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ORIGINAL PAPER

Morphological changes in an acidophilic bacterium induced by heavy metals

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Abstract The *Acidiphilium* strains inhabit acidic mine regions where they are subjected to occasional environmental stresses such as high and low temperatures, exposure to various heavy metals, etc. Change in morphology is one of the strategies that bacteria adopt to cope with environmental stresses; however, no study on this aspect has been reported in the case of *Acidiphilium* sp. This work is an attempt using the acidophilic heterotrophic bacterium *Acidiphilium symbioticum* H8. It was observed that the maximum alterations in size occurred when the bacterium was exposed to sub-inhibitory concentrations of Cu and Cd. Loosely packed coccobacillus-type normal cells formed characteristic chains of coccoidal lenticular shape with constrictions at the junctions between them in the presence of Cd; Cu induced transformation of cells to become round shaped; Ni caused the cells to aggregate, but Zn showed no effect. Respective metal depositions on the cell surface were confirmed by scanning electron microscopy equipped with energy dispersive X-ray analysis. Cell bound Ca^{2+} ions were replaced by these metal ions and measured by inductively coupled plasma mass spectrometry from the culture filtrate. Cell shape changed only after the addition of sub-inhibitory concentrations of the metals, but in growth inhibitory concentrations it was similar to the normal cells.

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Introduction

Biogeochemical activities started with the appearance of life on the earth as a result of interaction among atmosphere, biosphere, hydrosphere and lithosphere (Dopson et al. 2003). The acidophiles which grow optimally below pH 4 inhabit sulfide-rich, acidic mine regions (Hallberg and Johnson 2001). These bacteria are now exploited in biohydrometallurgical operations (Ehrlich and Brierly 1990; Rawlings 2002). The acidophiles isolated from industrial operations and natural ore-leaching sites consist of eubacteria and archaea, autotrophs and heterotrophs; they may be psychrotolerant, mesophile or thermophile (Ehrlich and Brierley 1990; Rawlings and Silver 1995). These bacteria are very often exposed to high concentrations of various heavy metal ions (density > 5 g/cm^3), the majority of which inhibit bacterial growth at low concentrations (Bruins et al. 2000). Some acidophiles, however, could tolerate relatively high concentrations of one or more heavy metals (Dopson et al. 2003). To cope with the occasional or frequent exposures to heavy metals, toxic organics and other environmental stresses, bacteria have evolved several strategies (Nies 2003; Baker-Austin and Dopson 2007), such as change in cellular morphology (Young 2003). Such changes induced by heavy metals were observed in the acidophilic heterotroph, *Acidocella* sp. GS19h strain (Chakravarty et al. 2007). Morphological changes were induced by metalloid oxyanions in phototrophic bacteria (Nepple et al. 1999), and by toxic organics in *Pseudomonas putida* and *Enterobacter* sp. (Neumann et al. 2005); temperature-induced morphological

variations in *Escherichia coli* (Bennett et al. 1992) and *Pseudomonas pseudoalcaigenes* (Shi and Xia 2003) are available. Conditional lethality of cell shape mutants of *Salmonella typhimurium* was reported (Costa and Anton 1999). Thus, unfavorable conditions, such as exposure to toxic metals, metalloids and organics, highly acidic or alkaline pH, suboptimal temperatures, induce stress responses exhibiting characteristic changes in cell morphology and assembly (Ramos et al. 2001; Young 2006). It is believed that stress responses protect vital processes and restore cellular homeostasis, as well as help to enhance cellular resistance against subsequent stress challenges (Storz and Hengge-Aronis 2000). Since the acidophiles of mine regions are confronted with frequent exposures to sub-lethal but high concentrations of various metals, it is likely that they also adopt this survival strategy of changing cell morphology under such situations. This specific aspect has not been addressed in the case of *Acidiphilium* strains inhabiting acidic mines. Previously, the effects of heavy metals on soil bacteria (Gogolev and Wilke 1997; Santamaría et al. 2003) and an *Acidocella* strain (Chakravarty et al. 2007) were reported. Here, we describe the morphological changes induced by some heavy metals in the Gram-negative, mesophilic, heterotrophic, acidophilic bacterium, *Acidiphilium symbioticum* H8 that was isolated from a culture of *Acidithiobacillus ferrooxidans* (Bhattacharya et al. 1991). The strain can tolerate high concentrations of heavy metals used in this work (Mahapatra and Banerjee 1996).

Materials and methods

Bacterial strain and growth conditions

Acidiphilium symbioticum (H8 strain) was isolated from a culture of *A. ferrooxidans* (Bhattacharya et al. 1991). The strain was routinely cultured aerobically at 30°C at 180 rpm on orbital shaker in MGY medium that contained (in 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). After adjusting the pH at 3.0 ± 0.1 with 10 N H_2SO_4 (Bhattacharya et al. 1991), the medium was sterilized at 110°C temperature and 10 lbs pressure for 30 min. Five percent (v/v) inoculum was used for routine subculturing. For conducting metal exposure experiments with the bacterial cells, late log phase culture (OD_{540} = ca. 0.65 with corresponding cell count of ca. 6×10^8 per ml) 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 (Mahapatra and Banerjee 1996). Each metal salt was added in a separate cell suspension containing previously harvested cells as described earlier, and allowed to grow at 30°C at 180 rpm on orbital shaker for 24 h. Cells were then separated and immediately used for measurement of cell size. Maximum concentrations of the metal salts used in this study were 500 mM $CdSO_4$, 100 mM $ZnSO_4$, 25 mM $NiSO_4$ and 12.5 mM $CuSO_4$.

Scanning electron microscopic study of whole cell

A scanning electron microscope equipped with an energy dispersive X-ray spectrometer (SEM-EDX) was used to observe the morphological changes induced in the test cell populations incubated in the presence of different metals. The samples for SEM were prepared following standard techniques (Lom and Weiser 1972). Following incubation, both control and stressed bacterial cells were harvested (4,000×*g*, 15 min, 4°C), washed with phosphate buffer (0.1 M, pH 7.2), and then fixed with glutaraldehyde (2.5%, v/v) in the same buffer for 3 h. The cells were then washed with 0.1 M phosphate buffer thrice—each for 15 min and post-fixed with 1 ml 1% (w/v) osmium tetroxide for 8 h at room temperature. The sample was then washed twice with buffer and dehydrated with graded alcohol. The Leica S440 Scanning Electron Microscope was used to observe cellular morphology. Elemental composition of selected areas was established using the energy dispersive X-ray (EDX) microanalysis system with Link ISIS (Oxford Instruments) attached with the microscope after the samples were carbon coated.

Morphometric analysis

To calculate cell volume (*V*) and surface area (*A*) by the following equations, normal and stressed bacterial cell dimensions were measured directly from the SEM photographs:

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

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

where *r* and *h* are radius and length of a cell in μm (Neumann et al. 2005). Mean cell dimensions of the test populations of the *Acidiphilium* strain were measured. Average cellular volume and surface area were calculated from 100 individual healthy bacterial cells per population. Cells undergoing division or showing deformation/rupture were not included.

Inductively coupled plasma mass spectrometric (ICP-MS) analysis

The amount of Ca^{2+} released from the cells was determined by the inductively coupled plasma mass spectrometer (ICP-MS). The method is rapid and sensitive for all elements, which can be detected at the level of 1 ng/l (Peng et al. 2004). Cells were harvested after incubation with a metal salt; the supernatant was passed through a 0.22 μm membrane filter (Millipore, USA) and the amount of Ca^{2+} in the filtrate was measured.

Results

Cell morphology

Scanning electron microscopy of *A. symbioticum* H8 showed that they exist as aggregates of loosely packed coccobacilli or as single cells in untreated culture with an average (n = 100) cell size of 1.0–1.3 μm × 0.4–0.7 μm. Cells with a smooth surface and a notch-like appearance at one end were clearly evident from micrographs (Fig. 1). Some dividing cells were found in the fields under microscope.

When the coccoidal cells were exposed separately in the presence of different metals, some distinct morphological features were evident from the SEM micrograph. Under all metal stressed conditions, dividing cells were found. When growing with Cd, the strain formed high aggregation of characteristic chains of coccoidal lenticular cells with constrictions at the junctions between cells. This filamentous appearance was measured having an average length of 4.5–6.5 μm. Rough cell surface and membrane indentations were clearly evident in this condition (Fig. 2a). The gradual increase of Cd concentration decreased the surface area/volume ratio (Fig. 3a), resulting the cells to be more elongated. Under Ni-stressed conditions, most of the cells had collapsed, formed a "donut-like" shape, and were clumped in such a way that precise measurement was difficult (Fig. 2b). The cells were slightly elongated with an average length of 1.6–1.8 μm and irregular electron dense area was found on the surface. It was observed from the micrographs that the cells were overlapped with each other. Like Cd, increasing concentrations of Ni decreased the surface area/volume ratio significantly (Fig. 3b). Less morphological disturbances were observed upon exposure to Zn. The coccobacillus shape was generally preserved and deformations/indentations of the cell surface were almost absent (Fig. 2c). It was loosely packed and the average length was 1.3 μm. A notch-like appearance was seen at one end. But in the presence of Cu, the rod shape was lost, and the strain was observed as a homogeneous

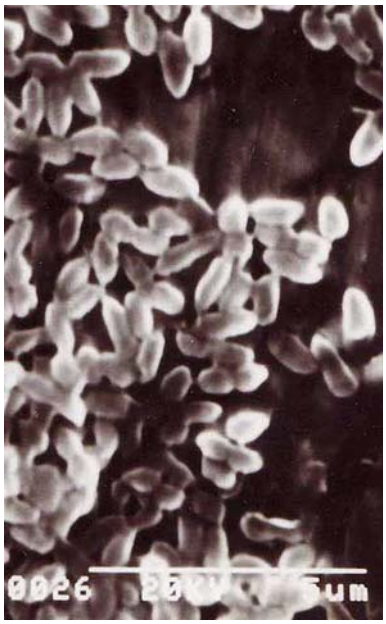

Fig. 1 Scanning electron micrograph of *Acidiphilium symbioticum* H8 cells grown at 30°C

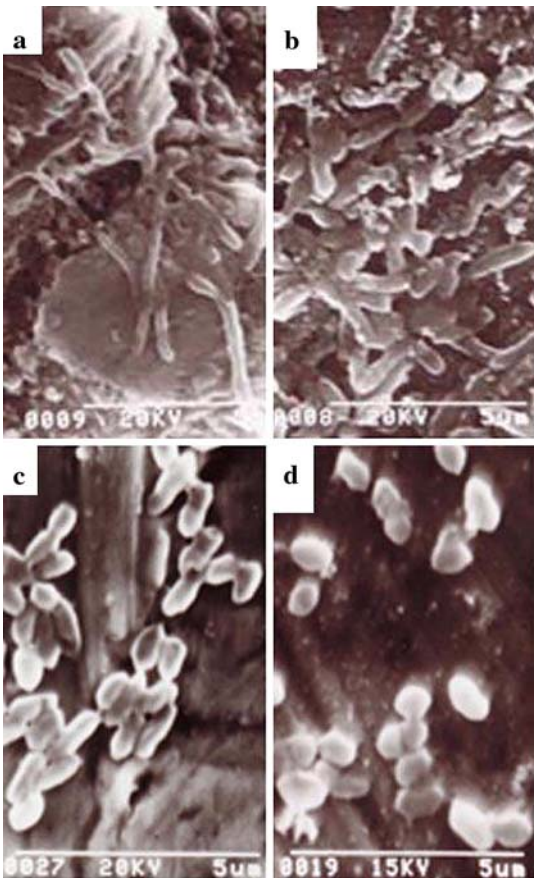

Fig. 2 Scanning electron micrographs of *Acidiphilium symbioticum* H8 cells after incubation with different metals: **a** Cd, **b** Ni, **c** Zn, **d** Cu

mixture of spherical and elongated cells in packed aggregation as well as individuals. Smooth cell surface having no membrane indentations was found with uniform electron dense area throughout the unicellular body of the strain. Average dimension of the cells was 1.0–1.1 μm by 0.4–0.6 μm (Fig. 2d). In Zn- and Cu-stressed cells, a gradual increase in metal concentrations did not affect significantly in the relative reduction of surface area/volume ratio of cells (Fig. 3c, d). 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

Metal stresses induced changes in cellular morphology and assembly of *A. symbioticum* H8 strain. Table 1 shows that all the cell dimensional parameters changed due to metal stress, and the resultant effect was reflected in cell volume. Cd induced maximum enlargement of cell volume, while Cu reduced their volume to a minimum. Such changes were noted only at growth sub-inhibitory concentrations of metals; no change in cell shape or assembly was evident when the cells were incubated with growth inhibitory concentrations of metals.

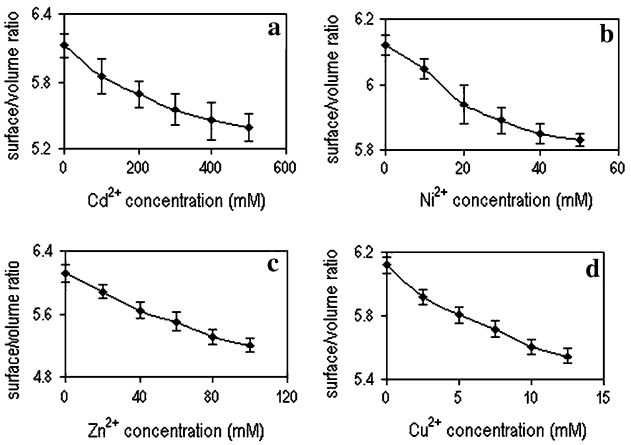

Fig. 3 Relative reduction of surface area/volume ratio in different metal concentrations: **a** Cd, **b** Ni, **c** Zn, **d** Cu

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 increased amount of this metal ion in the culture filtrate measured by the ICP-MS technique (Table 2). It is evident from the table that 1.8- to 2.5-fold Ca^{2+} was released from the metal-treated cells compared to that from the control cells; among the four metals, cadmium released the highest amount of Ca^{2+}.

Discussion

An increase in cell size observed in some phototrophic bacteria after exposure to metalloid oxyanions (e.g. chromate, selenate, arsenate) has been explained as a protection system for the bacteria exposed to a stressful environment (Nepple et al. 1999). Changes in cell morphology in response to heavy metals exposure noted in this study (Fig. 2 a–d) can be a similar protective mechanism of the bacterial strain used; such changes were reported previously in another acidophilic heterotroph, *Acidocella* sp. GS19h strain (Chakravarty et al. 2007). The relative decrease in cell surface with respect to cell volume (Table 1) plays the key role in the consequent reduction in

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

Metal stress	Amount of Ca (μg l^{-1})	Fold increase in Ca release
Nil (native cells)	17 ± 0.85	1
Cd	43 ± 1.91	2.52
Cu	38 ± 2.12	2.23
Ni	29 ± 2.91	1.70
Zn	32 ± 1.85	1.88

Results are presented as mean ± SD

Table 1 Dimensions of the normal and stressed *Acidiphilium symbioticum* H8 cells

Group (metal treated)	Length (μm)	Radius (μm)	Surface area (μm^2)	Volume (μm^3)	Surface/volume
Untreated	1.213 ± 0.008	0.447 ± 0.011	4.662 ± 0.021	0.761 ± 0.015	6.12
Cd	6.184 ± 0.025	0.394 ± 0.021	16.280 ± 0.027	3.016 ± 0.037	5.39
Cu	1.081 ± 0.022	0.540 ± 0.019	5.499 ± 0.031	0.989 ± 0.019	5.55
Ni	1.857 ± 0.043	0.420 ± 0.029	6.008 ± 0.028	1.029 ± 0.021	5.83
Zn	1.304 ± 0.020	0.543 ± 0.048	6.301 ± 0.031	1.209 ± 0.020	5.21

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

attachment/uptake sites on the cell surface for the heavy metals. This relative reduction of the cell surface–volume ratio is an effective way for the cells to lowering the toxic effects of environmental stress factors just by decreasing the attachable/exposed surface in relation to the whole cell volume (Neumann et al. 2005). Above explanation validates the observations that the cells maintain a higher than normal cell volume with respect to cell surface under stress conditions.

The mechanisms of heavy metals uptake and their resistance in bacteria have been well studied (Nies 1992; Brown et al. 1992; Ji and Silver 2005). One of the important mechanisms of resistance in Gram-negative bacteria is the RND (resistance, nodulation, cell division) protein mediated efflux of heavy metals through the bacterial cell membrane (Nies 2003); this mechanism operates also in acidophilic bacteria (Dopson et al. 2003). In case the number of RND proteins remains the same per cell, functioning of such efflux systems would be more effective if the cell surface area is reduced. Further, a reduction in the surface area will allow lower diffusion of heavy metal ions.

The EDX analysis of the cells confirmed depositions of the metals on cell envelop, and the deposited amount was estimated by atomic adsorption spectrometer (data not shown). Concomitantly, higher amount of Ca^{2+} was found in the culture filtrate of treated cells, which was estimated by ICP-MS (Table 2). Metal ions, especially Ca^{2+}, are reported to maintain the lipopolysaccharide assembly on the cell surface of *E. coli* (Kotra et al. 1999). Ca^{2+} plays an important role in cellular metabolism through 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). The divalent metal cations (Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+}), being structurally similar Ca^{2+} (Huheey et al. 1993), are proposed to replace Ca^{2+} from the binding sites because of similar ligand specificities.

Penicillin-binding-proteins (PBPs) located in the bacterial cell envelope, which bind penicillins and other β-lactam antibiotics, determine bacterial cell shape (Franklin and Snow 1981). Treatment of bacterial cells with a divalent metal dislodges cell bound Ca^{2+} with the treated divalent metals, thereby inactivating/modulating some of the functioning PBPs. This functional modification of normal PBPs' enzyme activity may activate other PBPs that do not function in normal cells or may change the activity of normal enzymes; in either case, cells undergo changes that decrease the surface area of metal-exposed cells in comparison to cell volume. Similarly, since each metal ion induced different cell morphology, it may be concluded that activity of each PBP was not affected in the same way by a metal. Like cephalexin, which inhibits septum formation, Cd^{2+} also induced filamentous growth of greatly elongated cells. Cu^{2+} stress, on the other hand, caused cells to assume an abnormal ovoid shape like that observed in the case of mecillinam (Fig. 2d).

In conclusion, it may be stated that *A. symbioticum* H8 strain circumvents the toxic effects of heavy metals at sublethal concentrations by reducing its surface area in respect of cell volume through alteration of cell structure. In all probability, the activities of PBPs are modulated by the heavy metal ion that replaces Ca^{2+} from the cell surface. At growth inhibitory concentration, the metals bind quickly with various intracellular proteins and ligands causing rapid cell death; thus no change in cell morphology was noted at growth inhibitory concentrations of the metals.

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