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Influence of Hydrostatic Pressure on the Effects of the Heavy Metal Cations of Manganese, Copper, Cobalt, and Nickel on the Growth of Three Deep-Sea Bacterial Isolates

EDWARD J. ARCURI AND HENRY L. EHRLICH*
Department of Biology, Rensselaer Polytechnic Institute, Troy, New York

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Increased hydrostatic pressure varied the 72-h growth yield of three bacterial isolates from the deep sea in the presence of heavy metal cations of Mn, Cu, Co, and Ni, depending on the bacterial isolate, the metal cation and its concentration, and the level of hydrostatic pressure. Above atmospheric, hydrostatic pressure was found to have one of the following four effects on the response of culture growth to a heavy metal cation. (i) It could be without effect; (ii) it could enhance inhibition by a metal cation; (iii) it could increase the 72-h growth yield by a metal cation; or (iv) it could protect against a growth inhibitory effect noted at a lower pressure. Possible reasons for these varied responses are discussed.

Bacteria have been found associated with deep-sea ferromanganese nodules (3). The nodules consist primarily of Fe and Mn, the average concentration of each in a Pacific Ocean nodule being 4.8 and 29.8%, respectively (6). These nodules also contain lesser amounts of Ni, Cu, and Co, the average concentrations of these elements being 1.4, 1.2, and 0.2%, respectively (6). Some of the bacteria associated with the concretions enzymatically catalyze the oxidation of Mn(II) whereas some others enzymatically catalyze the reduction of Mn(IV). They are, therefore, believed to play a role in nodule genesis in the deep sea (2).

The growth of nodules may be viewed as an accretion process in which the Mn(IV) oxides of nodules scavenge Mn^{2+} ions which are then biologically and chemically oxidized to Mn(IV) oxides. The accretion process also involves the scavenging of ferric iron and Cu^{2+}, Co^{2+}, and Ni^{2+} ions from seawater or interstitial water of the sediments by Mn(IV) oxides. Some in situ degradation or diagenesis of nodules may be viewed as a dissolution process in which Mn(IV) oxides are reduced biologically and chemically to Mn^{2+} ions with the concurrent release of other nodule components such as Cu^{2+}, Ni^{2+}, and Co^{2+} ions, but not Fe (4). Thus, the bacteria residing on nodules must be exposed to a flux of Mn^{2+}, Co^{2+}, Cu^{2+}, and Ni^{2+} ions, and it, therefore, becomes of interest to determine the effects of these ions on the bacteria. Yang and Ehrlich (8) investigated the effect of Mn^{2+}, Cu^{2+}, Ni^{2+}, and Co^{2+} ions on the growth of ferromanganese nodule bacteria at atmospheric pressure. However, since in situ the ferromanganese nodule bacteria are subjected to elevated hydrostatic pressure, its effect on metal ion interactions with the growing bacteria becomes of major importance and is the subject of this communication.

MATERIALS AND METHODS

Cultures. Culture BIII 39, which was isolated from a Pacific Ocean ferromanganese nodule, is a gram-negative, motile rod. It is also a Mn(II) oxidizer. Cultures BIII 32 and BIII 88 are also gram-negative motile rods. Culture BIII 32 was isolated from a Pacific Ocean ferromanganese nodule, whereas culture BIII 88 was isolated from Pacific Ocean sediment. Both cultures BIII 32 and BIII 88 are Mn(IV) reducers.

Stock cultures were maintained by monthly transfer on nutrient agar (Difco) slants, made up in full-strength, filtered seawater. These cultures were incubated and stored at 15°C. Working cultures were derived from a stock culture by transfer to fresh nutrient agar slants in test tubes and incubated for 24 h at 15°C.

Inoculum preparation. For experimental use, the cells of 24-h working slants were harvested in 5 ml of sterile sea water and washed three times by centrifugation at 2,100 × *g* for 7 min at 20°C with 5 ml of sterile seawater. The cell concentration of the suspension was then determined with a Petroff-Hausser counting chamber, after which the suspension was diluted with 3% sterile NaCl solution to give a final cell concentration of 5.0×10^3 cells/ml. This cell suspension was then used as an inoculum.

Measurement of microbial numbers. Viable cell counts were done by plating in triplicate on nutrient agar (Difco) made up in full-strength seawater, using the capping agar method of Ehrlich et al. (3) with the following modification. Samples (1 ml) of the cell suspension were added directly to the sur-

face of the basal agar (10 ml, gelled) in petri dishes. At that time, 3 ml of capping agar was poured onto the basal agar in each plate and distributed evenly. The capping agar was found to gel within 3 min.

Protocol for pressure experiments. To flasks containing 10.5 ml of autoclaved seawater, the following sterile solutions were added: 0.5 ml of 2.5% glucose, 0.5 ml of 0.625% peptone, and 0.5 ml of a desired metal ion (Mn^{2+}, Co^{2+}, Ni^{2+}, or Cu^{2+}) solution at an appropriate concentration. The temperature of these flasks was then lowered to 15°C prior to the addition of 0.5 ml of the diluted cell suspension. The final concentrations of glucose and peptone in the seawater solutions were 0.1 and 0.025%, respectively, and the final cell concentration was approximately 2.0×10^2 cells/ml. The final metal ion concentrations were 0, 0.1, 1.0, and 10.0 mg/liter for Mn^{2+}, Co^{2+}, or Ni^{2+}; or 0, 0.1, and 1.0 mg/liter for Cu^{2+}. A 10.0-mg/liter concentration of Cu^{2+} ion was not tested because of solubility limitations in seawater. Each of the various metal ion concentrations was tested in duplicate in each experiment. After mixing, the contents of each flask were then transferred aseptically to separate, sterile test tubes (14 by 85 mm), each containing a glass bead (1.5-mm radius), and stoppered with an autoclaved silicon stopper (size 00) in such a way that no air space existed between the medium and the stopper. The tubes were then placed in temperature-equilibrated (15°C) pressure cells, containing distilled water at 15°C. The cells were pressurized to the desired pressure by the method of ZoBell and Oppenheimer (10). Except for pressurization, the tubes for incubation at 1 atm were treated in the same manner as tubes for higher pressures. The pressure cells were incubated for 72 h at 15°C. Upon completion of incubation the tubes were removed from each pressure cell and their contents were mixed on a Vortex mixer. The amount of growth in each tube was then determined by plating in triplicate. Oxygen was not thought to be limiting to growth during 72 h at 1 atm because when tubes without added toxic metals were incubated with an air bubble below the stopper, the 72-h growth yield was not significantly different from that in the absence of an air bubble.

Chemicals. Analytical grade metal salts were used in this study. These included $MnSO_4 \cdot H_2O$, $Ni(NO_3)_2 \cdot 6H_2O$, $Co(NO_3)_2 \cdot 6H_2O$, and $CuSO_4 \cdot 5H_2O$.

RESULTS

The effects of various concentrations of Mn^{2+} on the cell yield of culture BIII 39 at 1, 272, 340, and 408 atm of hydrostatic pressure are shown in Fig. 1A. It can be seen that at 1, 272, 340, and 408 atm none of the concentrations of Mn^{2+} tested exerted any significant effect on the 72-h cell yield of culture BIII 39. It is to be noted that at 408 atm the organism was unable to grow and, in fact, exhibited a slight die-off in the presence and absence of added metal ion.

Figure 2A shows the effects of various concentrations of Mn^{2+} on culture BIII 88 at 1 and 340 atm. As in the case of culture BIII 39, none of the Mn^{2+} ion concentrations tested exerted any significant effect on the cell yield of culture BIII 88 at either pressure.

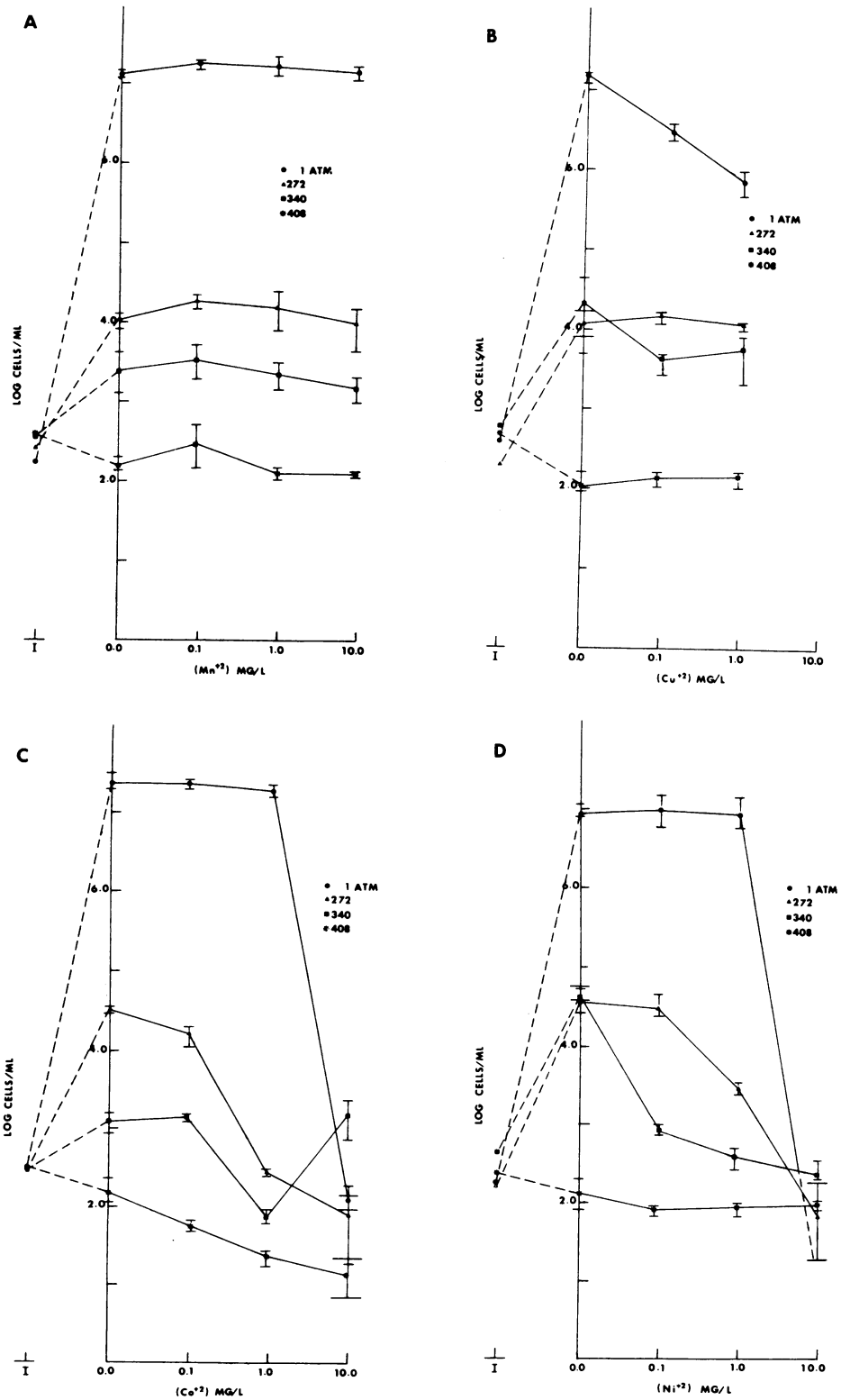
Figure 3A shows the effects of various concentrations of Mn^{2+} on the cell yield of culture BIII 32 at 1 and 340 atm. It can be seen that, although at 1 atm none of the metal ion concentrations exerted any significant effect on the cell yield of culture BIII 32, at 340 atm concentrations of 0.1, 1.0, and 10.0 mg of Mn^{2+} per liter caused 9.5, 12.4, and 8.8% increases in the cell yield, respectively. Although the deviation ranges of the plate counts, as represented by the range bars in Fig. 3A, may raise a question about the significance of these small stimulatory effects, the effects were readily reproduced in three independent experiments.

Figure 1B shows the effects of increased hydrostatic pressure on the interactions of Cu^{2+} with culture BIII 39. At 1 atm, concentrations of 0.1 and 1.0 mg of Cu^{2+} per liter exerted progressively greater depressing effects on the 72-h cell yields of the culture. However, upon increasing the pressure to 272 atm, the toxicity of both 0.1 and 1.0 mg of Cu^{2+} per liter disappeared. It may be inferred that the hydrostatic pressure at 272 atm was protecting the organism from the toxic effect of Cu^{2+} when compared to the toxic effect of Cu^{2+} observed at 1 atm. Upon increasing the pressure to 340 atm, toxic effects were again noted at 0.1 and 1.0 mg of Cu^{2+} per liter. Raising the hydrostatic pressure to 408 atm again eliminated the toxic effects of 0.1 and 1.0 mg of Cu^{2+} per liter, but it is important to note that at 408 atm the organism was unable to grow and exhibited a slight die-off in both the presence and absence of added Cu^{2+}.

The effects of increased hydrostatic pressure on the interactions of 0.1 and 1.0 mg of Cu^{2+} per liter with culture BIII 88 are given in Fig. 2A. It can be seen that, of the concentrations of Cu^{2+} tested, 1.0 mg of Cu^{2+} per liter exerted a slight toxic effect on the cell yield of culture BIII 88 at 1 atm, whereas 0.1 mg of Cu^{2+} per liter had no effect at that pressure. At 340 atm, 0.1 mg of Cu^{2+} per liter exerted, at best, a slight stimulatory effect on the cell yield of the organism, whereas 1.0 mg of Cu^{2+} per liter had no effect on the cell yield of culture BIII 88.

Figure 3A shows the effects of 1 and 340 atm of hydrostatic pressure on the interactions of Cu^{2+} with culture BIII 32. At 1 atm, an increasing toxicity with increasing metal ion concentration was observed. At 340 atm, the toxicity of 0.1 mg of Cu^{2+} per liter observed at 1 atm was no longer noted, whereas 1.0 mg of Cu^{2+} per liter caused a significant decrease in the 72-h cell yield.

The effects of increased hydrostatic pressure


A
B
C
D
LOG CELLS/ML
1 ATM
272
340
408
(Mn+2) MG/L
(Cu+2) MG/L
(Co+2) MG/L
(Ni+2) MG/L
0.0
0.1
1.0
10.0
2.0
4.0
6.0

on the interaction of Co^{2+} with culture BIII 39 are shown in Fig. 1C. At 1 atm, neither 0.1 nor 1.0 mg of Co^{2+} per liter exerted any significant effect on the cell yield of culture BIII 39, whereas 10.0 mg of Co^{2+} per liter had a marked toxic effect. At 272 atm, a slight decrease in the 72-h cell yield was noted at 0.1 mg of Co^{2+} per liter, whereas at 1.0 and 10.0 mg of Co^{2+} per liter progressively larger toxic effects were observed. Upon raising the pressure to 340 atm, no decrease in the cell yield was noted at 0.1 mg of Co^{2+} per liter, but a significant decrease in cell yield at 1.0 mg of Co^{2+} per liter was noted. Surprisingly, at 340 atm, no toxic effect was observed at a concentration of 10.0 mg of Co^{2+} per liter, as had been observed at both 1 and 272 atm. However, when the pressure was raised to 408 atm, a progressively increasing toxic effect with increasing Co^{2+} concentrations over the entire concentration range was observed. We do not at present understand this behavior at 340 atm. We were able to repeat the findings in two other independent experiments. A more detailed study of this phenomenon is needed for an explanation.

Figure 2B shows the effects of 1 and 340 atm of hydrostatic pressure on the interaction of Co^{2+} with culture BIII 88. At 1 atm, 0.1 mg of Co^{2+} per liter exerted no effect on the 72-h cell yield of culture BIII 88, whereas 1.0 mg of Co^{2+}

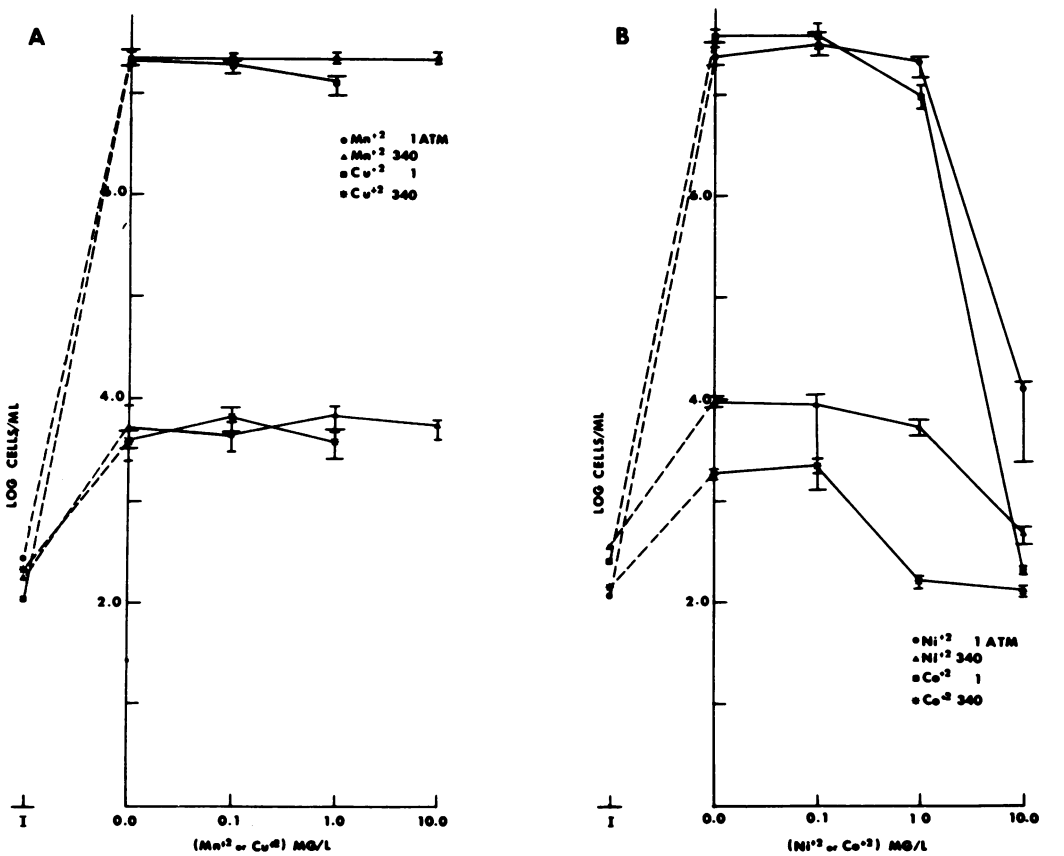


FIG. 2. *Effect of the divalent cations (A) Mn^{2+} and Cu^{2+} and (B) Co^{2+} and Ni^{2+} on the 72-h cell yield of culture BIII 88 at 1 and 340 atm of hydrostatic pressure. Each point represents the average of six plate counts. The bars represent the ranges over which the averages were computed. The initial cell concentration (I) is included as a basis for comparison with the cell concentration after 72 h.*

FIG. 1. *Effect of the divalent cations (A) Mn^{2+}, (B) Cu^{2+}, (C) Co^{2+}, and (D) Ni^{2+} on the 72-h cell yield of culture BIII 39 at 1, 272, 340, and 408 atm of hydrostatic pressure. Each point represents the average of six plate counts. The bars represent the ranges over which the averages were computed. The initial cell concentration (I) is included as a basis for comparison with the cell concentration after 72 h. The points in (C) at 1.0 and 10.0 mg of Co per liter were obtained by plating 2.0-ml samples of cell suspension instead of 1.0-ml samples (see Materials and Methods).*

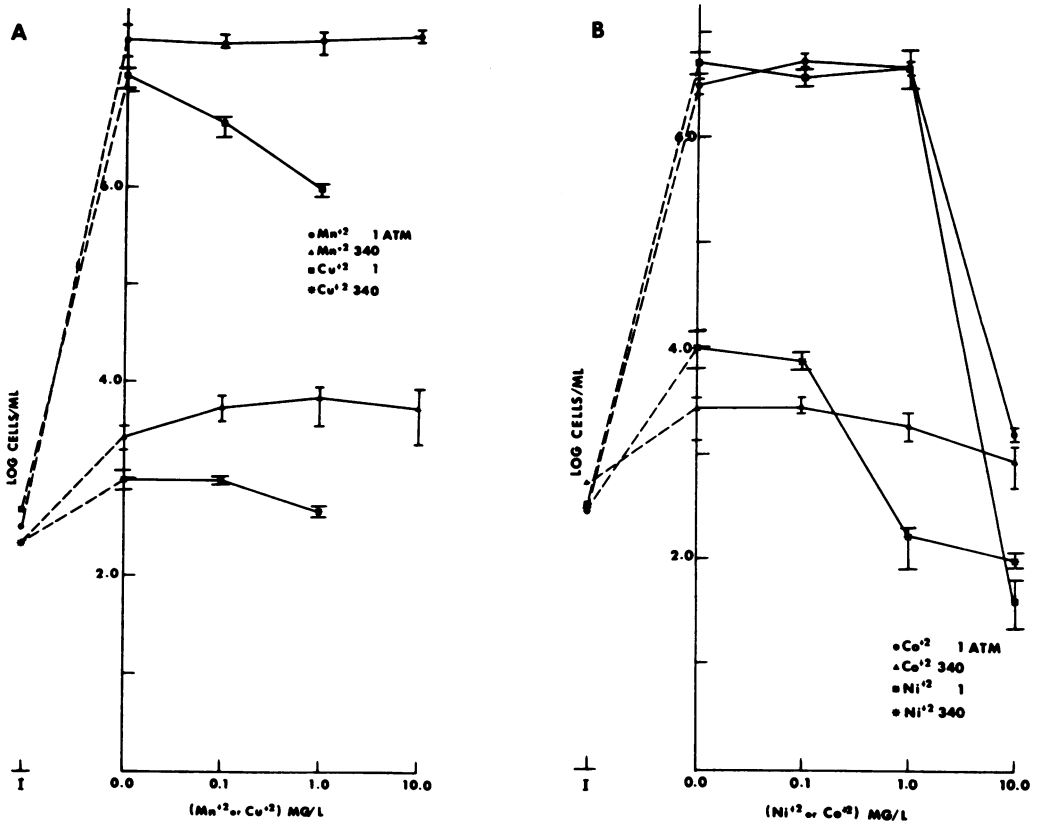


FIG. 3. *Effect of the divalent cations (A) Mn^{2+} and Cu^{2+} and (B) Co^{2+} and Ni^{2+} on the 72-h cell yield of culture BIII 32 at 1 and 340 atm of hydrostatic pressure. Each point represents the average of six plate counts. The bars represent the ranges over which the averages were computed. The initial cell concentration (I) is included as a basis for comparison with the cell concentrations after 72 h.*

per liter caused a small but significant decrease in cell yield, and 10.0 mg of Co^{2+} per liter caused a large decrease in cell yield. When the pressure was increased to 340 atm, 0.1 mg of Co^{2+} per liter caused no toxic effect, whereas both 1.0 and 10.0 mg of Co^{2+} per liter caused equally large decreases in cell yield.

The effects of 1 and 340 atm of hydrostatic pressure on the interaction of Co^{2+} with culture BIII 32 are shown in Fig. 3B. At 1 atm both 0.1 and 1.0 mg of Co^{2+} per liter exerted a slight stimulatory effect on the 72-h cell yield of culture BIII 32, whereas 10.0 mg of Co^{2+} per liter caused a large decrease in the cell yield of this organism. When these concentrations of Co^{2+} ion were tested at 340 atm, neither 0.1 nor 1.0 mg of Co^{2+} per liter exerted a significant effect on the cell yield of culture BIII 32, whereas 10.0 mg of Co^{2+} per liter caused a significant decrease in the 72-h cell yield.

Figure 1D shows the effects of increased hydrostatic pressure on the interaction of Ni^{2+} with culture BIII 39. It can be seen that an increase in toxicity occurred with increasing hydrostatic pressure. Thus, at 1 atm, only 10.0 mg of Ni^{2+} per liter exerted a toxic effect, whereas at 272 atm both 1.0 and 10.0 mg of Ni^{2+} per liter exerted a toxic effect. When the pressure was raised to 340 atm, all three of the metal ion concentrations tested exerted a significant toxic effect. However, at 408 atm, no toxicity was found at any of the metal ion concentrations tested. It should be noted that this organism was unable to grow at 408 atm and exhibited a slight die-off in both the absence and presence of added metal ion.

The effects of 1 and 340 atm of hydrostatic pressure on the interaction of Ni^{2+} with culture BIII 88 are shown in Fig. 2B. At 1 atm neither 0.1 nor 1.0 mg of Ni^{2+} exerted a very significant effect on the cell yield of culture BIII 88, whereas a large decrease in cell yield of culture BIII 88 was observed at 10.0 mg. At 340 atm, 0.1 mg of Ni^{2+} per liter exerted no toxic effect, whereas a slight toxic effect was observed at 1.0 mg of Ni^{2+} per liter. As was the case at 1 atm,

10.0 mg of Ni^{2+} per liter at 340 atm exerted a large toxic effect.

The effects of 1 and 340 atm of hydrostatic pressure on the interaction of Ni^{2+} with culture BIII 32 are shown in Fig. 3B. At 1 atm neither 0.1 nor 1.0 mg of Ni^{2+} per liter exerted a significant effect on the 72-h cell yield, whereas 10.0 mg of Ni^{2+} per liter caused a large decrease in cell yield. When the pressure was increased to 340 atm, 0.1 mg of Ni^{2+} per liter again exerted no effect on the cell yield, whereas both 1.0 and 10.0 mg of Ni^{2+} per liter caused significant decreases in the 72-h cell yield.

DISCUSSION

The results of this work indicate that the effects a metal ion exerts on the cell yield of a bacterium may be modified by pressure. We noted that, in the presence of potentially toxic metal ions, increased hydrostatic pressure may have one of the following four effects: (i) no change in the 72-h growth yield in response to a specific metal ion as compared to the response to the metal ion at 1 atm, (ii) a lowered 72-h growth yield (increased toxicity) when compared to the response to the metal ion at 1 atm, (iii) an increased 72-h growth yield when compared to the 72-h growth yield in the absence of the metal ion at a specific hydrostatic pressure, and (iv) a removal of growth inhibition (protective effect) when comparing the 72-h growth yield at an elevated hydrostatic pressure to that at a different pressure.

The observation that increasing hydrostatic pressure may totally reverse trends in metal ion effect from toxic to protective to toxic, as in the interactions of Cu^{2+} with culture BIII 39, seems to indicate that abrupt changes in cell response may be taking place at critical hydrostatic pressures rather than gradual changes with increasing hydrostatic pressure. These abrupt changes in response to metal ions at different pressures could be the results of pressure-induced conformational changes in cell wall or membrane proteins, or intracellular structures such as ribosomes (7), assuming these to be the sites of action of the metal ions, causing different reactive groups to be made available for interactions with the metal ions. The greater resistance of marine bacteria to heavy metal attack compared to terrestrial bacteria further suggests that some cellular structure is the site of heavy metal attack since it is believed that the basis for this difference in susceptibility is not biochemical or physiological (1, 5). In fact, it has been suggested that the difference in susceptibility lies in differences in cell wall and cell membrane structures and their various interactions (5).

The effect of the metal ions at 408 atm on cell yields of culture BIII 39 deserves closer consideration. We noted that culture BIII 39 was unable to grow at 408 atm in the absence of added metal ions and even exhibited a slight die-off. We also noted that the pressure induced toxicities of Cu^{2+} and Ni^{2+}, but not of Co^{2+}, disappeared at 408 atm. This could indicate that Cu^{2+} and Ni^{2+} but not Co^{2+} attack at a site or sites related to cell growth, such as sites of active cell wall synthesis. Since it has been reported that increased hydrostatic pressure prevents cell wall synthesis (9), it is possible that the absence of this process in some manner reduces the ability of the cells to bind heavy metals at a site critical to growth, thus reducing the toxic effects of the metals.

We conclude from the results presented here that the relative toxicities of the metal ions for growth of culture BIII 39 varied with pressure as follows: at 1 atm, $Cu^{2+} > Ni^{2+} = Co^{2+} >> Mn^{2+}$; at 272 atm, $Co^{2+} > Ni^{2+} > Cu^{2+} = Mn^{2+}$; at 340 atm, $Ni^{2+} > Co^{2+} \geq Cu^{2+} > Mn^{2+}$; and at 408 atm, $Co^{2+} >> Ni^{2+} = Cu^{2+} = Mn^{2+}$. For cultures BIII 88 and BIII 32, which were only tested at 1 and 340 atm, the relative toxicities of the metal ions varied as follows: for culture BIII 88 at 1 atm, $Co^{2+} = Ni^{2+} > Cu^{2+} = Mn^{2+}$, and at 340 atm, $Co^{2+} > Ni^{2+} > Cu^{2+} = Mn^{2+}$; for culture BIII 32 at 1 atm, $Cu^{2+} > Ni^{2+} = Co^{2+} > Mn^{2+}$, and at 340 atm $Ni^{2+} >> Co^{2+} = Cu^{2+} > Mn^{2+}$.

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