. Ingraham (University of California, Davis, CA). The silver-resistant *P. stutzeri* strain AG259 was maintained in Lennox L (LB) broth containing 0.5 mM $AgNO_3$. The silver-sensitive *P. stutzeri* JM303 strain was maintained in LB medium as no growth occurred on LB agar or in LB broth containing $AgNO_3$ at

a concentration as low as 0.05 mM. A type specimen of *P. stutzeri* (ATCC 17588) was purchased from the American Type Culture Collection (Rockville, MD) for taxonomic comparison. *P. stutzeri* strains were tested on the GN Biolog System (Biolog, Hayward, CA), according to manufacturer's specifications to confirm their identity. For long-term storage, cells were grown to late exponential phase (18 h), placed in 15% (v/v) sterile glycerol and stored at −80 °C.

Chemicals

Silver was used as a filter-sterilized (through 0.20 μm sterile nylon membrane) solution of $AgNO_3$. All media and solutions were prepared using ultrapure type I water from a Sybron/Barnstead Nanopure II Water System. Lennox L (LB) broth was obtained from Acumedia Manufacturers (Baltimore, MD). Janus Green B dye, PIPES (piperazine-*N*,*N*′-bis[2-ethanesulfonic acid]) buffer and sodium sulfide were purchased from Sigma (St Louis, MO).

Prior to use, all glassware and polypropylene flasks were washed in phosphate-free detergent, followed by a minimum of 1 h soaking in 10 mM EDTA, 5 rinses with deionized water and a minimum of 1 h in dilute (2%, v/v) HCl. Ultrapure water was used for the final five rinses.

Inocula and glassware preparation

Inoculum cultures of *P. stutzeri* were prepared by adding one loopful of cells from an LB agar slant to 10 ml of LB broth (pH 6.8), contained in 50 ml Erlenmeyer flasks at 28 °C, in the dark with shaking at 200 r.p.m. for 18 h. A 0.5 ml aliquot of each culture was added to a 250 ml Nalgene polypropylene flask containing 50 ml LB broth. Nalgene polypropylene flasks were used to reduce silver binding to container surfaces. Cultures were grown for 18 h at 28 °C with shaking at 200 r.p.m.

Dye displacement of surface-bound silver

Cells were harvested by centrifugation at 8000 × *g* for 10 min at 4 °C, washed twice in 5 mM sterile PIPES buffer and resuspended in sterile PIPES buffer. Aqueous Janus Green B dye was added to 18 ml of cell suspension (about Log 8 cells/ml) to a final concentration of 1 mM and the mixture incubated for 30 min at 28 °C. Cells were centrifuged at 8000 × *g* for 15 min, washed and resuspended in 20 ml of 5 mM PIPES and shaken at 120 r.p.m. at 28 °C. At specific intervals, 1.0 ml aliquots were removed, centrifuged for 30 s at 15000 × *g* and washed twice in 5 mM PIPES. Silver concentrations were determined using a Buck Scientific atomic absorption spectrophotometer as described by Slawson *et al.* (1992) after digestion in 6N ultrapure HNO_3 for 1 h at 80 °C. The concentrations of Janus Green B dye in supernatant fluids were determined using a Unicam spectrophotometer (Philips, Cambridge, UK) at 615 nm. Sample concentrations were compared with standard curves generated using 1–50 μmol Janus Green B. During dye exposure and silver accumulation experiments, the pH of the buffer remained at 6.8.

Analysis of low molecular weight cellular proteins

Duplicate cultures of *P. stutzeri* AG259 and JM303 were harvested after 18 h growth, washed three times in sterile 5 mM PIPES buffer, centrifuged at 8000 × *g* and resuspended in 2 ml of the same buffer. Cells were cooled on ice and disrupted by sonication with an Artek Model 300 Sonic Dismembrator at 60% capacity (30 s bursts, with 30 s cooling periods in between) until the suspensions were clear. Cell-free extracts were cleared by centrifugation at 8000 × *g* for 10 min at 4 °C. Cell extracts were also transferred to micro-spin filter units (300000 molecular weight cut-off; Diamed Supplies, Mississauga, Ontario, Canada) and centrifuged for up to 2 h at 2200 × *g* at 4 °C to remove high molecular weight material.

Protein concentrations in filtrates were determined spectrophotometrically with the Bio-Rad Protein Dye reagent using bovine serum albumin as the standard. Samples were read at 595 nm in a Unicam spectrophotometer. The protein content of each sample was diluted to 0.05 mg ml^{-1} prior to injection of a 0.1 ml sample. A Bio-Rad HPLC system equipped with a model 1740 UV/Vis detector was used for protein analysis. Protein fractionation was achieved on a Bio-Sil SEC-125 column (300 × 7.8 mm), eluted by degassed 0.05 M sodium sulfate and 0.02 M sodium phosphate (pH 6.8) buffer at a flow rate of 1 ml min^{-1}. Protein fractions were collected and analyzed for silver content using atomic absorption spectrophotometry.

Analysis of cellular polyphosphate content by nuclear magnetic resonance (NMR) spectroscopy

Cultures of *P. stutzeri* were grown for 18 h in LB broth, both in the absence and presence of $AgNO_3$ (strain JM303 was exposed to 0.05 mM silver, strain AG259 to 0.5 mM levels). Cells were harvested by centrifugation and approximately 2.5 g cells (wet weight) were resuspended in 10 ml fresh LB broth and transferred to a 15 mm NMR tube. D_2O was added to the cell suspensions to give a final concentration of 7% (v/v) and a 0.25 ml aliquot of SAG 471 antifoam (Harrison and Crosfield Canada Ltd., Calgary, Canada) was added to control foaming, as the samples were oxygenated by sparging with air at a rate of 40 ml min^{-1}. ^{31}P-NMR spectra were acquired in 15 min blocks with a recycle time of 1 s and a flip angle of 60° using a 15 mm ^{13}C–^{31}P dual-tuned switchable probe on a Bruker AM-400 wide-bore spectrometer, operating in the Fourier Transform mode. A capillary containing 0.2 M methylene diphosphonic acid (MDP) was used as a calibration standard and was set to −18.56 ppm. The decoupling power during acquisition was 2 W. To produce cell extracts, 2.5 g cells pregrown as described above, were harvested by centrifugation and resuspended in 10 ml PIPES buffer (5 mM, pH 8.0) containing 30 mM EDTA. Cells were cooled with dry ice and broken in three cycles

with glass beads in a Bead Mill. After removal of cell debris by centrifugation, D_2O was added to the supernatant to give a final concentration of 7% (v/v) prior to NMR analysis, essentially as described above, except that the samples were not sparged with air.

Qualitative analysis of H_2S production

Production of H_2S gas was determined qualitatively using the lead acetate strip test (MacFaddin 1976). LB broth (10 ml) was aseptically transferred to sterile 15 ml centrifuge tubes and individually inoculated with *P. stutzeri* strains. Lead acetate strips (Fisher Scientific, Toronto, Canada) were folded over the lip of the tube and secured with the cap leaving a space between the strip and the medium. Cultures were incubated at 28 °C with shaking at 100 r.p.m. for up to 3 weeks and monitored daily for blackening of the lead acetate strip, a positive test for H_2S production. Both silver-resistant and silver-sensitive *P. stutzeri* strains were grown in the presence and absence of $AgNO_3$. Additional treatments included LB broth amended with 0.02% (w/v) sodium thiosulfate and 0.02% (w/v) ferrous ammonium sulfate for comparative purposes.

Quantitative sulfide analysis

Production of H_2S and intracellular inorganic sulfide in *P. stutzeri* strains AG259 and JM303 was analyzed by the method of King & Morris (1967). Cells were grown at 28 °C with shaking at 200 r.p.m. for 18 h in 50 ml flasks containing 10 ml LB broth amended with 0.5 or 0.01 mM $AgNO_3$, for the silver-resistant AG259 and silver-sensitive JM303 strains, respectively. A 1% (v/v) inoculum was transferred to 5 ml LB broth contained in 50 ml flasks. All treatments were prepared in triplicate. Flasks were sealed with a gas tight Suba seal rubber stopper to prevent escape of liberated H_2S gas and incubated at 28 °C with shaking at 200 r.p.m. for 18 h. Flasks had serum stoppers fitted with polypropylene center-wells (Kontes Glassware Instruments, Vineland, NJ) which contained 0.5 ml zinc acetate solution (2.6% w/v in water) to trap H_2S.

The center-well was removed from the serum stopper after incubation and placed in a 15 ml polypropylene tube to which was added 0.7 ml of deoxygenated water. After brief mixing, 0.1 ml of 6% (w/v) NaOH was added and the contents mixed by vortexing for 1 min. To each tube was added 0.26 ml of diamine reagent [*N*,*N*-dimethyl-*p*-phenylenediamine; 0.1% (w/v) in 5N HCl] and the mixture shaken until clear. A 0.1 ml volume of 0.0115 M $FeCl_3$ in 0.6N HCl was added, the tubes vortexed for 1 min and incubated at 22 °C for 30 min, after which 0.85 ml of distilled water was added and the suspension mixed for 10 s by vortexing.

To determine inorganic acid-labile sulfide production, 1 ml of harvested and washed cells was mixed with 0.4 ml of 6% (w/v) NaOH and the mixture incubated for 15 min at 95 °C in 15 ml polypropylene tubes. After cooling at 22 °C for 30 min, 0.4 ml deoxygenated water and 0.5 ml zinc acetate solution (2.6% w/v in water) were added and the mixture shaken at 1000 r.p.m. for 1 min at 22 °C on a Thermolyne Sybron Type 50000 Maxi-Mix III shaker (Barnstead/Thermolyne, Dubuque, IA). A 0.25 ml sample of diamine reagent was added and shaken, and $FeCl_3$ and distilled water added as in the quantification of H_2S. The suspension was centrifuged at 4000 × *g* for 10 min. Samples were read for methylene blue content at 670 nm using a Unicam spectrophotometer against a reagent blank and a standard series containing 5–65 nmol Na_2S. The OD of methylene blue was proportional to sulfide concentration. The levels of H_2S gas and intracellular inorganic acid-labile sulfide produced were expressed on a gram dry weight cells basis. Dry weights were determined by filtering 1 ml of culture onto predried and preweighed 0.20 μm nylon filters, and drying at 80 °C overnight in a vacuum oven. All values are expressed as averages of three replicates. Statistical analyses were performed using the Costat Statistical Package (Cohort, Berkeley, CA). ANOVA and Student–Newman–Keuls tests were performed at the 95% confidence level.

Results and discussion

The Biolog GN identification protocol uses 95 different carbon sources to obtain substrate utilization data unique for each bacterial strain. Using this system, the silver-resistant *P. stutzeri* AG259 strain yielded a 90% similarity to the *P. stutzeri* type strain while the silver-sensitive JM303 strain exhibited 68.3% similarity. Of the 95 carbon substrates in the Biolog GN test, *P. stutzeri* strains AG259 and JM303 exhibited similar utilization patterns for 84 substrates, but different patterns for 11 carbon sources. The latter substrates are summarized in Table 1. The silver-resistant AG259 strain metabolized several carbon sources not metabolized by the silver-sensitive JM303 strain.

Table 1. Selected GN Biolog metabolic test reactions

Carbon source	*P. stutzeri* AG259	*P. stutzeri* JM303
Glycogen	+	−
D-Fructose	+	−
D-Psicose	+	−
D-Trehalose	+	−
D-Saccharic acid	+	−
L-Aspartic acid	+	−
L-Pyroglutamic acid	+	−
L-Serine	−	+
L-Threonine	−	+
γ-Amino butyric acid	+	−
Glycerol	+	−

+ denotes metabolism; − denotes no utilization of carbon substrate.

A method for the semi-quantitative assessment of metal ion binding to microbial cell surfaces was described by Savvaidis *et al.* (1990). This method is based on the affinity of cationic dyes for anionic cell surfaces and the ability of some metal ions to compete with and reverse dye binding. The ability of silver to displace Janus Green B dye from *P. stutzeri* AG259 and JM303 cells was tested. The dye concentration in the supernatant fluid reflects the amount of unbound dye after removal of cells. The silver-resistant *P. stutzeri* AG259 strain complexed dye more tightly than the silver-sensitive *P. stutzeri* JM303 strain, as shown by lower concentration of unbound Janus Green B dye in the supernatant fluid (Figure 1).

The amount of dye remaining in the supernatant after cells were exposed to 0.5 mM $AgNO_3$ was also measured. If silver displaces surface-bound dye, this would be detected by an increase in the dye concentration in the supernatant. This was observed with the silver-resistant *P. stutzeri* AG259 strain (Figure 1A), whereby the dye concentration after 30 min increased about 2 μmol. Therefore, the silver-sensitive *P. stutzeri* JM303 strain showed a reduced capacity to bind Janus Green B dye compared with the silver-resistant strain. For *P. stutzeri* AG259, a relatively constant dye concentration was measured in the supernatant over the incubation period. However, in the supernatant fluid of *P. stutzeri* JM303, the dye concentration decreased gradually (Figure 1B). Upon exposure to silver, the available dye concentration decreased, indicating an increase in dye binding affinity in the presence of the metal. Dye displacement by silver occurred in the silver-resistant strain, but not in the silver-sensitive strain. The silver-sensitive JM303 strain initially accumulated silver rapidly, followed by a slow decrease phase (Figure 1B).

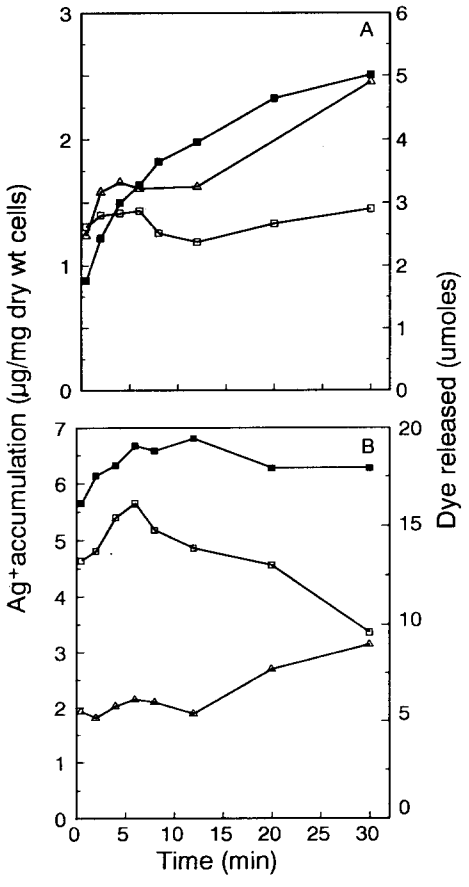

Figure 1. Displacement of surface bound Janus Green B dye by 0.5 mM $AgNO_3$ in resting cell suspensions of (**A**) *P. stutzeri* AG259 and (**B**) *P. stutzeri* JM303. (□) Janus Green B concentrations after exposure; (△) Janus Green B concentrations after subsequent exposure to 0.5 mM $AgNO_3$; (■) silver accumulation during exposure to Janus Green B.

The dye binding–displacement assay is suitable for measuring passive ion exchange at the cell surface. Silver accumulation in both *P. stutzeri* strains was decreased when negatively charged sites on the cell surface were unavailable (Figure 1). The difference in dye binding affinity between the two *P. stutzeri* strains may reflect a difference in their response to silver exposure. If specific entry sites for silver were blocked by dye, silver uptake may be inhibited.

The possibility that silver-resistance in *P. stutzeri* AG259 may be related to the presence of cysteine-rich, low molecular weight metal binding protein(s) similar to those reported by Higham *et al.* (1984) was investigated. Protein standards ranged from 1250 to 670000 Da (Figures 2A and 3A). The cell-free extracts of both *P. stutzeri* strains showed similar protein groups. The silver-sensitive JM303 strain produced high molecular weight proteins during exposure to silver (Figure 3E). All protein groups produced in the silver-resistant strain could be accounted for in the silver-sensitive strain (Figures 2 and 3). Higham *et al.* (1984) isolated three low molecular weight (6700, 6900 and 3600 Da) Cd^{2+}-binding cysteine-rich proteins from *P. putida*. Since proteins of molecular weight lower than 17000 Da were not evident in cell-free extracts of either *P. stutzeri* strain, it is unlikely that metallothionein(s) was produced.

Cell-free extracts were also passed through a molecular sieve (300000 Da) prior to analysis to remove high molecular weight proteins and non-protein material. Proteins in the 6000–8000 Da range, typical of metallothionein (Thomas *et al.* 1986), were not evident (Figure 4). The results suggested

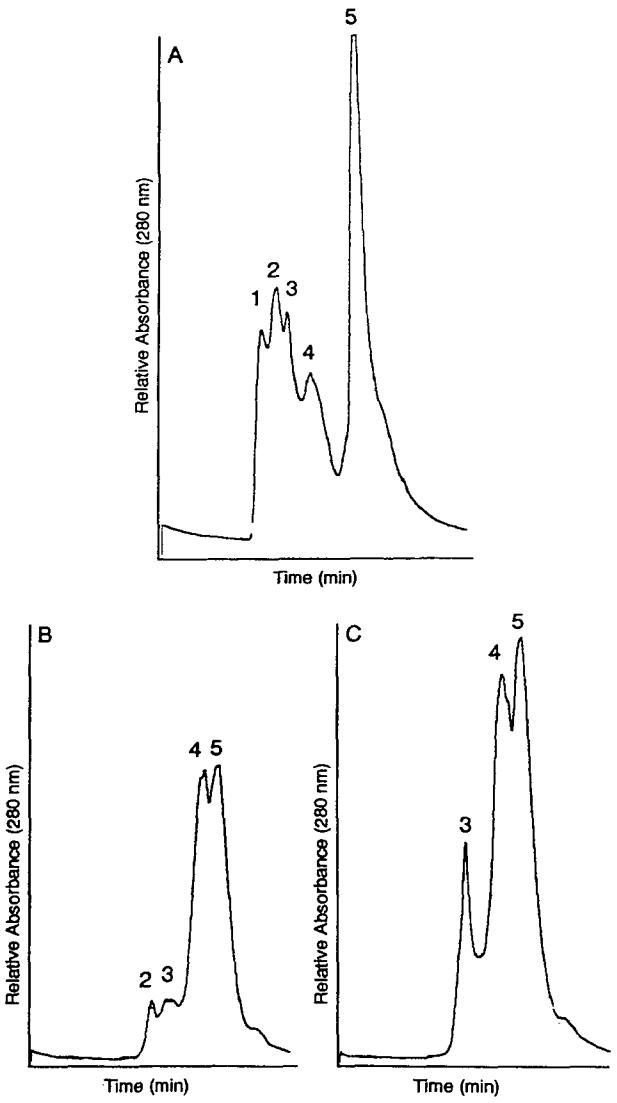

Figure 2. HPLC protein profiles of (**A**) protein standards (Da): 1, 670 000; 2, 158 000; 3, 44 000; 4, 17 500; 5, 1350. Cell-free extracts of *P. stutzeri* AG259 prepared from (**B**) cells grown in LB broth and (**C**) cells grown in LB broth containing 0.5 mM $AgNO_3$. Time (x-axis) for separation was about 25 min.

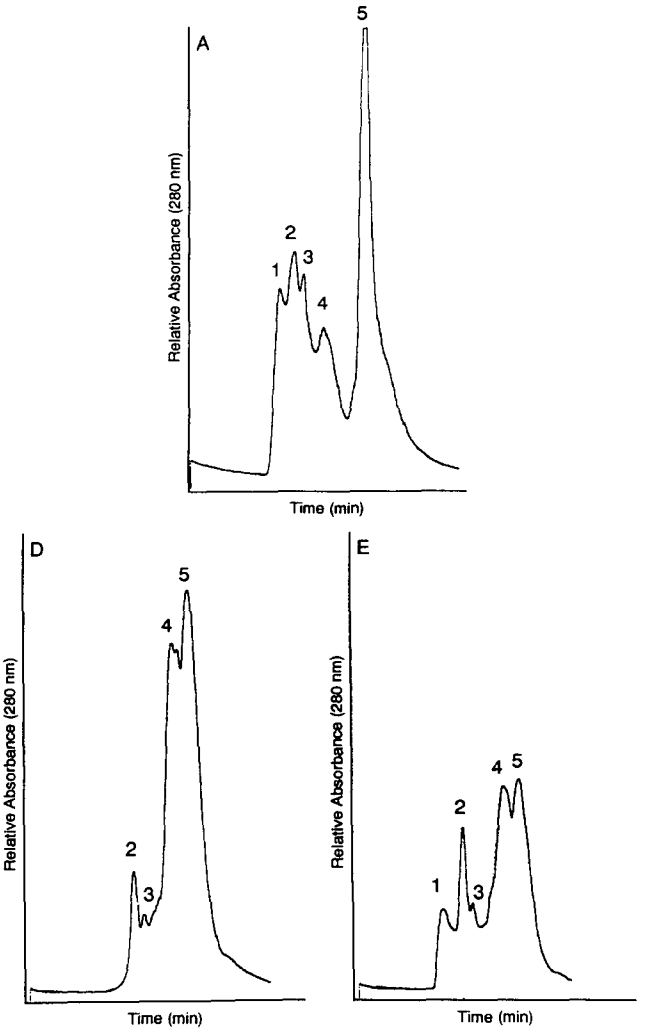

Figure 3. HPLC protein profiles of (**A**) protein standards as in Fig. 2. Cell-free extracts of *P. stutzeri* JM303 prepared from (**D**) cells grown in LB broth and (**E**) cells grown in LB broth containing 0.01 mM $AgNO_3$. Time (x-axis) for separation was about 25 min.

that silver resistance in *P. stutzeri* was not due to low molecular weight metal-binding protein(s).

Protein fractions collected from HPLC analyses for *P. stutzeri* AG259 and JM303 strains were analyzed for silver content by atomic absorption spectrophotometry. This technique has been used by Higham *et al.* (1984) to detect Cd^{2+}-binding proteins in a *Pseudomonas putida* strain. In protein samples from strain AG259 grown in the presence of silver, low amounts of silver, less than 0.25 $\mu g\,ml^{-1}$, were detected (data not shown). Other protein fractions from different treatments did not contain detectable silver. It was concluded that silver was not present in high concentration in the protein fractions.

The possibility that silver resistance in whole cells of *P. stutzeri* AG259 may be associated with production of intracellular polyphosphate was examined by NMR spectroscopy. The assignment of various phosphate-specific peaks was based on previously published spectra involving yeast (Navon *et al.* 1979, den Hollander *et al.* 1981, Nicolay *et al.* 1982, Lohmeier-Vogel *et al.* 1989) and bacteria (Lohmeier-Vogel *et al.* 1986). In both the presence and absence of silver, high levels of sugar phosphomonoesters were visible in the oxygenated cell suspensions, as well as a large peak due to inorganic Pi in the medium (Figure 5). Aside from some

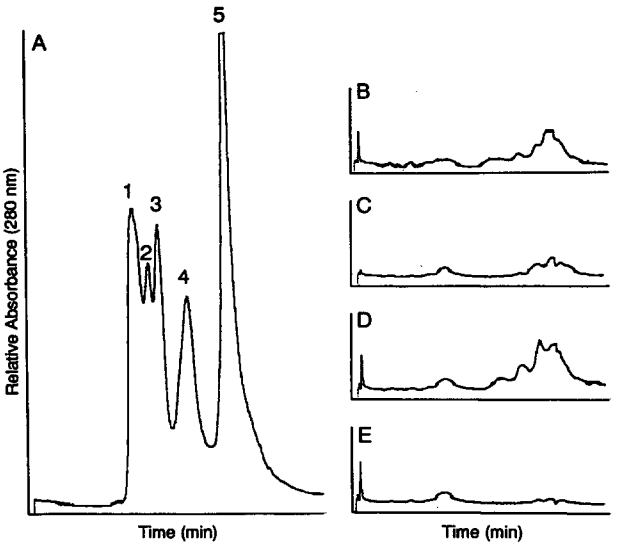

Figure 4. HPLC protein profiles of (**A**) protein standards as in Figure 2. Cell-free extracts prepared from (**B**) *P. stutzeri* AG259 cells grown in LB broth, (**C**) *P. stutzeri* AG259 cells grown in LB broth containing 0.5 mM $AgNO_3$, (**D**) *P. stutzeri* JM303 cells grown in LB broth and (**E**) *P. stutzeri* JM303 cells grown in LB broth containing 0.01 mM $AgNO_3$. All samples were passed through a 300 000 Da molecular sieve prior to HPLC analysis. Time (x-axis) for separation was about 25 min.

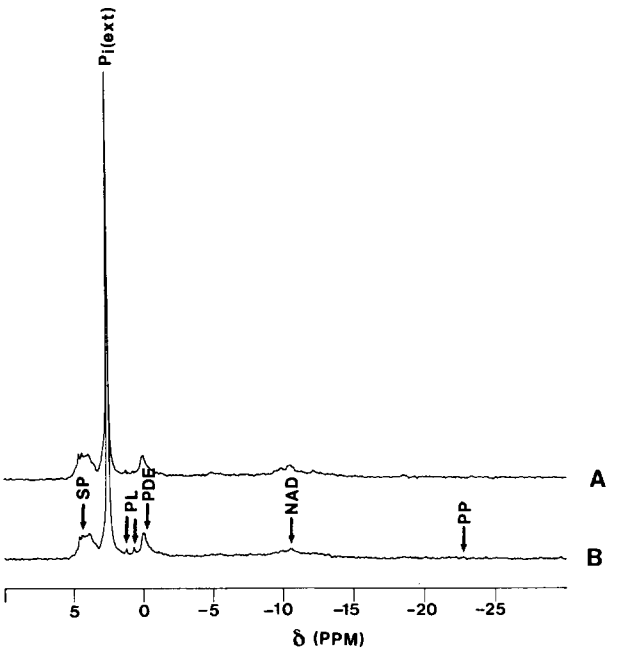

Figure 5. Typical 15 min ^{31}P-NMR spectra of oxygenated cell suspensions of *P. stutzeri* AG259 pregrown (**A**) in the absence of silver and (**B**) in the presence of 0.5 mM $AgNO_3$. SP, phosphomonoesters (mainly sugarphosphates); $Pi_{(ext)}$, extracellular Pi; PL, phospholipid components; PDE, phosphodiester resonances associated with cell wall components; NAD, location of nicotinamide adenine dinucleotide (phosphate); PP, polyphosphate resonances.

phospholipid and cell wall associated material, there was little evidence for any other intracellular phosphorylated metabolites, especially the compound of interest, polyphosphate. *P. stutzeri* JM303 was also analyzed by NMR (results not shown) and gave essentially identical spectra.

The technique of ^{31}P-NMR is able to visualize only mobile phosphorylated metabolites (Lundberg *et al.* 1990). The possibility exists that intracellular polyphosphate could be complexed with silver as an NMR-invisible silver–polyphosphate precipitate in *P. stutzeri* AG259. For this reason, extracts were prepared by disrupting the cells with glass beads in the presence of EDTA, which should chelate the silver and solubilize the polyphosphate component. However, even under these conditions, polyphosphate was not visible in the ^{31}P-NMR spectra (Figure 6), although previously invisible ATP resonances did appear in the spectra of the extracts, as well as a peak most likely attributable to pyrophosphate. However, the results with cell extracts showed no discernible difference between the ^{31}P-NMR spectra of silver-resistant and silver-sensitive *P. stutzeri* strains. As such, it is unlikely that intracellular polyphosphate is involved in binding silver. Results indicated that silver resistance in *P. stutzeri* AG259

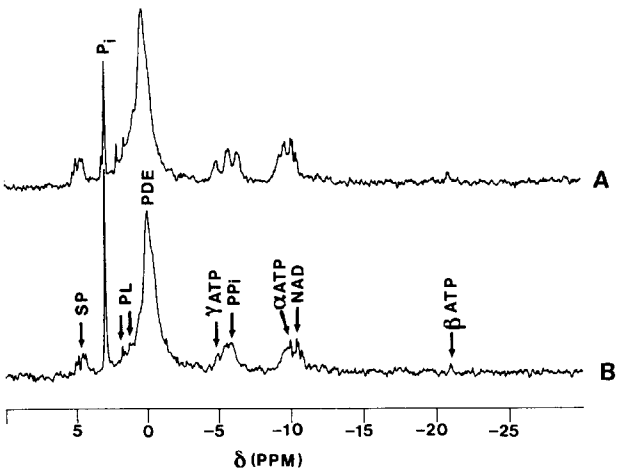

Figure 6. Typical 15 min ^{31}P-NMR spectra of cell extracts of *P. stutzeri* AG259 pregrown (**A**) in the absence of silver and (**B**) in the presence of 0.5 mM $AgNO_3$. Abbreviations are the same as in Figure 5. ATP α, β and γ resonances, as well as pyrophosphate peaks (PPi) are visible in these spectra.

was not due to either protein or intracellular polyphosphate production. Further investigation focused on the role of H_2S.

P. stutzeri may be unique among pseudomonads in being able to produce H_2S gas (MacFaddin 1976). This ability was first determined qualitatively in the *P. stutzeri* strains using the lead acetate test (MacFaddin 1976). This method is based on the reaction of H_2S with lead acetate to produce lead sulfide, water and acetate. Sulfate was supplemented in some growth media to determine if H_2S production could be enhanced. In general, the silver-sensitive JM303 strain produced blackening of the lead acetate strips sooner than the silver-resistant strain (Table 2). Addition of sulfate did not significantly alter H_2S production in AG259 cells, but in JM303 cells, H_2S evolution decreased as compared with treatments not amended with sulfate.

In addition to blackening of the lead acetate strip, a dark precipitate was produced in the bottom of some of the culture tubes. Cells of both strains grown in unsupplemented LB medium did not produce any black precipitate in tubes. In LB broth supplemented with sulfate, a black precipitate developed in the tube containing silver-resistant cells, but this was not as distinct as with the silver-sensitive JM303 strain. In LB broth amended with silver only, no precipitate was detected with the silver-sensitive strain, but a black precipitate was formed by the silver-resistant strain. Both strains formed a black precipitate in tubes amended with silver and sulfate. Growth of the silver-resistant *P. stutzeri* AG259 strain was associated with a darkening of the broth. H_2S production, as detected by the lead acetate test, was more pronounced in culture tubes that did not contain a precipitate.

Some of the black precipitates were collected, after decanting cells from the tubes, and analyzed for silver content (Table 3). In treatments without silver (A and B), silver concentrations were low (below 0.5 $\mu g\,ml^{-1}$) for both strains. In treatments containing silver (C and D), AG259 cells exhibited higher silver levels in both the supernatant and precipitate compared with JM303 cells. In the silver-sensitive JM303 strain, silver levels in the supernatant were higher than those in the precipitate. In the silver-resistant *P. stutzeri* AG259 strain, the precipitate contained higher silver levels than the supernatant in LB broth amended with silver. However, the addition of sulfate reduced this effect.

Further analyses were performed to quantify H_2S and intracellular acid-labile sulfide production by both *P. stutzeri* strains. Quantitative measurements of H_2S produced confirmed qualitative findings from the lead acetate strip test. The silver-sensitive strain generally produced higher levels of H_2S than the silver-resistant strain and this effect was more pronounced in the presence of silver (Table 4).

Table 2. Qualitative analysis of H_2S production in silver-resistant *P. stutzeri* AG259 and silver-sensitive *P. stutzeri* JM303[a]

P. stutzeri strain	Treatment[b]	Relative blackening of lead acetate strip over time (days)					
		2	4	5	6	7	14
AG259	A	−	−	−	−	+	++
	B	−	−	−	−	−	−
	C	−	−	−	−	−	+
	D	−	−	−	−	−	−
JM303	A	−	+	++	++	++	+++
	B	−	−	−	−	−	+
	C	−	+	++	++	++	+++
	D	−	−	−	−	−	+

[a]All treatments were performed in duplicate for each strain.
[b]Treatments represent the growth medium and were as follows:
A: LB broth.
B: LB + 0.02% (w/v) sodium thiosulfate + 0.02% (w/v) ferrous ammonium sulfate.
C: LB + $AgNO_3$ (AG259, 0.5 mM; JM303, 0.01 mM).
D: LB + 0.02% (w/v) sodium thiosulfate + 0.02% (w/v) ferrous ammonium sulfate + $AgNO_3$ (AG259, 0.5 mM; JM303, 0.01 mM).
−, no change in lead acetate paper.
+, slight blackening of lead acetate paper.
++, moderate blackening of lead acetate paper.
+++, very dark blackening of lead acetate paper.

Table 3. Silver concentrations present in supernatant and precipitate during qualitative assay for H_2S production[a]

P. stutzeri strain	Treatment	Silver concentration ($\mu g\,ml^{-1}$)	
		supernatant	precipitate
AG259	A	0.43	ND
	B	0.44	0.18
	C	12.34	36.20
	D	20.15	17.46
JM303	A	ND	0.10
	B	0.13	0.12
	C	1.62	0.22
	D	1.21	0.16

[a]Values were means of two determinations.
A: LB broth.
B: LB + 0.02% (w/v) sodium thiosulphate + 0.02% (w/v) ferrous ammonium sulfate.
C: LB + $AgNO_3$ (AG259, 0.5 mM; JM303, 0.01 mM).
D: LB + 0.02% (w/v) sodium thiosulphate + 0.02% (w/v) ferrous ammonium sulfate + $AgNO_3$ (AG259, 0.5 mM; JM303, 0.01 mM).
ND, not detected.

Table 4. Quantification of H_2S and intracellular acid-labile sulfide production in *P. stutzeri* AG259 and *P. stutzeri* JM303 after 18 h incubation

P. stutzeri strain	Treatment	Sulfide production[a] (nmol S^{2-} mg^{-1} dry cell wt)	
		H_2S	-SH
AG259	LB	10.5 (b,c)	49.2 (b)
AG259	LB + 0.5 mM Ag	0.98 (c)	39.7 (b)
JM303	LB	44.9 (b)	77.7 (a)
JM303	LB + 0.01 mM Ag	115.3 (a)	14.3 (c)

[a]Values were means of triplicate treatments.
[b]Non-significant (95% level) ranges (followed by same letter) were obtained from Student–Newman–Keuls Test.

During repeated trials, the silver-resistant *P. stutzeri* AG259 strain generally produced higher levels of inorganic acid-labile sulfide than the silver-sensitive JM303 strain. Once again, this effect was more pronounced in the presence of the metal. However, cells of both strains generally showed a reduction in sulfide production when grown in the presence of silver (Table 4) and this effect was enhanced during incubation up to 48 h (results not shown). However, *P. stutzeri* strain AG259 did not produce significantly different (95% level) concentrations of H_2S in LB broth with or without silver amendment even though the value measured was 10-fold lower (Table 4). During incubation, sulfide may be too tightly bound to silver to be displaced using zinc acetate. In this assay, precise measurement of inorganic acid-labile sulfide was difficult in the presence of silver, as the cationic metal may interfere with the coupling reaction (King & Morris 1967). The 10-fold decrease was not significantly different as a result of variability in the data. The analysis of H_2S in the presence of a metal like silver is difficult due to the high affinity of the metal for H_2S. In addition, treatments amended with silver showed a general darkening of the medium, which was more pronounced with the silver-resistant *P. stutzeri* AG259 strain. Since the method of King & Morris (1967) quantifies sulfide through methylene blue production, blackening of the medium may interfere with the colorimetric reaction.

To determine the effect of silver on sulfide quantification, a sulfide standard curve was prepared in the presence of 0.5 mM $AgNO_3$ and compared with a control series in the absence of silver. The presence of silver significantly affected the detection of measurable sulfide (data not shown). King & Morris (1967) suggested the coupling reaction between zinc sulfide and the diamine reagent may be inhibited in the presence of heavy metals. Therefore, quantification of H_2S may be more reliable since the metal does not interfere with the test reaction.

As both silver-resistant and silver-sensitive *P. stutzeri* strains produced H_2S, it was expected that quantification of H_2S would be possible. However, as indicated during the qualitative assay, H_2S produced by the silver-resistant AG259 strain may be trapping silver, forming insoluble AgS. This type of metal sequestration is common in sulfate-reducing bacteria (Brierley *et al.* 1985). Therefore, during quantitative assays, it was expected that with the silver-resistant AG259 cells, inorganic acid-labile sulfide levels would be higher than H_2S levels. This effect was observed in cells after 18 h incubation (Table 4). In comparison, intracellular acid-labile sulfide levels in the silver-sensitive JM303 strain were expected to be lower than the silver-resistant strain. Further quantitative analyses were not pursued as the silver and the darkened medium interfered with the test reactions.

Immobilization of silver by sulfide may account in part for resistance of strain AG259 to this metal. After 18 h incubation (Table 4), inorganic acid-labile sulfide was reduced in the presence (39.8 nmol mg^{-1} dry weight) of silver compared with cells grown in the absence (49.2 nmol mg^{-1} dry weight) of the metal. However, as indicated earlier, these values were not significantly different at the 95% level. Instead, higher sulfide levels would be required to immobilize the metal, however, if the AgS complex could not be dissociated in the presence of zinc acetate, the lowered sulfide levels would reflect the amount available for measurement.

Aiking *et al.* (1982) reported that adaptation to cadmium by *Klebsiella aerogenes* proceeded via formation of cadmium sulfide. They observed electron-dense granules on the cell surface of cultures grown in the presence of cadmium. Granules were absent in cultures grown without the metal (Aiking *et al.* 1982). The presence of the granules supported their conclusion that formation of CdS was an important detoxification mechanism for this microorganism (Aiking *et al.* 1982). Pooley (1982) also demonstrated AgS particle formation during studies on the ability of *Thiobacillus ferroxidans* and *T. thiooxidans* to accumulate silver.

Pan-Hou & Imura (1981) observed that H_2S has a role in mercury resistance in *Clostridium cochlearium* T-2. The production of H_2S in this strain

Table 5. H_2S production in selected metal-resistant bacteria

Bacteria	Metal resistance	H_2S production		Source
		cited[a]	reported	
P. stutzeri AG259	Ag	(+)	+	Slawson *et al.* (1992)
C. intermedius	Ag	−	+	Goddard & Bull (1989)
E. cloacae	Ag	−		Nakahara *et al.* (1989)
E. coli R1	Ag	−	+	Starodub & Trevors (1989)
Bacillus sp.	Hg	NA	+	Belliveau *et al.* (1991)
P. putida	Cd[b]	−		Higham *et al.* (1984)
K. aerogenes	Cd	−	+	Aiking *et al.* (1982)
C. cochlearium	Hg	(+)	+	Pan-Hou & Imura (1981)
T. ferroxidans	Ag	NA		Pooley (1982)
T. thiooxidans	Ag	NA		Pooley (1982)

[a]Cited from Holt (1984), unless otherwise stated.
[b]Cited from MacFaddin (1976).
NA, not reported specifically for this microorganism in Holt (1984).
(+) represents a variable ability to produce H_2S.

inactivated inorganic mercury. A detoxification mechanism involving decomposition of methylmercury was also evident. Both activities were associated with the presence of a plasmid (Pan-Hou & Imura 1981). The ability of a silver-resistant *E. coli* R1 strain isolated from a burn patient to produce H_2S was compared with a silver-sensitive strain and both were positive using the lead acetate strip test. However, dense silver particles were found to be associated with the silver-sensitive strain in this study (Starodub & Trevors 1989). However, since *E. coli* R1 did not accumulate silver, this indicates that the silver resistance mechanism in this microorganism differs from that in *P. stutzeri* AG259.

The ability of metal-resistant strains to produce H_2S is summarized in Table 5. Almost all strains were reported to produce H_2S. It is possible that in bacteria which have a metal resistance associated with sulfide production, the resistance mechanism may be related to the ability to produce, or enhance production of hydrogen sulfide.

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