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Vitreoscilla hemoglobin renders *Enterobacter aerogenes* highly susceptible to heavy metals

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Abstract

When expressed in heterologous microorganisms *Vitreoscilla* hemoglobin (VHb) acts as oxygen storage and causes a higher oxygen uptake. In this study, the effect of this protein on growth, sensitivity and antioxidant properties of *Enterobacter aerogenes* exposed to metal stress was investigated. The strain expressing VHb was more sensitive to mercury and cadmium as the minimal inhibitory concentration (MIC) for these metals was up to 2-fold lower in this strain than the host and the recombinant strain carrying a comparable plasmid. At lower concentrations than MIC, the metals partially limited growth and caused an inhibition proportional to metal concentration applied. The growth pattern of VHb expressing strain was also distinctly different from other two non-hemoglobin strains. The hemoglobin containing strain showed substantially higher superoxide dismuates (SOD) activity than the non-hemoglobin strains, while catalase levels were similar in all strains. All strains exposed to copper, however, showed similar MIC values, growth patterns, and SOD and catalase levels.

Introduction

Microbes encounter metals/heavy metals of various kinds in their environment and it is, therefore, not surprising that they should interact with them, sometimes to their benefit, at other times to their detriment. Although as micronutrients, some metals, e.g., Mn, Cu, Zn, Mo, and Ni are essential or beneficial for microorganisms, plants, and animals, others including Hg and Cd have no known biological function (Osborn *et al.* 1997; Roane and Pepper 2000). Yet, all metals at high concentrations are toxic to microorganisms, inhibiting enzymatic activities, disrupting membrane functions, and damaging nucleic acids. To cope with these, microorganisms employ a large variety of elaborated strategies. The effects and mechanisms of metal stress on microorganisms have been the subject of many studies, reviewed by Ehrlich (1997) and Nies (1999). Cells respond to heavy metals by exhibiting a loss of reductive reserve (such as glutathione) and displaying evidence of oxidant stress. When this occurs, there is denaturation of proteins especially those that contain heme groups (as cytochromes and hemoglobin) through the enzymatic cleavage of porphyrin resulting in the liberation of free iron, a free radical generator (Stocker 1990).

The *in vivo* production of free radicals can be both enzymatic and non-enzymatic. Aerobic respiratory metabolism of bacteria (and also other organisms) optimally results in four-electron reduction of O_2 to H_2O. A variable percentage of O_2 reduction, however, occurs initially via either one, two, and three-electron

reduction to $O_2^{\bullet -}$, H_2O_2, and $^{\bullet}OH$, respectively. Reactive oxygen species are more active than the triplet oxygen molecule present in our atmosphere. Various extrinsic and intrinsic agents such as light, heat and metals form these free radicals and active oxygen species. In *Escherichia coli* these oxidants arise primarily from the autoxidation of components of its respiratory chain (Messner and Imlay 1999). Superoxide radical and H_2O_2 are moderately reactive in aqueous solutions, but they both contribute to the formation of the highly reactive $^{\bullet}OH$ radical via the Fenton ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^-$) and Haber-Weiss ($H_2O_2 + O_2^{\bullet -} \rightarrow O_2 + {}^{\bullet}OH + OH^-$) reactions. It is known that, internal generation of these reactive species results in damage to DNA, proteins, and lipids (Aldsworth *et al.* 1999). Aerobic organisms are protected from oxidative stress induced by free radicals and active oxygen species by an array of defense systems. Most bacteria, including *E. coli*, contain superoxide dismutase (SOD) and catalase as means of eliminating $O_2^{\bullet -}$ and H_2O_2, respectively, before these reactive oxygen species react with metal ions to produce $^{\bullet}OH$ (McCormick *et al.* 1998). Thus, cells have the ability to convert a pro-oxidant insult from heavy metals into radical scavengers and antioxidants.

It is known that excess oxygen and oxygen starvation both cause stress in bacteria, through the production of harmful free radicals. While, high oxygen concentration results in generation of reactive oxygen species and potentially lethal damage to membranes and DNA, hypoxia causes in a failure to generate sufficient ATP to maintain essential cellular functions. In previous studies, we have shown that, *Enterobacter aerogenes* and *Pseudomonas aeruginosa* engineered with *Vitreoscilla* hemoglobin (VHb) gene (*vgb*) had higher oxygen uptake rates (Geckil *et al.* 2001) than the *vgb*$^-$ counterparts. Also, the ability of VHb to complement deficiencies of terminal cytochrome oxidases in *E. coli* suggests that this hemoglobin can receive electrons during respiration (Dikshit *et al.* 1992). From an evolutionary point of view, VHb has been suggested to function in 'detoxification' rather than in 'respiration', as is the case for mammalian hemoglobin (Minning *et al.* 1999). The aim of this study was to investigate the effect of an efficient oxygen uptake (and presumably detoxifying) system (VHb/*vgb*) on growth and antioxidant enzyme status of *E. aerogenes* under heavy metal imposed oxidative stress. The heavy metals selected were bivalent metal cations Cu, Cd, and Hg, with a different range of toxicities. While Cu is an essential micronutrient, Cd and Hg have no known physiological functions and are widely recognized as the most toxic heavy metal species in our environment.

Materials and methods

Chemicals, enzymes, and growth medium

Ferricytochrome *c*, xanthine oxidase (Grade I), SOD (bovine erythrocyte), and xanthine were purchased from Sigma. The chloride salt formulations of metals ($CdCl_2.H_2O$, $CuCl_2.2H_2O$, $HgCl_2$) were from Merck Chemicals. Stock solutions were prepared in doubly distilled water, sterilized at 120 °C for 15 min and stored in the dark. Hydrogen peroxide (a 30% solution, v/v) was from Merck Chemicals Co. All chemicals were of analytical reagent grade. Luria Broth (LB, pH 7.0) medium was prepared as described (Miller, 1972), except that peptone was used in place of tryptone. Where indicated, ampicillin was added to LB (LBamp) at concentration of 100 μg/ml for *E. aerogenes* recombinants. The glassware used was acid (1 N HCl) washed and rinsed several times with distilled water before use to avoid metal contamination. Culture medium and stock solutions of metals were autoclaved (at 120 °C for 25 min) separately and aliquots of appropriate metal stock solutions were added to culture media to give specified final metal concentrations.

Bacterial strains and growth conditions

The bacterial host used throughout this study was *E. aerogenes* (NRRL B-427), obtained from the USDA culture collection in Peoria, IL. Plasmid pUC8:15 (Dikshit and Webster 1988) has *vgb* (0.6 kb) inserted into vector pUC8 (Messing 1983) on a *Vitreoscilla* DNA fragment of 2.3 kb. Transformation of pUC8 and pUC8:15 into *E. aerogenes* was by a modification of the $CaCl_2$ method of (Cohen *et al.* 1972). The recombinant strains of *E. aerogenes* bearing the plasmid pUC8 or pUC8:15 were designated as 'Ea[pUC8]' and 'Ea[pUC8:15]', respectively, (Geckil *et al.* 2003). A 1/100 inoculum of overnight culture of each strain grown in LB or LBamp was prepared in 20 ml fresh LB (in 125 ml flasks) and grown to about $A_{600} = 0.05$ (about 1 h of incubation) to which sub-MIC concentrations ($CuCl_2 \cdot 2H_2O$, 3.125 mM; $CdCl_2 \cdot H_2O$, 0.625 mM; and $HgCl_2$, 5×10^{-3} mM) of heavy metals were added. Incubation continued at 37 °C in a water-shaker (200 rpm) for an additional 10 h

period. Samples for determining cell mass (A_{600}) and viable cell number were taken at 2 h intervals during incubation.

Determination of MIC

Aliquots of freshly grown cultures were used to inoculate tubes containing 5 ml medium with a graded series of increasing metal concentrations. The MIC (the traditional means to define the resistance level of a microorganism to a toxicant) was defined as the lowest concentration of metal that inhibited cell growth at 37 °C in a 200-rpm water-bath for a 24 h incubation period.

Growth inhibition levels on diffusion zones of metals

A semi-quantitative plate agar plate diffusion method was carried out to determine the level of growth inhibition by heavy metals used. A 50 μl overnight culture of each strain was inoculated into 5 ml LB and incubated (37 °C, 200 rpm) to about $A_{600} = 1.0$ where 500 μl was spread over agar plates and taken into incubator for 20 min drying. Ten μl of different dilutions of stock solutions of each heavy metal was applied onto the center of the plates and growth on diffusion zone was evaluated after 24 h of incubation compared to controls receiving 10 μl sterilized distilled water.

Preparation of cell-free extracts for catalase and SOD assays

The inoculation and growth conditions of cells in the presence of heavy metals were the same as for the growth studies above, except that cells were harvested after 24 h of incubation. Cells were harvested by centrifugation (10 000 rpm, 10 min) and the pellet was washed with 50 mM KPi buffer (0.02 M KH_2PO_4 and 0.03 M $Na_2HPO_4 \cdot 2H_2O$), pH 7.0. While the cell pellet for catalase was suspended in the same buffer, cell pellet for SOD was suspended in 50 mM KPi buffer containing 0.1 mM EDTA, pH 7.8. Cell pellets for both enzymes were re-suspended in ice-cold aliquots of their buffer to $A_{600} = 10$ and disrupted with an ultrasonifier (Bronson sonifier 450) by 6 cycles, 20-s sonications and 40-s pauses on ice. The extract was centrifuged (15 000 $\times$ *g*, 10 min, 4 °C) and cell-free supernatants were subjected to enzyme assays without further delay.

Catalase and SOD assay

Catalase activity was measured by the method of Aebi (1984). The rate of disappearance of H_2O_2 was measured spectrophotometrically at 240 nm ($\varepsilon_{240} = 40\ M^{-1}\ cm^{-1}$). One unit of catalase activity is defined as the amount of enzyme catalyzing the degradation of 1 μmol of H_2O_2/min at 25 °C. Superoxide dismutase activity in extracts was determined by measuring the inhibition of cytochrome *c* reduction using xanthine/xanthine oxidase $O_2^{\bullet -}$ generating system at 550 nm (McCord and Fridovich 1969). One unit of SOD activity was defined as the amount of enzyme that inhibits the rate of cyt *c* reduction by 50% at 25 °C. Specific activity was defined as the amount of the enzyme causing half maximum inhibition of cyt *c* reduction and expressed as U mg^{-1} protein. Total protein for both enzyme assays was determined colorimetrically (Lowry *et al.* 1951), using bovine serum albumin as the standard.

Results

Metal susceptibility and growth profiles of E. aerogenes *and its vgb$^-$ and vgb$^+$ recombinants*

The inhibitory effect of Cu^{2+}, Cd^{2+}, and Hg^{2+}, in the form of their chloride salt formulations ($CdCl_2 \cdot H_2O$, $CuCl_2 \cdot 2H_2O$, $HgCl_2$), on *E. aerogenes* and its recombinants grown in aerobic shake-flasks was investigated. Cells were grown in the presence of sub-MIC concentrations of metals, determined from our preliminary experiments. At lower concentrations than MICs, there was a decrease in both viable cell number (colony forming unit, cfu) and total cell mass (measured as A_{600}) proportional to the metal concentration applied (data not shown). The VHb expressing strain (Ea[pUC8:15]) showed substantially lower MICs for Cd and Hg than non-*vgb* strains. The MIC for Cu, however, was similar for all three strains. The 24 h MICs for growth of *E. aerogenes* and its *vgb*$^-$ recombinant (Ea[pUC8]) in LB medium were 2.5 mM and 0.04 mM, for Cd and Hg, respectively. The strain Ea[pUC8:15], however, had MIC values 1.5 mM and 0.02 mM for Cd and Hg, respectively. All three strains showed a 24 h MIC of 6.25 mM for Cu. Similar results were also observed on agar diffusion plates (Table 1). The concentrations of metals required to inhibit cell growth on solid medium to a level similar to growth inhibition in liquid medium were, however, higher for all three metals. The growth responses of *E. aeogenes* and its *vgb*$^-$ and *vgb*$^+$ recombinants to the presence of 3.125 mM Cu, 0.625 mM Cd, and 0.005 mM Hg in

Table 1. Growth inhibition of *E. aerogenes* and its recombinants on the diffusion zone of metals at various concentrations, using a semi-quantitative agar plate diffusion method

Strain	Cu (mM) control	0.1	0.5	1.0	5.0	10
Ea	+++++	+++++	+++++	+++++	+++−−	−−−−−
Ea[pUC8]	+++++	+++++	+++++	+++++	+++−−	−−−−−
Ea[pUC8:15]	+++++	+++++	+++++	+++++	+++−−	−−−−−
	Cd (mM) control	0.05	0.25	0.5	2.5	5.0
Ea	+++++	+++++	+++++	++++−	+−−−−	−−−−−
Ea[pUC8]	+++++	+++++	+++++	++++−	+−−−−	−−−−−
Ea[pUC8:15]	+++++	+++++	++++−	+++−−	−−−−−	−−−−−
	Hg (mM) control	0.001	0.005	0.01	0.05	0.1
Ea	+++++	+++++	+++++	+++−−	−−−−−	−−−−−
Ea[pUC8]	+++++	+++++	+++++	+++−−	−−−−−	−−−−−
Ea[pUC8:15]	+++++	+++++	++++−	++−−−	−−−−−	−−−−−

LB medium are given in Figures 1–3. The host strain, *E. aerogenes*, showed slightly better growth (measured either as viable cell number or total cell mass) than recombinant strains to the presence of Cu. Contrary to the effect of Cu, growth of Ea[pUC8:15] was severely affected by both Cd and Hg compared with host or *vgb*$^-$ recombinant strain.

Determined as viable cell number or total cell mass (A_{600}), the VHb expressing strain was rendered highly susceptible to killing or growth inhibition by Cd (Figure 2) and Hg (Figure 3). There was >20 %decrease in viable cell number for Ea[pUC8:15] during the first 2 h of incubation in the presence of Cd. No such effect was observed for either host strain or Ea[pUC8] both of which showed slight increases in viable cell numbers. At the end of incubation period (10 h), the non-*vgb* strains showed more than 5-fold higher viable cell number than that of *vgb*-bearing strain.

The growth inhibitory effect of mercury showed similar pattern to that of Cd. The effect of this metal on VHb expressing strain was even more extensive than on non-*vgb* strains. Addition of Hg into culture medium caused an about 4-fold decrease in viable cell number of this strain in initial 4 h of growth, an effect that was not observed for either *E. aerogenes* or Ea[pUC8]. The later two strains showed 40-fold and almost 2-orders of magnitude, respectively, higher viable cell number than Ea[pUC8:15] at 10 h of incubation period.

VHb effect on the expression of catalase and SOD in the presence of heavy metals

The metal effect on the activities of catalase and SOD is presented in Table 2. SOD activities of all three strains grown in the presence of Cu were similar. The activity of this enzyme, however, was substantially higher in VHb expressing strain (Ea[pUC8:15]) than both non-*vgb* strains in the presence of either Cd or Hg. The *vgb*-bearing strain showed 70% and about 40% higher SOD activity than that of host strain and *vgb*$^-$ strain, respectively, in the presence of Cd. This difference was even more evident with Hg, which caused almost a 2-fold increase in SOD activity of VHb expressing strain compared with other two non-*vgb* strains showing similar level of activity. The catalase activity of all three strains, however, was similar in the presence of these metals.

Discussion

Despite the abundance of data on the characteristics of the hemoglobins and myoglobin of eukaryotic origin, the biological function of *Vitreoscilla* hemoglobin (VHb), the only well-characterized bacterial hemoglobin, has remained elusive. In this study, the effect of oxidative stress imposed by heavy metals on growth properties and activities of two key antioxidant enzymes (catalase and SOD) of *E. aerogenes* and

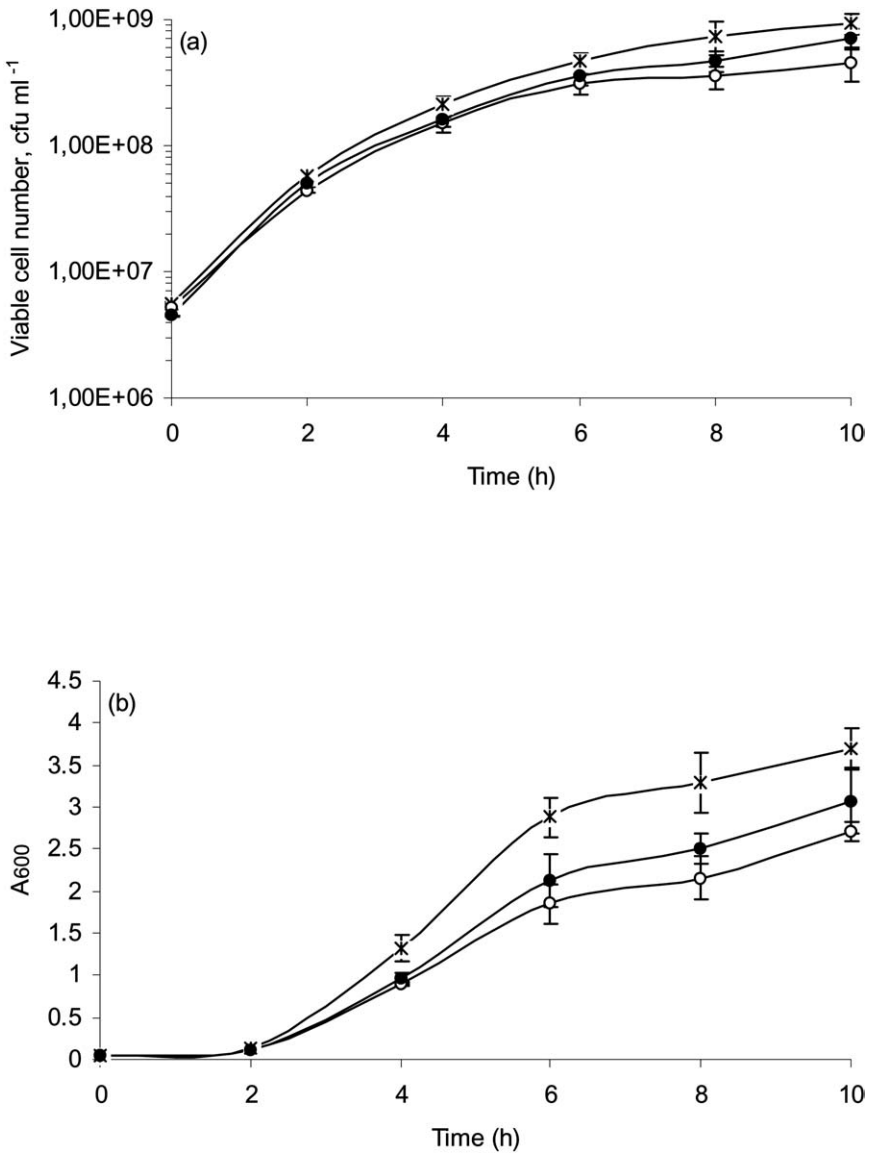

Figure 1. Effect of copper on viable cell number (a) and total cell mass (b) of exponentially growing *E. aerogenes* (⁎), Ea[pUC8] (○) and Ea[pUC8:15] (●). Cells were grown in LB to about $A_{600} = 0.05$ where 3.125 mM Cu was added and incubation continued in a shaking water-bath (200 rpm) at 37 °C. Each data point is the average of three independent experiments with error bars indicating standard deviations (σ_{n-1}); where not visible they are smaller than diameters of the points.

its *vgb*$^-$ and *vgb*$^+$ recombinants was investigated. To our knowledge, we present here the first study that assesses the heavy metal susceptibility and antioxidant enzymes activity of a bacterium (*E. aerogenes*) carrying a recombinant bacterial hemoglobin system, VHb/*vgb*. The metals selected for this study were Cu, Cd and Hg, all three bivalent metal cations with diverse functions or toxicity levels.

The results clearly showed that the presence of VHb makes *E. aerogenes* highly susceptible to killing by Cd and Hg, but not by Cu. Measured as viable cell number or total cell mass (as A_{600}), the growth pattern of VHb expressing strain was also distinctly different from non-*vgb* strains in the presence of Cd and Hg. At sub-MIC concentrations, these metals had substantial growth inhibiting effects on VHb expressing strain compared with either host strain or the strain carrying a comparable plasmid with no *vgb* insert (Ea[pUC8). The *vgb* strain (Ea[pUC8:15]) had more than 1.5-fold and 2-fold lower MIC values for Cd and

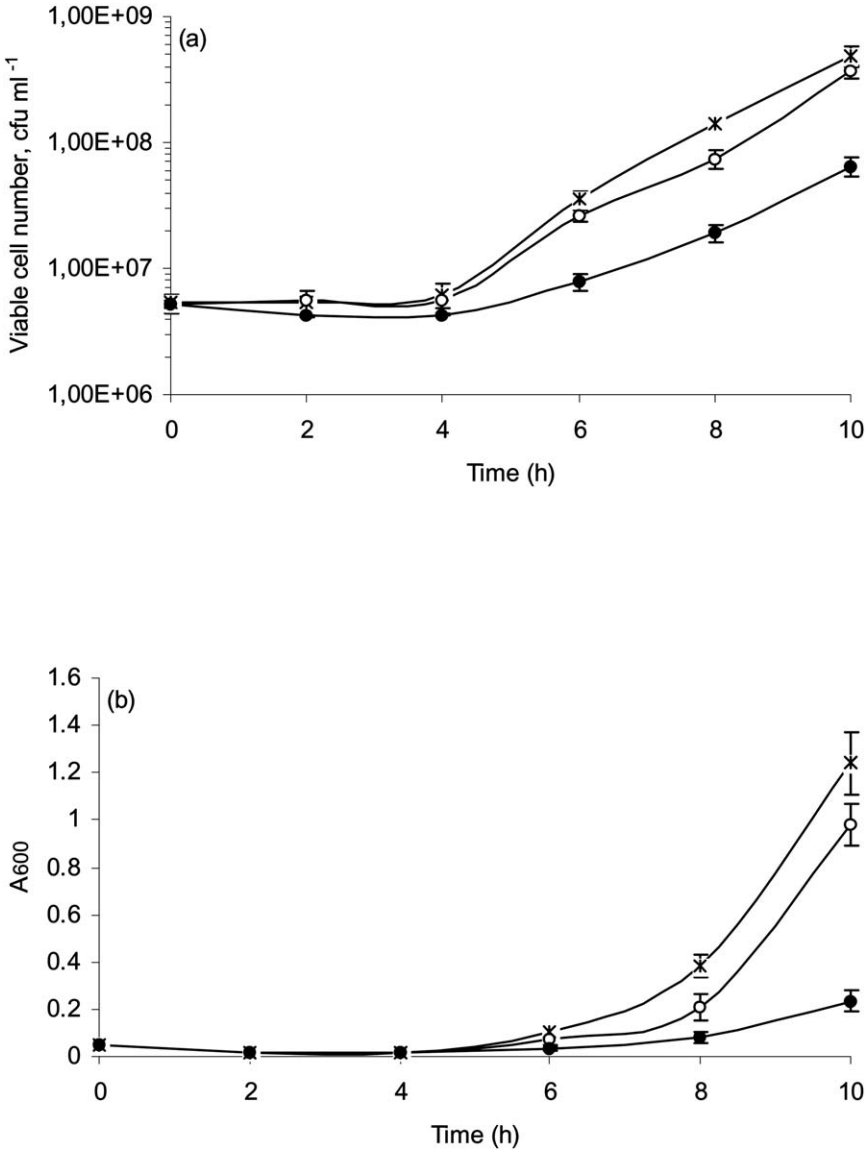

Figure 2. Effect of cadmium on viable cell number (a) and total cell mass (b) of exponentially growing *E. aerogenes* (⁎), Ea[pUC8] (○) and Ea[pUC8:15] (●). Cells were grown in LB to about $A_{600} = 0.05$ where 0.625 mM Cd was added and incubation continued in a shaking water-bath (200 rpm) at 37 °C. Each data point is the average of three independent experiments with error bars indicating standard deviations (σ_{n-1}); where not visible they are smaller than diameters of the points.

Hg, respectively, compared to that of the host strain and *vgb*$^{-}$ recombinant strain both with similar MIC values for these metals. Substantial growth inhibition of Ea[pUC8:15] strain by Cd and Hg could be due to strong respiration (Geckil *et al.* 2003) of this strain and interference of these metals with hemoglobin function, both contributing synergistically to an elevated oxidative stress. The same effects, however, was not observed with Cu. In this context, the minimal inhibitory concentration for Cu was similar for all three strains, which showed also similar growth patterns to the presence of sub-MIC concentrations of this metal. In line with other studies (Hassen *et al.* 1998; Nies 1999), mercury was determined to be the most toxic metal with 24 h MIC value more than 60-fold and 2 orders of magnitude lower than MICs for Cd, and Cu, respectively. Same was also true for the effect of Hg on growth patterns of cells. The concentration of Hg causing a similar growth inhibition to other two metals was several hundred (625)-fold lower than Cu and more

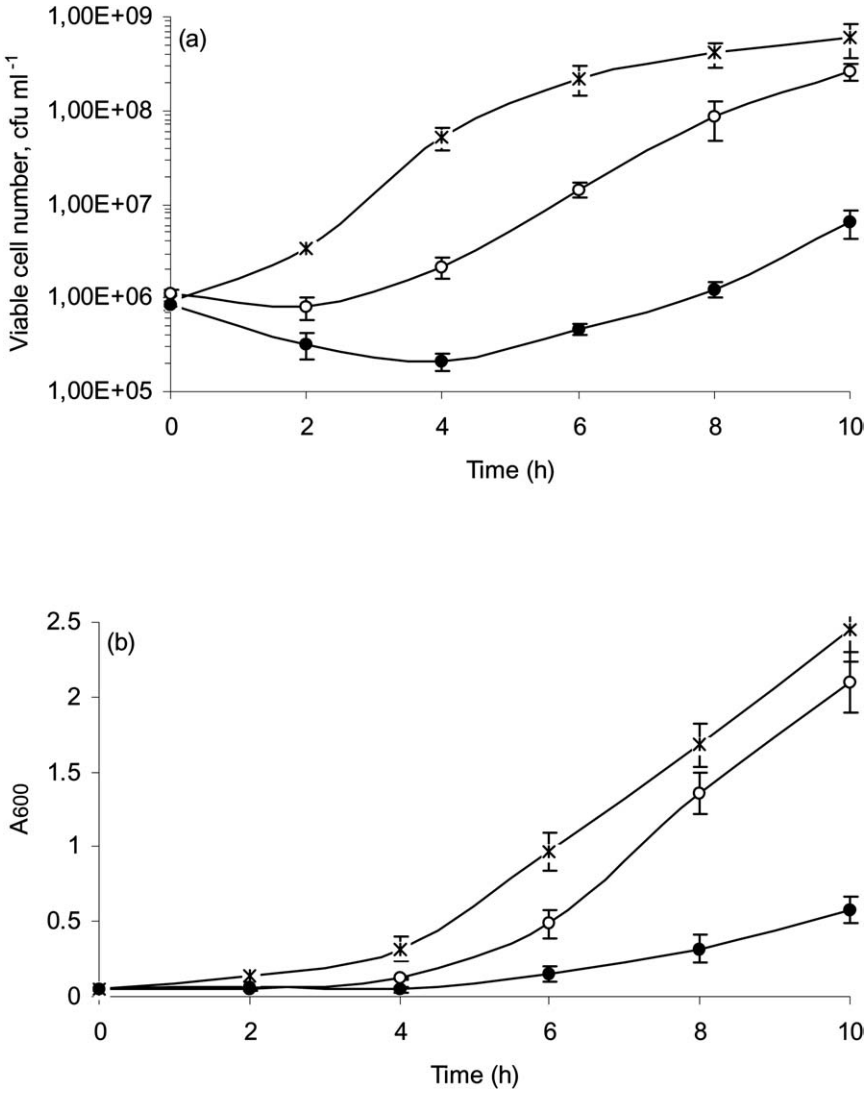

Figure 3. Effect of mercury on viable cell number (a) and total cell mass (b) of exponentially growing *E. aerogenes* (⁕), Ea[pUC8] (○) and Ea[pUC8:15] (●). Cells were grown in LB to about $A_{600} = 0.05$ where 0.005 mM Hg was added and incubation continued in a shaking water-bath (200 rpm) at 37 °C. Each data point is the average of three independent experiments with error bars indicating standard deviations (σ_{n-1}); where not visible they are smaller than diameters of the points.

than two orders of magnitude (125-fold) lower than lower than Cd. One other possible explanation for the extensive growth restricting effect of Cd and Hg but not that of Cu on hemoglobin expressing strain might reside in different redox potential of this strain. It has been shown that VHb expressing bacteria generally have a more oxidized interior than the VHb^- counterparts (Tsai *et al.* 1995), which could well have effects on rates of key processes including energy requiring multiple transport systems existing for the transport of these metals (Bornet *et al.* 2003; De Freitas *et al.* 2003; Nies 2003).

The MIC values of three metals for *E. aerogenes* and its recombinants grown in LB were substantially (4–6 fold) higher than the of MIC values for the same metals reported for *E. coli* grown in a mineral salts medium (Nies 1999). This difference in two such closely related bacteria may be attributable to the difference in medium composition used with each of the two organisms, because it has been found that medium composition is an important factor affecting metal toxicity for bacteria (Konopka and Zakharova 1999). There are significant interactions between the chemical milieu and heavy metals, which affect the

Table 2. Oxidative stress enzymes in cell-free extracts of *E. aerogenes* and its vgb^- and vgb^+ recombinants grown in LB in the presence of heavy metals for 24 h (37 °C, 200 rpm). The metal concentrations used were 3.125 mM, 0.625 mM and 5×10^{-3} mM for Cu, Cd and Hg, respectively. Each value is the average of two trials in duplicate with ± SDEVs (σ_{n-1}) in the parenthesis

Metal	Strain	Specific activity (units (mg protein)$^{-1}$) Catalase	SOD
Cu	*E. aerogenes*	28 (2)	25(5)
	Ea[pUC8]	25(1)	23(2)
	Ea[pUC8:15]	27(1)	28(5)
Cd	*E. aerogenes*	33(6)	25(4)
	Ea[pUC8]	31(5)	31(2)
	Ea[pUC8:15]	27(2)	42(7)
Hg	*E. aerogenes*	29(1)	23(1)
	Ea[pUC8]	28(3)	23(4)
	Ea[pUC8:15]	25(3)	46(5)

bioavailability of metal ions and therefore the capacity of microbes to grow. Thus, it is thought that the biological response to a dissolved metal is a function of the concentration of free-metal ion. In this context, cells in liquid LB were more sensitive than cells on solid medium (LB agar) to the same concentrations of heavy metals used, a result that might be explained by bioavailability of metal ions to growing cells.

To find out whether growth inhibition by heavy metals was the result of oxidative stress, antioxidative systems were analyzed. The activities of two key enzymes, catalase and SOD, of antioxidant response were increased by several fold compared with activities of the same enzymes in non-stressed cells or in cells exposed to oxidative stress by exogenously added hydrogen peroxide which, contrary to heavy metals, was determined to even repress the production of catalase and SOD activity (Geckil *et al.* 2003). Thus, the oxidative stress generated by heavy metals has opposing effects in terms of antioxidant response to that of hydrogen peroxide. This was even more evident in the case of SOD, which is known to be induced as the first line of defense against the generation of toxic oxygen species (Lenartova *et al.* 1998). All three strains had similar activities for both catalase and SOD in the presence of Cu. While the catalase activity was also similar in these strains in the presence of Cd or Hg, the hemoglobin containing strain ((Ea[pUC8:15]) showed substantially higher SOD activity than *E. aerogenes* and Ea[pUC8]. This indicates that the VHb in combination with cadmium or mercury enhanced the oxidative stress for *E. aerogenes* possibly inducing mainly the SoxRS regulon, as the catalase activity was not influenced. Although, there is need for further studies to explain the function of VHb resulting induction of SOD (but not catalase) activity in *vgb*-recombinant strain in the presence of Cd and Hg, one explanation might be that the higher oxygen uptake by VHb^-expressing strain (Geckil *et al.* 2001; 2003; 2004) might cause higher generation of reactive oxygen species. The presence of heavy metals further induces the production of these reactive oxygen radicals, resulting in turn, induction of SOD as the first line of defense. Cadmium and Hg posses much higher affinity to thiol (-SH) groups of proteins and reductive reserves (e.g., glutathione) in the cell than the latter which has an electrochemical potential well within the physiological range of the cell (Nies 1999). Studies with Hg have demonstrated that it exerts oxidative stress via H_2O_2 generation, glutathione depletion, and reactivity with membrane bound protein thiols and these may lead to lipid peroxidation (Lenartova *et al.* 1998). To elucidate the role of VHb in making cells highly sensitive to Cd and Hg, but not to Cu, and in contributing to a higher SOD activity but not catalase further more complete studies are necessary and future studies will be aimed at identifying this role of VHb.

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