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# Silver, copper, lead and zinc accumulation by *Pseudomonas stutzeri* AG259 and *Streptomyces albus*: electron microscopy and energy dispersive X-ray studies

B. Mattuschka, G. Straube & J. T. Trevors*

*Institute of Bioengineering, Martin-Luther University of Halle, Merseburg, Germany and *Department of Environmental Biology, University of Guelph, Ontario, Canada*

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**Metal accumulation by a silver-resistant *Pseudomonas stutzeri* AG259 strain and a *Streptomyces albus* strain was investigated in a mixed metal solution of silver, copper, lead and zinc. The location of silver, lead and copper on cells was determined by transmission electron microscopy coupled with an X-ray analysis system. In *P. stutzeri* cells silver was detected as dense deposits on the cells. Copper and lead were distributed over the cells. *S. albus* accumulated these metals only on part of cells with a higher concentration per cell than in *P. stutzeri*.**

**Keywords:** accumulation, copper, electron microscopy, lead, *Pseudomonas stutzeri*, silver, *Streptomyces albus*, X-ray analysis, zinc

## Introduction

The high affinity of metal ions to microbial cells has been documented by several researchers (Hutchins *et al.* 1986, Gadd 1989, Hughes & Poole 1989, Mullen *et al.* 1989). Various microbial species have been studied for their ability to accumulate metals, e.g. algae, yeasts, fungi and bacteria (Kuyucak & Volesky 1988, Trevors & Cotter 1990, White & Gadd 1990, Junghans & Straube 1991, Cotoras *et al.* 1992, Farah & Sneddon 1992). The mechanism of metal complexation to microbial cells depends on the ultrastructure of the microorganism (Gram-positive or Gram-negative), growth phase of cells and environmental conditions, such as ion concentration, physical and chemical properties of metallic ions, nutrient availability, and pH. The energy-independent binding of metals to microbial cell surfaces, energy-dependent metal transport and complexation of metals to extracellular capsules are three main mechanisms of microbial metal binding (Beveridge 1986, Scott & Palmer 1988, Ford & Mitchell 1992, Slawson *et al.* 1992a).

Address for correspondence: B. Mattuschka, Institute of Bioengineering, Martin-Luther University of Halle, Merseburg 06217, Germany.

Electron microscopy is a useful method to study metal complexation to cells. For example, the intracellular accumulation of germanium in *Pseudomonas putida* cells was examined by electron microscopy (Klapcinska & Chmielowski 1986). The difference in silver accumulation between silver-resistant and -sensitive strains of *Eschericha coli* was shown by transmission electron microscopy (Starodub & Trevors 1989). The location of cadmium and the chemical nature of bound cadmium in *Klebsiella aerogenes* and a *Citrobacter* sp. were investigated using electron microscopy and X-ray analysis (Aiking *et al.* 1984, Macaskie *et al.* 1987). The staining effect of metals on cells increases the contrast of the cells and makes the location of some metals visible. Electron microscopy coupled with an X-ray detection system gives the possibility to analyze the elemental distribution in different areas of specimens. Wavelength dispersive X-ray microanalysis makes use of the fact that atoms, when struck by electrons from an external source, yield X-rays which are characteristic of those atoms (Chandler 1977).

*Pseudomonas stutzeri* AG259 was investigated in connection with silver resistance mechanisms and silver accumulation (Haefeli *et al.* 1984, Slawson *et al.* 1990, 1992b). The investigation of the

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Gram-positive *Streptomyces albus* in metal uptake was carried out in connection with former experiments with a *Streptomyces* strain (unpublished) which showed a high uptake capacity for silver, lead, chromium and selectivity for these metal ions. Actinomycetes were investigated for selective uranium sorption from mixed metal solutions (Nakajima *et al.* 1981). As compared with other species of microorganisms, actinomycetes removed higher amounts of uranium. For example, *Streptomyces griseoflavus* accumulated 143.52 mg $g^{-1}$ dry weight cells. High copper adsorption (5.3 mmol $g^{-1}$ dry weight cells) from a mixed metal solution of copper, uranium, lead, mercury, cadmium, zinc, nickel, cobalt and manganese was observed with *S. albus* (Nakjima & Sakaguchi 1986).

In our case, the parallel investigation of the Gram-negative *P. stutzeri* and the Gram-positive *S. albus* was carried out to study possible differences in metal accumulation.

## Materials and methods

### *Microorganisms*

*P. stutzeri* AG259 was maintained in Lennox L (LB) broth containing 0.5 mmol $l^{-1}$ silver nitrate at 28 °C. Cultures were grown in 100 ml of broth contained in a 500 ml Erlenmeyer flask for 18 h on a rotary shaker at 120 r.p.m. at 28 °C. For long-term storage the culture was maintained at −80 °C in 20% (v/v) sterile glycerol.

*S. albus* was obtained from the Department of Microbiology culture collection at the University of Guelph. *S. albus* was grown in 100 ml nutrient broth contained in 500 ml Erlenmeyer flasks amended with 1% (w/v) maltose for 42 h at 28 °C on a rotary shaker at 120 r.p.m.

### *Chemicals*

The metal solutions used in accumulation experiments were prepared with nitrate salts of silver copper, lead and zinc in 5.0 mmol $l^{-1}$ MES buffer (morpholinoethansulfonic acid) at pH 5.5. MES buffer exhibits neglible binding to metal ions and therefore is useful in metal accumulation studies. Each of the four metals was used at a concentration of 0.5 or 2.5 mmol $l^{-1}$ in a mixture for accumulation experiments. Ultrapure 6.0 M nitric acid was used for desorption of metals from the surface of cells.

### *Metal accumulation by cells*

*P. stutzeri* and *S. albus* cells were harvested by centrifugation at 5000 × *g* for 10 min at 4 °C. Cells were washed twice with MES buffer (pH 5.5) and harvested as before. Cells were suspended in the same buffer with double the cell concentration desired in the accumulation experiment. Cell density was determined by optical density at 600 nm and drying of samples at 105 °C for 24 h to determine the dry mass.

Cell suspensions were mixed in proportion 1:1 with metal solutions to yield an initial concentration of 0.5 mmol $l^{-1}$ of each metal and a cell mass of 3.0 g $l^{-1}$ dry weight/l. The mixture was contained in a 250 ml Erlenmeyer flask and shaken at 120 r.p.m. in the dark. Parallel 1 ml samples were periodically removed from flasks and centrifuged at 5000 × *g* for 5 min. The supernatant fluid was removed and stored at 4 °C for metal analysis. Cell pellets were washed once with MES buffer and digested in ultrapure 6 M $HNO_3$ for 1 h at 80 °C. Metal concentrations were measured using an acetylene flame atomic absorption spectrophotometer as described by Slawson *et al.* (1992a,b).

### *Electron microscopy and energy dispersive X-ray analysis*

Cells from metal accumulation studies and control cells not exposed to metals were fixed in 2% glutaraldehyde at 4 °C over night and embedded in 2% (w/v) Nobel Agar. The dehydration procedure was carried out with an acetone series (30, 50, 70, 95 and 100% acetone). Agar embedded samples were treated for 15 min with each of the acetone concentrations. The samples were kept over night in EPON 812 dissolved in acetone to 50%. The samples were then embedded in complete EPON 812 (plus 3% DMP 30 as hardener). Samples were dried at 60 °C over 2 days. Thin sections (60 nm thick) were prepared using an ultramicrotone equipped with a diamond knife. Sections were collected on carbon-Formwar coated 200 mesh copper or nickel grids. Electron microscopy was carried out with a Philips EM 300 electron microscope at 60 kV. The EM 400T electron microscope was equipped with an EDAX energy dispersive X-ray detector used for elemental analysis. The investigations were performed with an electron beam diameter of 0.4 $\mu$m and counts were collected for 100 s.

## Results

*P. stutzeri* and *S. albus* cells were exposed to a metal mixture of silver, lead, copper and zinc ions. The metal removal from aqueous solution is shown in Figures 1 and 2. The high accumulation rate reached equilibrium between 0.5 and 1.0 h. Metal accumulation after 3 h by *P. stutzeri* and *S. albus* is also summarized in Table 1.

Electron microscopy studies were carried out to determine where metal ions were located on cells after exposure to a mixed solution of silver, copper, lead and zinc. Ultrathin sections of *P. stutzeri* and *S. albus* cells not exposed and exposed to metal solutions are shown in Figures 3 and 4, respectively. The contact of cells with a mixed solution of 0.5 mmol $l^{-1}$ silver, copper, lead and zinc caused only minimal low staining of the cells (Figure 3B).

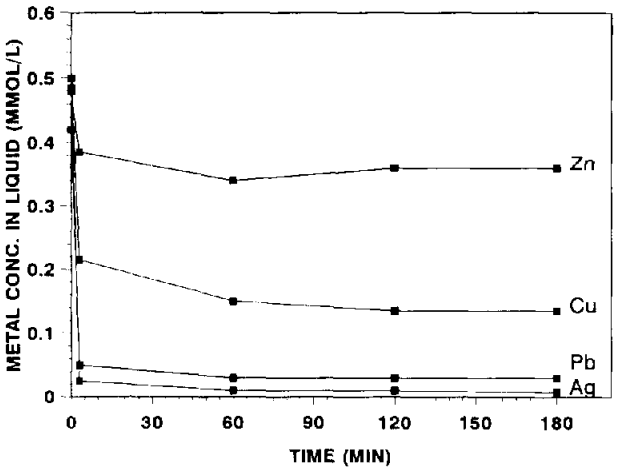


**Figure 1.** Metal accumulation removal by *P. stutzeri* AG259 from a mixed metal solution of 0.5 mmol $l^{-1}$ silver, copper, lead and zinc.

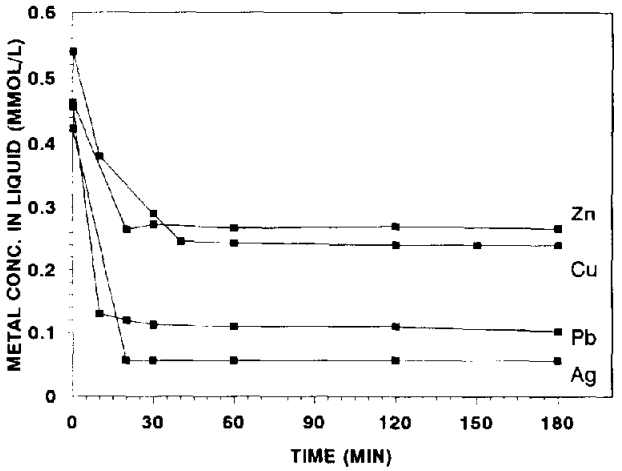


**Figure 2.** Metal accumulation by *S. albus* from a mixed metal solution of 0.5 mmol $l^{-1}$ of each silver, copper, lead and zinc.

The use of a more concentrated metal solution (2.5 mmol $l^{-1}$ of each metal) improved the staining effect (Figure 3C and D). Representative energy dispersive X-ray analysis graphs for *P. stutzeri* and *S. albus* samples are shown in Figures 5 and 6.

The analysis of whole *P. stutzeri* cells revealed that lead, copper and silver accumulated on the cells (Figure 5). The distribution of lead and copper seemed to be over the entire cell. Silver was concentrated in dense deposits on the cell wall as well as outside cells. The X-ray pattern of dense deposits revealed a silver peak and a weak copper peak (Figure 5C).

It was observed that only a part of *S. albus* cells accumulated high amounts of the metals. The dense cell wall areas of metal loaded cells were analyzed as lead, copper and silver. The spectra in Figure 6(B) shows high lead peaks (peaks from different electron stages), a low copper peak and a lower silver peak. The copper peaks in Figure 6(a and d) were caused by the cooper grid, whereas in Figure 6(b and c) nickel grids were used. The X-ray analysis revealed no information about zinc, because its signal was likely masked by the copper and nickel signals.

## Discussion

Silver resistance and accumulation were described by Slawson *et al.* (1992a,b) with the silver-resistant *P. stutzeri* AG259 in comparison with a silver-sensitive *P. stutzeri* strain JM303. Energy dispersive X-ray analyses revealed the association of dense silver deposits with the silver-resistant strain, but not with the silver-sensitive strain. Silver uptake by the resistant strain was determined to be about 20 $\mu g\, mg^{-1}$ dry weight (185 $\mu mol\, g^{-1}$ dry weight) (Slawson *et al.* 1992a,b).

In the present study metal accumulation from a mixed solution of silver, copper, lead and zinc, and localization of metals on the strain *P. stutzeri* AG259 was demonstrated. Accumulation of silver from a mixed metal solution was 142 $\mu mol\ Ag\ g^{-1}$ dry weight of *P. stutzeri* AG259. The amount of silver accumulated decreased compared with silver accumulation from silver solution only. This decrease was most likely because of the additional accumulation of other metal ions. It is noteworthy that silver was the metal accumulated to the highest level compared with lead, copper and zinc. This may be related to the fact that *P. stutzeri* AG259 is resistant to silver via a yet unexplained plasmid encoded mechanism. The electron micrograph in Figure 3(C) shows that only certain cells accumulated silver in high dense particles (black circles). It was not possible to analyze one part of the cell wall or only part of a cell, because of the width of the electron beam. Because of the location of electron dense areas, we assume that lead and copper were distributed over the entire cell whereas silver was concentrated in dense deposits on the cell or was found outside of cells, possibly on extracellular material as metal–sulfide complexes.

Large metallic deposits of elemental gold on *Bacillus subtilis* cell walls have been described (Beveridge & Murray 1976). The process of metal accumulation was a two-step deposition with, first, stoichoimetric binding of metal to reactive group within the wall and, second, nucleation of the deposition of more metal at the same location. Silver

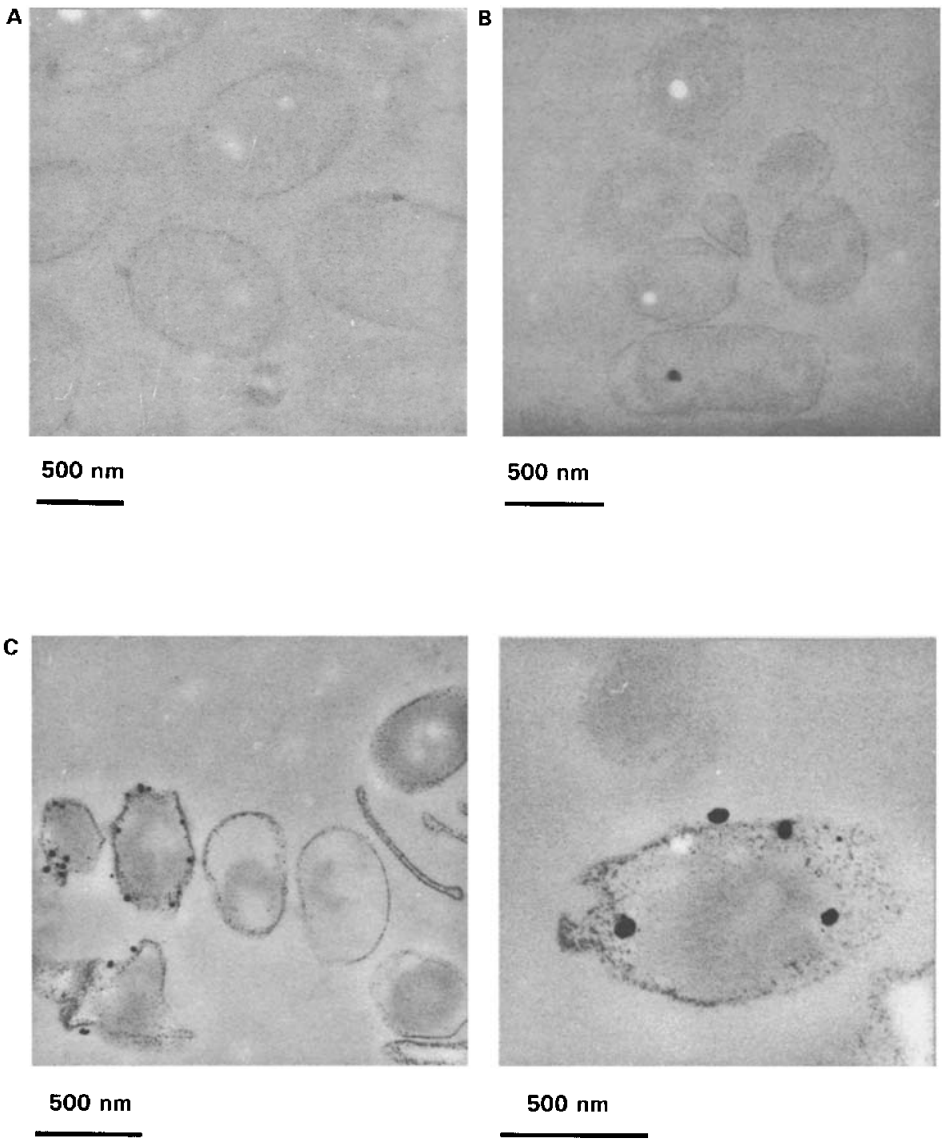


**Figure 3.** Electron microscopy analyses of thin-sections of *P. stutzeri* AG259. (**A**) Not exposed to metal, (**B**) after 3 h exposure to a mixed metal solution of 0.5 mmol $l^{-1}$ silver, copper, lead and zinc, and (**C**) after 3 h exposure to a 2.5 mmol $l^{-1}$ metal solution of each metal.

accumulation as dense deposits was also observed in *Citrobacter intermedius* B6 (Goddard & Bull 1989). The silver accumulation capacity was about 250 $\mu$mol $g^{-1}$ dry weight cells in the presence of 0.5 mmol $l^{-1}$ initial silver concentration. The dense silver deposits were located mostly on the cell wall and to some extent outside the cells.

The observed affinity of metals to *S. albus* cells was (Ag > Pb > Cu > Zn) was similar to previous reports. The fungus *Phoma* sp. bound metals from mixed solution in the following order of the amount of uptake: Ag > Cu > Pb > Ni > Cd (Pighi & Schinner 1989). In general, 32 fungi accumulated silver selectively from a multi-metal solution. Silver accumulation by *Phoma* sp., tolerating 1 mmol $l^{-1}$ Ag, was higher with increasing mycelium age. The metal uptake was determined for different Actinomycetes with an affinity of Pb, Cu > Zn, Co, Ni, Cd (Nakajima & Sakaguchi 1986). An explanation for the similar affinity of metal ions to different micro-

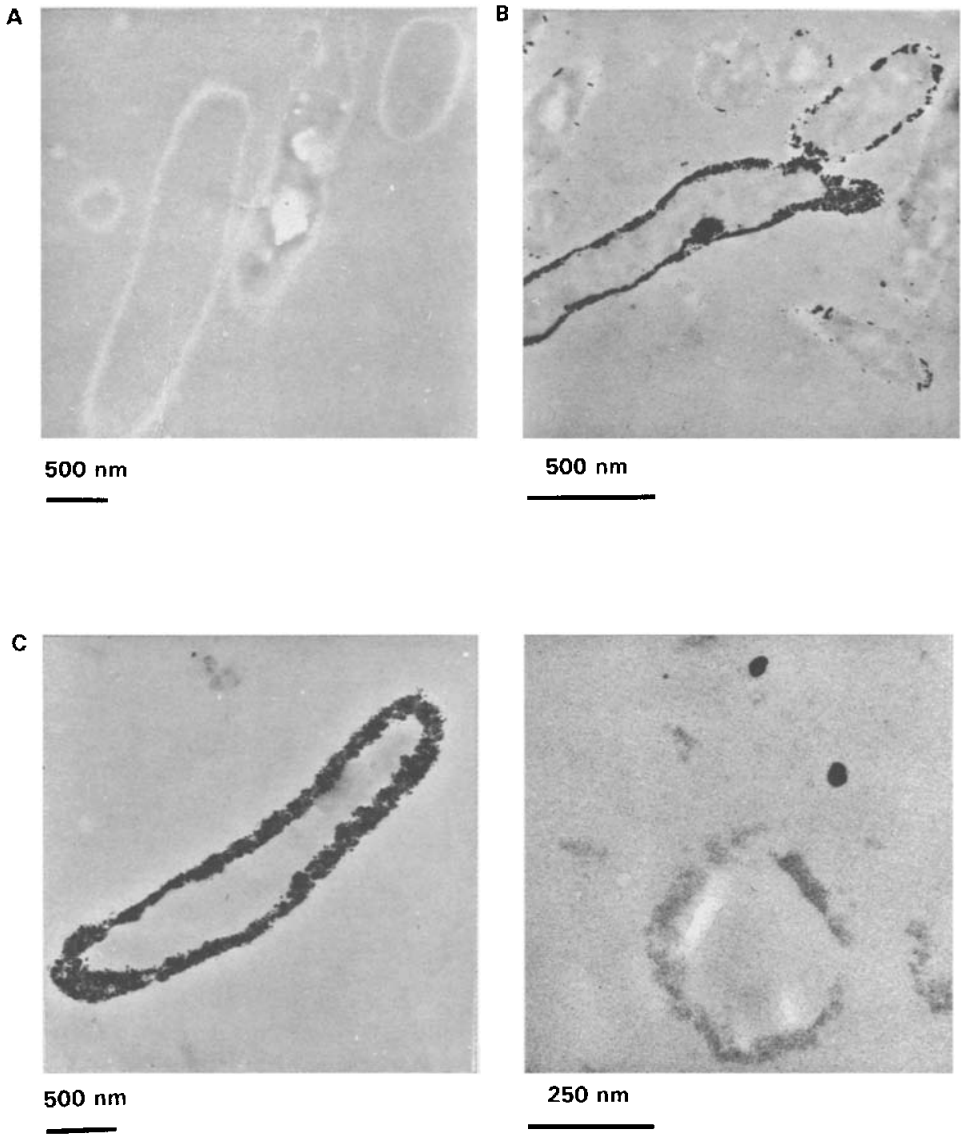


**Figure 4.** Electron microscopy analyses of thin-sectioned cells of *S. albus* (**A**) Not exposed to metal, (**B**) *S. albus* with dense silver deposit outside of a cell, and (**C**) after 3 h exposure to a mixed metal solution of 0.5 mmol $l^{-1}$ silver, copper, lead and zinc; right: dense deposits analyzed as silver (Figure 6c).

organisms might be the independence of the process from type of microorganism strain. Uranium and lead uptake was tested with the strain *Streptomyces longwoodensis* (Friis & Myers-Keith 1986). Older cells (> 14 h) accumulated about double the amount of uranium of younger cells (< 14 h). Lead uptake was determined to be 0.070 g $g^{-1}$ dry weight (340 $\mu$mol $g^{-1}$ dry weight) in the presence of 0.5 mmol $l^{-1}$ lead, pH 3 and 0.3 g $l^{-1}$ cell mass.

The electron micrographs revealed the difference between cells of *S. albus* not exposed to the metal mixture and cells exposed to the four metals (Figure 4). Metal-exposed *S. albus* cells exhibited three cell types: cells without metal staining, cell walls with a few small dense areas and fully stained cell envelopes with a dense structure. The stained cells might be dead cells, which have no resistance or protection mechanisms, so the metal ions bind in higher amounts on charged sites on the cell walls. Electron microscopy examination revealed that the cell wall

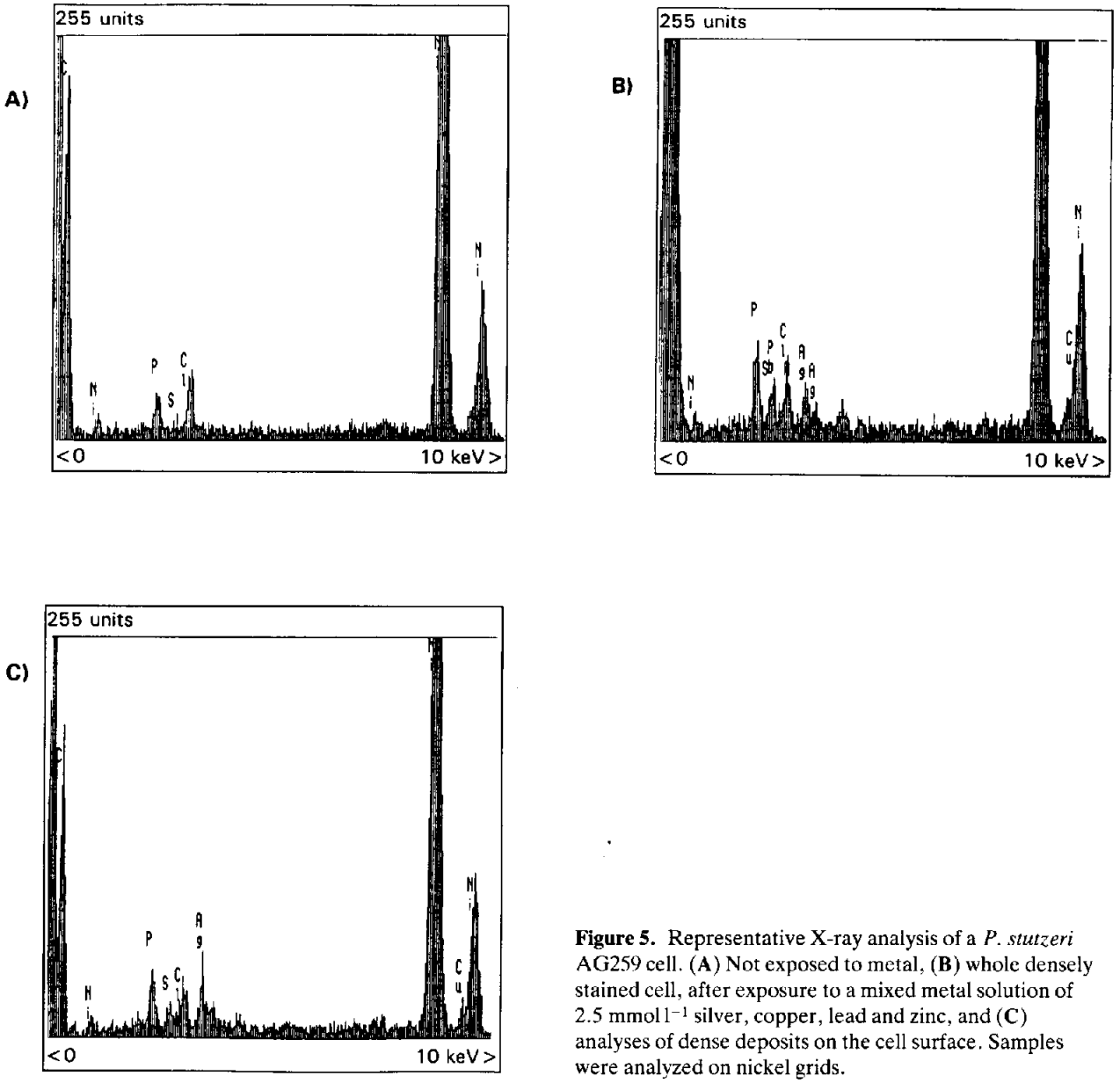


**Figure 5.** Representative X-ray analysis of a *P. stutzeri* AG259 cell. (**A**) Not exposed to metal, (**B**) whole densely stained cell, after exposure to a mixed metal solution of 2.5 mmol $l^{-1}$ silver, copper, lead and zinc, and (**C**) analyses of dense deposits on the cell surface. Samples were analyzed on nickel grids.

of the algae or yeasts was the main location for metal deposition (Kuyucak & Volesky 1988). It was described that the dead biomass of yeasts tends to sequester and retain the metals better, probably due to the absence of active ion transport mechanisms.

Both *P. stutzeri* and *S. albus* accumulated metals mainly on the cell wall. *P. stutzeri* cells with a higher stained cytoplasmic area might bind metals also inside the cell. Silver deposits were not observed on *S. albus* like on *P. stutzeri*. The fact that only a portion of *S. albus* cells accumulated higher amounts of metal is interesting. In contrast, all *P. stutzeri* cells were stained with lower copper and lead levels than *S. albus*.

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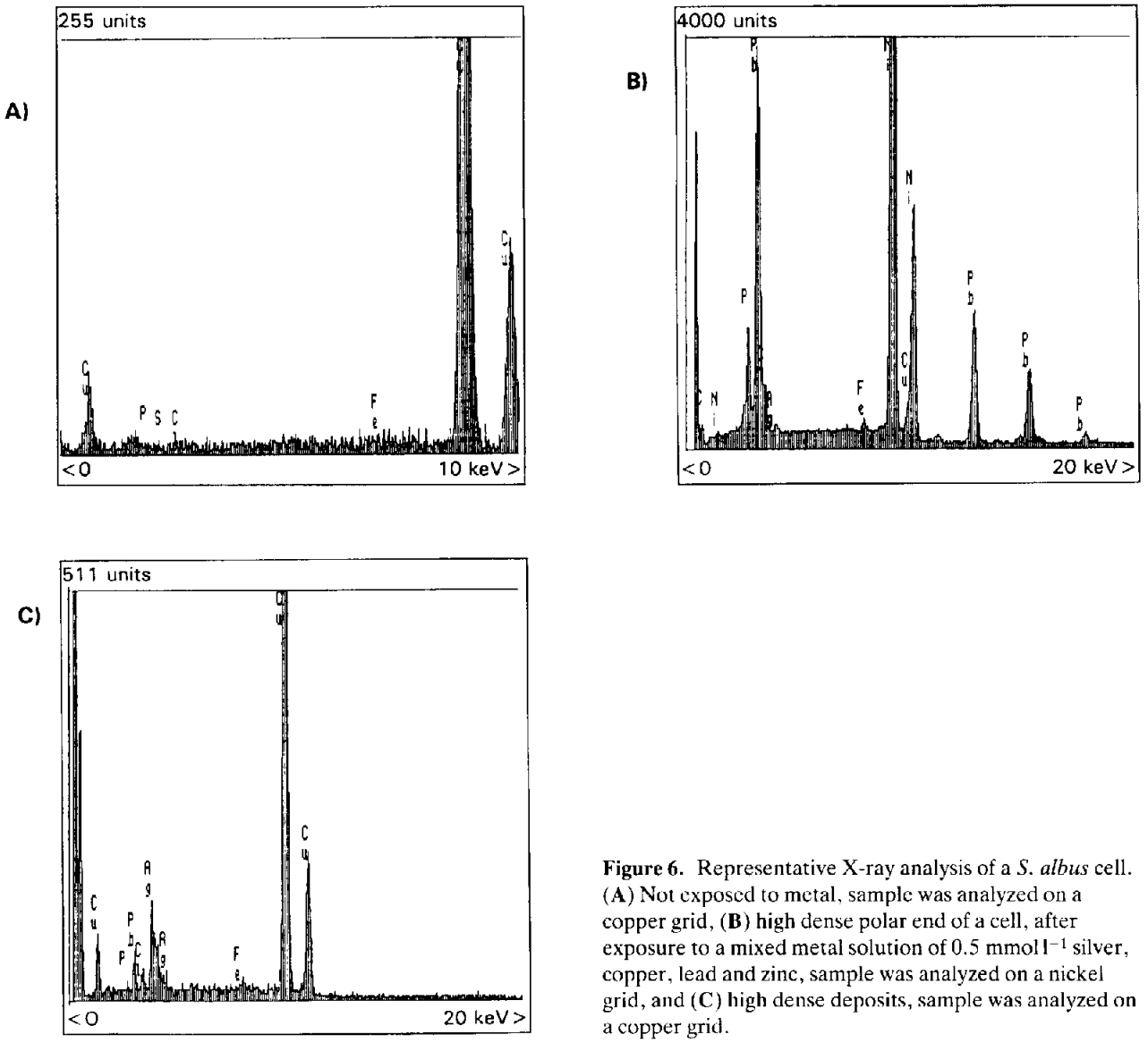


**Figure 6.** Representative X-ray analysis of a *S. albus* cell. (**A**) Not exposed to metal, sample was analyzed on a copper grid, (**B**) high dense polar end of a cell, after exposure to a mixed metal solution of 0.5 mmol $l^{-1}$ silver, copper, lead and zinc, sample was analyzed on a nickel grid, and (**C**) high dense deposits, sample was analyzed on a copper grid.

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