Short Communication

EFFECT OF ZINC ON MORPHOLOGY AND ULTRASTRUCTURE OF *PSEUDOMONAS* *STUTZERI* RS34

RENU BHAGAT AND SHEELA SRIVASTAVA*

*Department of Genetics, University of Delhi South Campus,
New Delhi-110021, India*

(Received April 14, 1994; Accepted June 6, 1994)

The use of microbial cells as biosorbents for heavy metals offers a potential alternative to existing methods for decontamination and/or recovery of metals from a variety of industrial process streams. Extensive studies have been carried out on the mechanisms of accumulation of metals by microbes (4, 13). Some of these investigations have shown that morphological and ultrastructural changes form a major class of response of a microbial cell to overcome the metal stress (5, 15).

Cells of *Pseudomonas stutzeri* RS34 are capable of sequestering zinc (Zn) on the cell wall and this bioaccumulation capacity can be exploited for recovery of Zn (2). Zinc is known to be a structural component of the cell wall of the Gram-negative bacteria (3). We report here the morphological and ultrastructural adaptations induced in *P. stutzeri* RS34 in response to Zn. Wavelength dispersive X-ray analysis confirmed the distribution and deposition of Zn on the cell surface. *P. stutzeri* RS34 can thus be employed for decontamination and removal of metal(s) from mine effluents and ore tailings.

The bacterium, *P. stutzeri* RS34, isolated from an industrially polluted area was used for this study. Cells were grown in a defined minimal medium (1). One molar stock of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ was prepared and diluted appropriately for the required final concentration in the media. Complex medium used, whenever required, was Luria-Bertani Agar (LB Agar).

Cells were grown in liquid media with or without zinc at 37°C in a New Brunswick Scientific Shaker Incubator (Edison, NJ, U.S.A.), at 200 rpm until saturation (16–18 h). The cells were harvested by centrifugation at 6,000 rpm for 20 min, washed several times with minimal medium so as to remove non-specifically

* Address reprint requests to: Dr. Sheela Srivastava, Department of Genetics, University of Delhi South Campus, Benito Juarez Road, New Delhi-110021, India.

bound Zn and then prepared for electron microscopic analyses.

A drop of thick suspension of washed cells was placed on a coverslip and a thin smear was made on small coverslips. Cells were fixed in glutaraldehyde (2.5%) for 1 h rinsed with 0.1 M phosphate buffer 2–3 times, dehydrated in ascending grades of acetone and finally washed with dried acetone. Specimens after being processed for critical point drying were mounted on stubs with a silver glue for conduction. The cells were examined through a Scanning Electron Microscope (Philips SEM-50\B, The Netherlands).

Wavelength dispersive X-ray analysis (WDAX) was performed with whole cell preparations using Electron Probe Microanalyzer (CAMECA S $\times$ 50, France). The analysis was performed at 25 kV with an electron beam diameter of 0.5 μ m. For the measurement, the cells were tentatively divided into three regions, the two apices (S1 and S3) and the central portion (S2). The data of WDAX were statistically analyzed by the method of randomized complete block design analysis as given by Snedecor and Cochran (12).

For transmission electron microscopy, pellets of sedimented cells washed with saline were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 h at 4°C, and washed thrice with the same buffer. Dehydration was carried out in an ascending series of acetone followed by toluene. Specimens were infiltrated with 3:1 (v/v) mixture of toluene and Araldite [50% epoxyresin + 50% DDSA, dodec-enyl succinic anhydride] for 2 h, followed by pure Araldite, and were finally embedded in beam capsule with Araldite+accelerator [trimethylamino methyl phenol (DMP-30)] and polymerized at 50°C for 24 h and then at 60°C for 48 h. Ultrathin sections were cut with a Ultracut Microtome E (Reichert Jung) stained with uranyl acetate and lead citrate, and examined in a transmission electron microscope (CM-10, Philips). Toluidine blue stain was used to identify polyphosphate granules (9).

Electron micrographs of Zn-treated (2 mM) cells, showed a number of morphological changes which were not seen in control cells (Fig. 1a). About 50% of the treated cells had protuberances on their outer surface. These cells showed thickened areas and appeared to be joined forming clusters (Fig. 1b). The size of zinc treated cells, however, was not significantly different from that of the control.

Morphological and ultrastructural changes are common microbial responses induced by metal Cu²⁺ and Co²⁺ in *Cunninghamella blakesleeana* (15). In *P. stutzeri* strain RS34 also, the protuberances seen on the surface of the cell might be used to accommodate high levels of zinc by increasing the surface area. WDAX analysis was carried out to determine the pattern of distribution of zinc. More Zn was detected at the two apices of the cell (S1 and S3) as compared to the central portion (S2) (Table 1). The analysis of variance shows that the three positions were heterogenous at the 5% level of significance. Further, the amount of Zn at S1 and S3 was significantly higher than S2, but the difference between S1 and S3 was not significant. Zn deposition throughout the cell surface of *P. stutzeri* is in accordance with the results of Pb-exposed *Penicillium chrysogenum* and silver-

Fig. 1. Scanning electron micrographs of *Pseudomonas stutzeri* RS34 grown for 16 h: control cells (a) and Zn-treated (2 mM) cells (b).

Extensions are seen on zinc-treated cells. Bar = 1 μ m.

Table 1. WDAX analysis for distribution of Zn on the cell surface of *Pseudomonas stutzeri* RS34.

Different areas of the cells	Zn counts/10,000 s/cell ^a
Apex 1 (S1)	232.5
Central (S2)	202.5
Apex 2 (S3)	225.37

^a Average of 3 independent sets.

SE (m) = ± 6.378 .

treated Gram-negative and Gram-positive bacteria where EDAX confirmed sequestration of Pb and Ag, respectively (7, 8). The presence of Cd deposits on the cell surface of yeast was also confirmed by EDAX (16).

To investigate the ultrastructural changes, thin sections of the zinc-treated and control cells were examined through a transmission electron microscope. Bacteria cultured in normal growth medium (control) appeared to be structurally intact (Fig. 2a). Sections of Zn-treated (7.5 mM) cells, on the other hand, were found to be markedly different in having protuberances on the surface, and were seen to be surrounded by a defined layer (Fig. 2b). The cells were clustered with incomplete separation after division (Fig. 2c) and contained electron-dense aggregates (Fig. 2d). Large intracellular vacuoles (V) and other abnormalities like electron-dense aggregates (EDA) and cytoplasmic granules were also observed. Electron-dense particles in Zn-treated cells stained red with toluidine blue and, therefore, were identified as polyphosphate granules (PPG) (Fig. 2b, c, d).

The blebs or protuberances of the weakened membranes induced by metal and

Fig. 2. Transmission electron micrographs of thin sections of control (a); Zn-treated cells (b, c, d).

Polyphosphate granules (PPG), electron-dense aggregates (EDA), and vacuolization (V) are seen in Zn-treated (7.5 mM) cells. Bar=0.1 μ m.

polymyxin B have been observed in freeze-etched preparations of *Salmonella typhimurium* and *Escherichia coli* (10). In thin sections, the tendency of Zn^{2+} -treated cells to remain attached may indicate structural impairment of the separation mechanism. In *P. putida* Cd^{2+} -adapted cells, this response has been explained by the altered outer membrane structure or the inhibition of autolysis involved in cell separation (5). While a high concentration of Cd induces changes in the membrane structure (such as blebbing) in *P. putida* (5), the proliferating Cd-accommodated cells of *E. coli*, are morphologically indistinguishable from cells unexposed to Cd^{2+} (6).

Deposition of uranium in *Saccharomyces cerevisiae* and intracellular accumulation in *P. aeruginosa* does not require apparent structural changes (13). The whole-cell preparations of *P. tabaci* grown in Ni^{2+} have a plasmolysed appearance at late stationary phase different from that of control (11). Electron microscopic examination of sections of *Enterobacter* grown in the presence of HgCl_2 showed a wide range of morphological abnormalities like pleomorphism, electron-dense particles, irregular cell division and plasmolysis (14).

In conclusion, exposure to zinc in *P. stutzeri* RS34, induces distinct ultra-structural and morphological adaptations. Zn-treated *P. stutzeri* cells show typical responses to metal such as intense vacuolization, appearance of electron-dense aggregates and polyphosphate granules which are also known to provide intracellular metal binding sites. Wavelength dispersive X-ray analysis showed that Zn is deposited on the surface although no regular pattern could be observed.

The financial assistance to R. B. from University Grants Commission is gratefully acknowledged. Authors are thankful to Dr. Ashok Kappor of SSPL, Delhi for his help in WDAX analysis. Sincere appreciation is also due to Mr. Rajiv Chawla, J. T. P. A., UDSC, New Delhi for helping in the preparation of the manuscript.

REFERENCES

- 1) Bhagat, R. and Srivastava, S., Growth response of *Pseudomonas stutzeri* RS34 to ethylenediamine-tetracetic (EDTA) and its interaction with zinc. *Indian J. Exp. Biol.*, **31**, 590–594 (1993).
- 2) Bhagat, R. and Srivastava, S., Biorecovery of zinc by *Pseudomonas stutzeri* RS34. In *Biohydrometallurgical Technologies*, ed. by Torma, A. E., Apel, M. L., and Brierley, C. L., TMS publications, Warrendale, Pennsylvania (1993), p. 209–217.
- 3) Eagon, R. G., Cell wall associated inorganic substances from *Pseudomonas aeruginosa*. *Can. J. Microbiol.*, **15**, 235–236 (1969).
- 4) Gadd, G. M., Metal tolerance. In *Microbiology of Extreme Environment*, ed. by Clive, E., McGraw-Hill Publishing Company, New York (1990), p. 178–210.
- 5) Higham, D. P., Sadler, P. J., and Scawen, M. D., Effect of cadmium on the morphology, membrane integrity and permeability of *Pseudomonas putida*. *J. Gen. Microbiol.*, **132**, 1475–1482 (1986).
- 6) Mitra, R. S., Gray, R. H., Chin, B., and Berstein, I. A., Molecular mechanisms of accommodation in *Escherichia coli* to toxic levels of cadmium. *J. Bacteriol.*, **121**, 1180–1188 (1975).
- 7) Mullen, M. D., Wolf, D. C., Ferris, D. C., Beveridge, T. J., Flemming, C. A., and Bailey, G. W., Bacterial sorption of heavy metals. *Appl. Environ. Microbiol.*, **52**, 3143–3149 (1989).
- 8) Niu, H., Xu, X. S., and Wang, J. H., Removal of lead from aqueous solutions by *Penicillium* biomass. *Biotechnol. Bioeng.*, **42**, 785–787 (1993).
- 9) Norris, J. R. and Swain, H., Staining bacteria. In *Methods in Microbiology 5A*, Academic Press, New York (1971), p. 105–134.
- 10) Schindler, P. R. G. and Teuber, M., Action of polymyxin B on bacterial membranes: Morphological changes in the cytoplasm and in the outer membrane of *Salmonella typhimurium* and *Escherichia coli* B. *Antimicrob. Agents Chemother.*, **8**, 95–104 (1975).
- 11) Sige, D. C. and Al-Rabae, R. H., Nickel toxicity in *Pseudomonas tabaci*: Single cell and bulk sample analysis of bacteria cultured at high concentration. *Protoplasma*, **130**, 171–185 (1986).
- 12) Snedecor, G. W. and Cochran, W. S., Statistical Methods, The Iowa State University Press, Ames, Iowa (1982), p. 407–412.

- 13) Strandberg, G. W., Shumate, S. E., and Parott, J. R., Microbial cells as biosorbents for heavy metals: Accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*. *Appl. Environ. Microbiol.* **41**, 237–245 (1981).
- 14) Vaituzis, Z., Nelson, J. D., Jr., Wan, L. W., and Cotwell, R. R., Effects of mercuric chloride on growth and morphology of selected strains of mercury resistant bacteria. *Appl. Microbiol.*, **29**, 275–286 (1975).
- 15) Venkateswerlu, G., Yoder, M. J., and Stotzky, G., Morphological ultrastructural changes induced in *Cunninghamella blakesleeana* by copper and cobalt. *Appl. Microbial. Biotechnol.*, **31**, 204–210 (1989).
- 16) Volesky, B., May, H., and Holan, Z. R., Cadmium biosorption by *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.*, **41**, 826–829 (1993).