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Morphological Changes in an *Acidocella* Strain in Response to Heavy Metal Stress

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Abstract: Many bacteria adapt to environmental stresses through morphological changes. The normal flora of acidic mines are often subject to various stresses, especially exposures to toxic heavy metals; however, a study conducting the effects of these metals on the morphology of any bacterial species of such regions has not been reported. This work is an attempt to fill the lacunae using an acidophilic heterotrophic bacterium *Acidocella* sp. GS19h strain, an isolate of Indian copper mine, as the test strain. Morphological alterations were induced when the bacterial cells were incubated with sub-inhibitory concentrations of some heavy metals (e.g., Cd, Cu, Ni and Zn). Loosely packed coccobacillus type normal cells formed characteristic chains of coccidal lenticular shape with constrictions at the junctions between them in presence of Cd or Ni; Cu induced transformation of cells to becoming round-shaped, while Zn turned the cells filamentous and aggregated. Respective metal depositions on the cell surface were confirmed by SEM-EDX. Release of more Ca^{2+} ion, measured by ICP-MS, in the culture filtrates of metal-stressed cells suggests replacement of cell-bound Ca^{2+} by these metal ions.

Key words: Microbial adaptation, heavy metal, cell morphology, SEM, ICP-MS

INTRODUCTION

Environments that very often contain high levels of dissolved metals include active and disused mines, where the production of acidic mine and rock drainages is catalysed by the action of microorganisms (Dopson *et al*., 2003). A specialist group of bacteria, which grow optimally below pH 4 and known as acidophiles, inhabit such regions (Hallberg and Johnson, 2001). The acidophiles isolated from industrial operations and natural ore-leaching sites consist of eubacteria and archea, autotrophs and heterotrophs; they can live under psychrophilic, mesophilic and thermophilic conditions (Rawlings and Silver, 1995).

Although the interactions of microorganisms and minerals have been occurring in nature since the beginning of life on the earth (Ehrlich and Brierly, 1990; Rawlings, 2002), but majority of heavy metals (density>5) exhibit toxicity to all kinds of living beings including bacteria at relatively low concentrations (Bruins *et al*., 2000). Bacteria utilize different strategies to adapt to varying environmental situations including exposures to high concentrations of heavy metals. One of the

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strategies that bacteria adapt to cope with stress conditions is the change in morphology. Such changes were observed in phototrophic bacteria on exposure to metalloid oxyanions (Nepple *et al*., 1999) and in *Pseudomonas putida* and *Enterobacter* sp. in presence of toxic organic compounds (Neumann *et al*., 2005); temperature induced morphological changes in *Escherichia coli* (Bennet *et al*., 1992) and *Pseudomonas pseudoalcaigenes* (Shi and Xia, 2003) were reported. Conditional lethality of cell shape mutations in *Salmonella typhimurium* (Costa and Anton, 1999) is another interesting example. Thus unfavorable conditions like exposures to toxic metals/metalloids or organic solvents, highly acidic or alkaline pH, high and low temperature typically induce a stress response exhibiting characteristic changes in the cell shape and assembly. The stress responses help to protect vital processes and to restore cellular homeostasis, as well as increase cellular resistance against subsequent stress challenges (Storz and Hengge-Aronis, 2000). The acidophiles of mine regions are frequently exposed to sub-lethal but high concentration of various metals and it is supposed that these bacteria also have evolved some survival strategies like changes in cell morphology under such situations. This specific aspect has not been addressed in case of bacteria inhabiting acidic mines though the effects of heavy metals on few other soil bacteria have been reported (Gogolev and Wilke, 1997; Santamaría *et al*., 2003). In this study we describe the morphological changes induced by some heavy metals in an acidophilic bacterium *Acidocella* sp. GS19h that was isolated from an Indian copper mine (Banerjee *et al*., 1996) and can tolerate high concentration of heavy metals used in this study (Ghosh *et al*., 1997). The strain is a Gram-negative, mesophilic, heterotroph.

MATERIALS AND METHODS

This study was conducted in the addressed institute during 2006; some parameters, such as electron micrographs were taken elsewhere (indicated in Acknowledgements).

Bacterial Strain and Growth Conditions

Acidocella sp. GS19h strain was isolated from the19th level soil sample of an Indian copper mine (Banerjee *et al*., 1996). The strain was maintained and routinely cultured aerobically at 30°C at 180 rpm on orbital shaker in MGY medium that contained (g L^{-1}) KCl (0.1), $MgSO_4.7H_2O$ (0.25), $(NH_4)_2SO_4$ (2.0), K_2HPO_4 (0.25), glucose (1.0) and yeast extract (0.1); medium pH was adjusted at 3.0±0.1 with 10 N H_2SO_4 (Bhattacharya *et al*., 1991). For conducting metal exposure experiments with the bacterial cells, late log phase culture (OD_{540} = ca. 0.62 with corresponding cell count of ca. 6×10^8 mL^{-1}) was harvested.

Incubation under Metal Stress

Bacterial cells were kept under metal stress in MGY medium containing a heavy metal salt; concentration of a metal salt was selected on the basis of its minimum inhibitory concentration (Ghosh *et al*., 1997). Each metal salt was added in a separate cell suspension containing previously harvested cells as described above and allowed to grow at 30°C for 24 h. Cells were then separated and immediately used for measurement of cell size. Following concentrations of the metal salts were used in this study: 500 mM $CdSO_4$, 500 mM $ZnSO_4$, 100 mM $NiSO_4$ and 12.5 mM $CuSO_4$.

Scanning Electron Microscopic Study of Whole Cell

Morphological changes induced in the test cell populations incubated in presence of different metals were examined by SEM. The samples for SEM were prepared following standard techniques (Lom and Weiser, 1972). Leica S440 Scanning Electron Microscope was used to observe cellular morphology. Elemental composition of selected areas was established using Energy Dispersive X-ray (EDX) microanalysis system with Link ISIS (Oxford Instruments) attached with the microscope after the samples were carbon coated.

Morphometric Analysis

Normal and stressed bacterial cell dimensions were measured directly from the SEM photographs to calculate cell Volumes (V) and surface Area (A) by the following equation:

$$V\ (\mu m^3) = \pi r^2 h$$
$$A\ (\mu m^2) = 2\ \pi r^2 + 2\ \pi r\ h$$

where r and h represent radius (or width) and length of cells in μm (Neumann *et al*., 2005). The mean cell dimensions of all test populations of the *Acidocella* strain were measured. Average cellular volumes and surface areas were calculated by using 100 individual bacterial cells per population. Cells showing division or deformation/rupture were not included.

Inductively Coupled Plasma Mass Spectrometric (ICP-MS) Analysis

Inductively Coupled Plasma Mass Spectrometer (ICP-MS) was used to determine the amount of Ca^{2+} released from the cells. This is a rapid and sensitive method of determination for all elements and is sensitive enough to detect 1 ng L^{-1} of an element (Peng *et al*., 2004). After incubation with a metal salt, cells were harvested, the filtrate was passed through a 0.22 μm filter and the amount of Ca^{2+} in the supernatant was measured.

RESULTS

Cell Morphology

Scanning electron microscopy of the *Acidocella* sp. GS19h cells shows that they exist as aggregates of loosely packed coccobacilli or as singles in metal untreated culture with an average (n = 100) cell size of 1.0-1.5 μm by 0.4-0.7 μm (Fig. 1). Electron dense area was noticed at the mid surface of the cell body; in comparison, two ends of the cells were electron light at localized area. Cells contained some membrane indentations that appear as black patch on the surface (Fig. 1). Some dividing cells were found in the fields under microscope.

When the cells were exposed separately in presence of different metals, some special morphological features were distinctly evident from the SEM micrograph. In all metal stressed conditions, dividing cells were found as well as cells with membrane indentations or rough surface. The

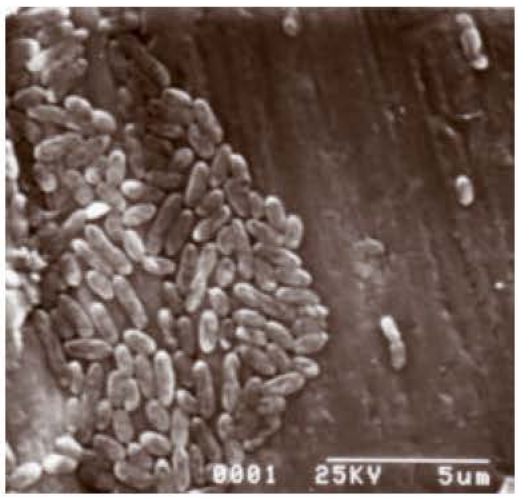

Fig. 1: Scanning electron microscopic pictures of *Acidocella* GS19 h cells grown at 30°C

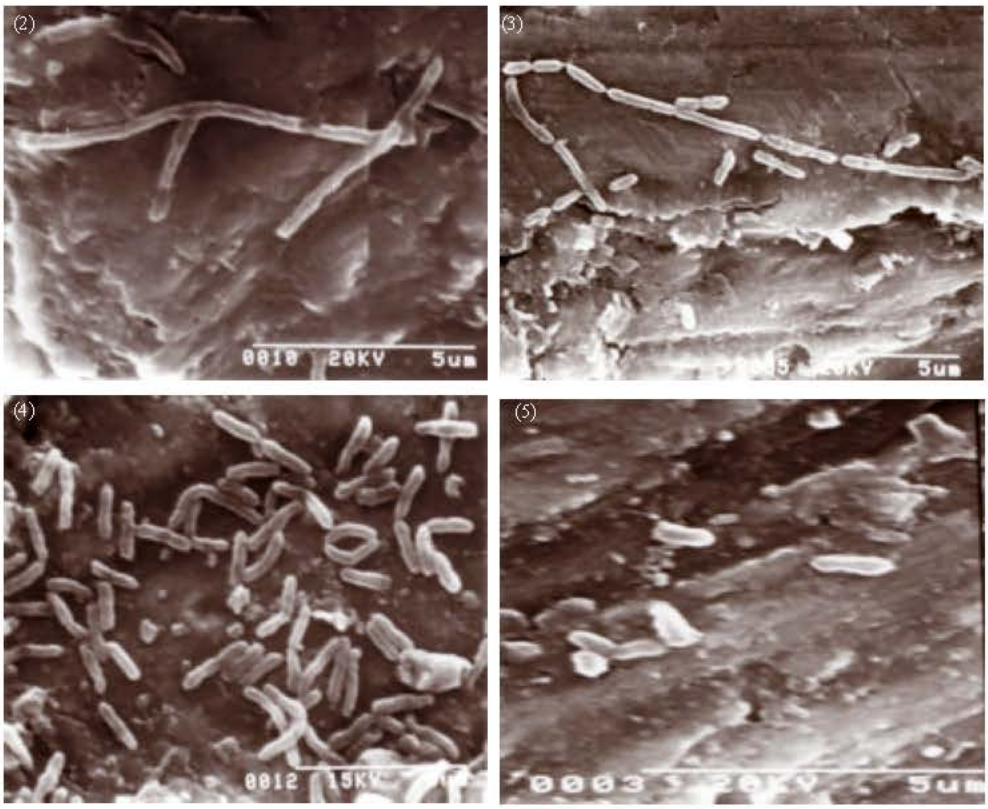

Fig. 2-5: Scanning electron microscopic pictures of *Acidocella* GS19 h cells after incubation with different metals: (2) Cd; (3) Ni; (4) Zn and (5) Cu

cells were mostly aggregated and in elongated form. Occasionally, in presence of Zn or Cu, blistering was also seen. When grown with Cd or Ni, the strain formed characteristic chains of coccidal lenticular cells with constrictions at the junctions between cells. In Cd stressed cells, this filamentous appearance was measured having an average length of 6-7 μm (Fig. 2), whereas an average length of 3.0-5.0 μm was found in case of Ni (Fig. 3). Cells became elongated in Zn supplemented medium with an average length of 3.0-3.5 μm. In this filamentous appearance, cells were also present in aggregated form though the number of long chains (three cells or more) decreased (Fig. 4). But in presence of Cu, the rod shape was lost and the strain was observed as a mixed population of spherical and elongated cells in packed aggregation as well as in individual form (Fig. 5). Uniform electron dense area on the surface of the cell was seen. There were some sticky appearances as evident from their overlapping nature with each other. Some cells showed even rougher surface structure and blister-like protrusions. In all cases, EDX spectra gave the evidence of metal deposition on the cell surface (data not shown).

Morphometric Analysis and Comparative Study of Treated and Untreated Cells

The dimensions of *Acidocella* GS19h strain changed due to metal stress. The cell dimensions, presented in Table 1, reveal that all the dimensional parameters were changed and the resultant effect was reflected in cell volume. The maximum and minimum cell volume was observed when the bacterium

Table 1: Dimension of the normal and stressed *Acidocella* GS19 h cells

Group	Width (μm)	Length (μm)	Radius (μm)	Surface area (μm^2)	Volume (μm^3)
Untreated	0.657±0.049	0.789±0.006	0.197±0.009	1.219±0.015	0.096±0.013
In Cd	0.319±0.024	8.804±0.021	0.212±0.025	12.003±0.022	1.24±0.043
In Cu	0.468±0.051	0.995±0.027	0.265±0.016	2.097±0.019	0.219±0.025
In Ni	0.344±0.062	5.689±0.043	0.172±0.031	6.330±0.034	0.529±0.013
In Zn	0.394±0.058	3.157±0.025	0.197±0.051	4.148±0.036	0.384±0.020

The width, length and radius of the cells are presented as mean (n = 100) ±SD

Table 2: Amount of Ca^{2+} released from the cells due to metal stress

Metal	Amount of Ca ($\mu g\ L^{-1}$)	Fold increase in Ca^{2+} release
Nil (Native cells)	24±1.15	1
Cd	42±0.98	1.75
Cu	38±1.08	1.58
Ni	31±1.21	1.29
Zn	35±1.41	1.46

Results are presented as mean±SD

was stressed with Cd and Cu, respectively. It was of interest to note that no change in cell shape or assembly was evident when the cells were incubated with growth inhibitory concentrations of metals.

Inductively Coupled Plasma Mass Spectrometric (ICP-MS) Analysis

Deposition of the metals on cell surface caused release of Ca^{2+}, which is reflected from the amount of this metal ion in the culture filtrate measured by ICP-MS technique (Table 2). It is evident from the Table 2 that 1.3 to 1.7 fold Ca^{2+} was released from the metal-treated cells compared to that from the control cells and among the four metals cadmium released the highest amount of Ca^{2+}.

DISCUSSION

Morphological changes like increased cell size observed in some phototrophic bacteria after exposure to the metalloid oxyanions (e.g., chromate, selenate, arsenate etc.) was described as a protection system for bacteria facing a stressful environment (Nepple *et al*., 1999). The changes in cell morphology as a response to heavy metal ions exposure observed in this study (Fig. 2-5) can be explained similarly because of the relative reduction of cell surface with respect to cell volume (Table 1) with consequent decrease in attachment/uptake sites for the heavy metals. This relative reduction of the cell surface-volume ratio presents an effective mechanism for the cells to reduce the toxic effects of environmental stress factors just by reducing the attachable/exposed surface in relation to the whole cell volume (Neumann *et al*., 2005). These observations explain why higher than normal cell volume with respect to cell surface is better under stress conditions.

The mechanisms of uptake and resistance to heavy metals in bacteria have been thoroughly studied (Nies, 1992; Brown *et al*., 1992; Ji and Silver, 2005). One of the well-studied and established mechanisms of heavy metal resistance in Gram-negative bacteria is the RND (resistance, nodulation, cell division) protein mediated effluxing of heavy metals through the bacterial cell membrane (Nies, 2003); this mechanism of metal resistance operates also in acidophilic bacteria (Dopson *et al*., 2003). It is obvious that functioning of such efflux systems would be more effective if the overall membrane surface is reduced. This leads to a reduction in the surface area that allows lower diffusion.

In this study, EDX analysis confirmed depositions of the metals on cell envelop and the amount of deposition was estimated by atomic adsorption spectrometer (data not shown). Along with this, higher amount of Ca^{2+} was found in the culture filtrate, which was estimated by ICP-MS (Table 2). It has been reported that metal ions, especially Ca^{2+}, maintain the lipopolysaccharide assembly on the surface of *E. coli*-a gram-negative bacterium (Kotra *et al*., 1999). Ca^{2+} plays many important roles in cellular metabolism and function after binding with a variety of proteins (Kuroki *et al*., 1989; da Silva and Reinach, 1991; Skelton *et al*., 1994; Ikura, 1996; Pidcock and Moore, 2001). As the divalent metal

cations (Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}) are structurally similar Ca^{2+} (Huheey *et al*., 1993), it may be proposed that they replace Ca^{2+} from the binding sites because of their similar ligand specificities.

The shape of bacterial cells is determined by several enzymes known as penicillin-binding-proteins (PBPs), which are located in the bacterial cell envelope. Penicillin and other β-lactam antibiotics that bind with one or more these enzymes with different affinity, thus induce abnormal cell morphology in bacterial cells (Franklin and Snow, 1981). Replacement of Ca^{2+} with another divalent metal ion probably inactivates some of the functioning PBPs thus shaping the bacterial cell structure differently for each replacement metal ion. It is quite possible that the new metal ion replacing Ca^{2+} activates other PBPs that do not function in normal metal-unexposed cells or it modulates the activity of the normal enzymes; in either case, cells undergo such changes that reduce the surface area of metal-exposed cells in comparison to cell volume. Since each metal ion induced different cell morphology, it may be concluded that the PBPs were not affected in the same way by each metal ion. Compounds such as cephalexin inhibit the formation of septum causing filamentous growth of greatly elongated cells. Similar effects were observed when the cells were incubated with Cd^{2+} and Ni^{2+} and partly in case of Zn^{2+}. On the other hand, mecillinam causes cells to assume an abnormal ovoid shape like that observed in case of Cu stress (Fig. 5).

In conclusion, it may be stated that the acidophilic bacterium *Acidocella* sp. strain GS19h circumvents the toxic effects of heavy metals at sublethal concentration by reducing its surface area in respect of cell volume. This change is effected through alteration of cell structure that is in all probability caused by PBPs, the activities of which are modulated by the heavy metal ion that replaces Ca^{2+} from the cell surface. At growth inhibitory concentration of the metals, the metal binds quickly with various intracellular proteins and other compounds causing rapid cell death and thus no change in cell morphology was observed at growth inhibitory concentration of the metals.

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