

The Sensitivity of the Respiratory Chain of Bacteria and Yeast to Cadmium

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Abstract

Growth of *Paracoccus denitrificans* bacteria and *Candida utilis* yeast cells in the presence of increasing concentrations of cadmium led to an inhibition of cell multiplication. *P. denitrificans* was more resistant to the effect of cadmium than *C. utilis*. The pattern of cytochrome biosynthesis showed that cadmium inhibited cytochromes *b* and *c* synthesis in both organisms. In contrast while cadmium totally inhibited the biosynthesis of cytochrome *c* oxidase in *C. utilis*, it had a stimulatory effect on the biosynthesis of this enzyme in *P. denitrificans*. The comparison of colony size in cadmium-treated cells and in the control showed a remarkable decrease in the colony size of cadmium treated cells. The effect of increasing cadmium concentrations on colony count in *Candida utilis* showed that from 0.006 to 0.125 mM cadmium the number of colonies was slightly but consistently higher than the control. At 0.25 mM cadmium the number of colonies decreased dramatically, reaching zero for 0.5 mM cadmium. This is the first reported evidence for an inhibitory (in eukaryote) and stimulatory effect (in prokaryote) of cadmium on the biosynthesis of Cytochrome *c* oxidase. Moreover, our observations provide a better understanding of metal-microorganism interactions for bioremediation of metal contamination of the environment.

Keywords: *Cadmium, Respiratory chain, Paracoccus denitrificans, Candida utilis, Bioremediation.*

Introduction

Heavy metals have become widespread environmental toxicants which form a serious hazard to the public health and a threat to most life form (WHO, 1992, 1991, 1990). One of the biotechnological methods currently used to dispose of toxic waste, e.g. heavy metals, is bioremediation which uses microorganisms and plants (Hegedus *et al.*, 2001, Guerinot, 2002) to remove heavy metals from water or industrial waste. For this purpose certain species of microorganisms have been found to accumulate large quantities of metals (For review see ref. (Moo-Young, 1985, Rehn *et al.*, 1989, Kamely *et al.*, 1991). Metal cell interactions both at cellular and molecular level should be studied due to the fact that the removal of heavy metals by microorganisms exposes the biological system itself to a real poisoning. Furthermore the basic aspect of metals and biological systems interactions may lead to more efficient use of microorganisms. cadmium is one of the major environmental contaminants which has been shown to affect humans, animals and plants (Muller *et al.*, 1991, Habeebu *et al.*, 1998). Since as early as 1963, several Cd²⁺ compounds were shown to have a carcinogenic potential. It has been suggested that cadmium excreted its carcinogenic effect by causing breakage of DNA strand (Müller, *et al.*, 1991; Boadi *et al.*, 1992) but the precise mechanism remains unknown (Waalkes, 2000). *P. denitrificans* is a denitrifying bacterium which has the capacity of removing nitrate from soil and as such it interferes with soil fertility. Its response to the presence of elevated amounts of cadmium in the environment is of high interest. *Candida utilis* yeast cell, an eukaryotic microorganism, is used like other strains of yeast for various biotechnological applications (Jacobson *et al.*, 1989, Deak, 1991). Since cadmium is an inorganic toxicant of great environmental concern, its effect on any biotechnological application should be investigated.

Thus purpose of the present investigation was to study and compare the effect of cadmium on the biosynthesis of respiratory chain components of *P. denitrificans*, a prokaryotic organism, and of *C. utilis*, an eukaryotic organism. Such a study would provide a better understanding of the effect of metals on key cellular metabolism and further knowledge for the efficient use of these and perhaps other microorganisms in biotechnology.

Material and methods

P. denitrificans (ATCC 17741) was grown in enriched medium containing peptone (4g/1) yeast extract (2g/1) supplemented with Na₂HPO₄ (1g/1) and of pH adjusted to 6.8 by sulfuric acid.

C. utilis (ATCC 8025) was cultured in enriched medium adjusted at pH 6.8 containing peptone (5g/1), yeast extract (5g/1), and supplement with KH₂PO₄(1g/1), (NH₄)₂SO₄(2g/1), (NH₄)H₂PO₄(6g/1), NaCl(0.1g/1). 1% Glucose was used as substrate for both organisms. The cells were cultured for 24 hrs at 33°C (*P. denitrificans*) or 28°C (*C. utilis*) in the presence of various concentrations of cadmium chloride as indicated in the legend of the figures. The cytochromes content of the respiratory chain was determined from dithionite-reduced minus H₂O₂-oxidized difference spectra using the extinction coefficients for cytochrome oxidase, cytochromes *c* and *b* as described in reference (Keyhani *et al.*, 1971). Since there is a stoichiometry between functional cytochromes for oxidation-reduction, even a “nonfunctional” cytochrome can be detected under our experimental condition, e.g. chemical reduction by dithionite, which is not the case by studying the enzymatic activities.

The amount of cells collected under different experimental conditions are expressed as grams per liter (wet weight). To study the effect of cadmium on colony size, the yeast was diluted and cultured in yeast extract-peptone-agar medium in petri dishes. The colonies were counted after 48hrs of culture.

Ultra structure studies of *P. denitrificans* and *C. utilis* were performed as follows: *P. denitrificans* cells were doubly fixed with glutaraldehyde and osmium tetroxide according to the method described (Keyhani, 1980). After dehydration and embedding in Epon, thin sections of the fixed cells were prepared. They were then doubly stained with uranyl acetate and lead citrate and examined in a Philips EM 300 electron microscope. *C. utilis* cells were treated as described earlier (Keyhani, 1980). Briefly, the cells were first fixed in potassium permanganate, dehydrated and embedded in Epon. Then thin sections were prepared, stained with lead citrate and examined in a Philips EM 300 electron microscope.

Results

Comparison between P. denitrificans and C. utilis cell structure

Before describing the biochemical changes induced by cadmium it is necessary to consider the fine structure of *P. denitrificans* and *C. utilis* and to identify possible sites of metal-cell components interactions. Fig. 1 shows the fine structure of *P. denitrificans*. The cell wall surrounds the plasma membrane and the cytoplasm and maintains the shape of the cell. Moreover, it protects the plasma membrane from rupture due to the high internal osmotic pressure generated by the cytoplasm. In *P. denitrificans*, like other Gram-negative bacteria, the inner region of the wall is a thin layer of peptidoglycans and directly protects the inner membrane. The outer region of the wall is commonly referred to as the outer membrane. The region between the outer and inner membrane is called the periplasmic space and is found only in Gram-negative bacteria. In the periplasmic space various soluble proteins such as transport proteins, soluble cytochrome *c*, peroxidase and catalase have been found (Stouthamer, 1991). The outer membrane, in addition to membranous proteins, contains a canal forming protein called porin. The inner leaflet of the outer membrane consists of phospholipids. In contrast, the outer leaflet of the outer membrane derives exclusively from lipopolysaccharides. The inner membrane, which is equivalent of the plasma membrane in the eukaryotic cells, lies directly outside the cytoplasm. The inner membrane contains the respiratory chain and is the site of oxidative phosphorylation. In addition, the inner membrane is responsible for synthesis of complex lipids, final stage of peptidoglycans biosynthesis, the synthesis of lipopolysaccharides, capsular polysaccharides, transport proteins, secretion of proteins, motility and chemotaxis. The cytoplasm contains DNA, RNA and protein synthesizing complexes and a variety of proteins, enzymes, precursors and metabolites essential for bacterial growth and survival (Lodish *et al.*, 1991 Timbrell, 2002).

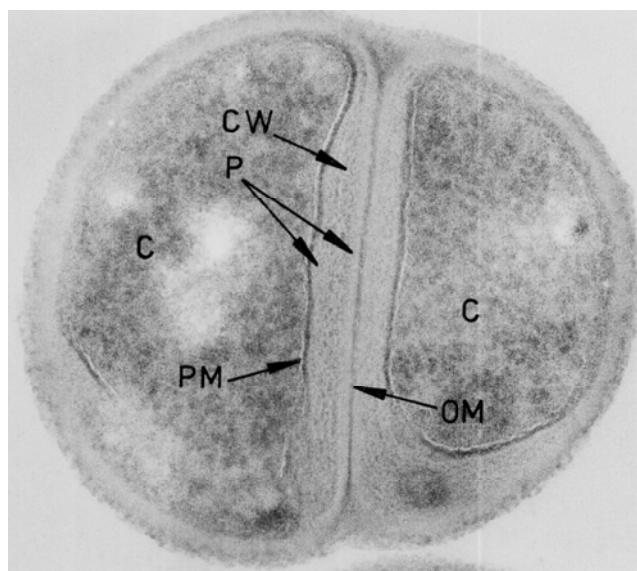


Figure 1 -Thin section showing the fine structure of *Paracoccus denitrificans*. The dividing bacteria shows its various components. C: cytoplasm containing the chromosome; PM: plasma membrane or inner membrane; OM: outer membrane. P: periplasmic space. Magnification=95000. Courtesy Laboratory for Life Sciences.

Fig.2 shows the fine structure of yeast *C. utilis*. In the yeast the cell wall is usually much thicker than that of prokaryotic cells and can often be seen even with the light microscope, although electron microscopy is necessary to obtain any detail. In Fig. 2 the thick wall of *C. utilis* yeast cell is well illustrated. Like in prokaryotes the cell wall in eukaryotes confers shape and rigidity to their cells. Beneath the cell wall and surrounding the cytoplasm, the plasma membrane is found. In the cytoplasm various organelles such as mitochondria, Golgi apparatus, endoplasmic reticulum and nucleus are found. The mitochondria are the site of the respiratory chain and participate in oxidative phosphorylation. Transport and excretion of proteins is much more complicated than in bacteria and is mediated through Golgi apparatus. DNA is located in the nucleus separated from the cytoplasm by nuclear envelope. From ultra structural studies the possible sites of action of metals can be concluded as follows:

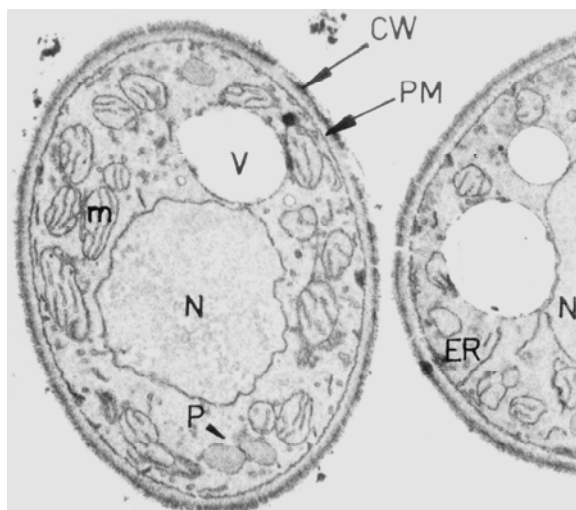


Figure 2 - Thin section showing essential components of the yeast cell *C. utilis*. Mitochondria (m) are dispersed in the cytoplasm. N: nucleus; P: peroxisome ER: endoplasmic reticulum: Vacuole; PM: plasma membrane; CW: cell wall Magnification X 18000 Courtesy Laboratory for Life Sciences.

Table 1- Comparison between various compartments of *P. denitrificans* and *C. utilis* that might be targeted by cadmium.

	<i>P. denitrificans</i>	<i>C. utilis</i>
Capsule	-	-
Outer membrane	+	-
Periplasmic space	+	-
Cell Wall	+	+
Plasma Membrane	+	+
Cytosol	-	+
Mitochondria	-	+
Cytomembranes	-	+
(ER and Golgi)	-	+
Peroxisomes	-	+
Nucleus	-	+

Although metallothioneins are the preferential sites for the storage of metals in the cytoplasm, all cellular organelles and components including cell wall, DNA, RNA and various cytosolic proteins might be involved in metal-cell interactions and thus participating in the process of removal of metal from contaminated environment. (Gupa *et al.*, 1993, Polak *et al.*, 2003).

Effect of cadmium on cell growth

Figures 3 and 4 show the effect of increasing concentrations of cadmium on cell yield in *P. denitrificans* and *C. utilis*, respectively.

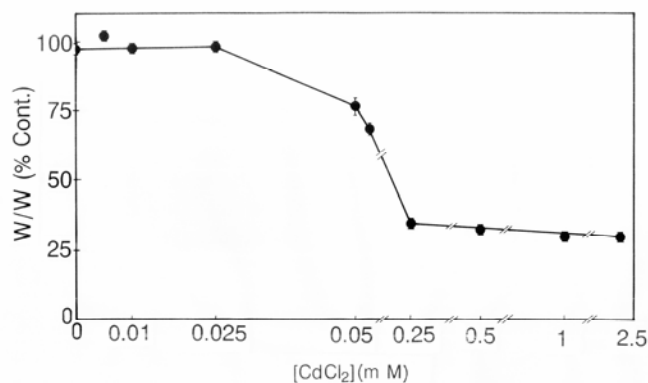


Figure 3 - Yield of *P. denitrificans* grown for 24hrs in media containing increasing cadmium concentrations.

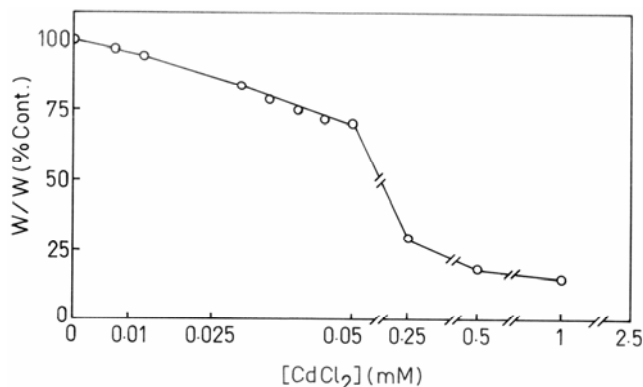


Figure 4 - Yield of *C. utilis* grown for 24hrs in media containing increasing cadmium concentrations. Compared to Fig .1, *C. utilis* is more sensitive to cadmium.

In *P. denitrificans* the effect of cadmium on cell growth appears to be multiphasic: up to 0.025mM cadmium, there is no effect on cell yield, from 0.025mM cadmium there is a small decrease in cell growth and from 0.05 to 0.25mM cadmium there is a sharp decline in cell growth followed by a slow decrease in cell growth from 0.25 to 2.5mM cadmium. In contrast, in *C. utilis* there is a sharp decline in cell growth even at very low concentrations of cadmium (up to 0.0625 mM) followed by a slow decrease in cell growth from 0.125 to 1mM cadmium.

Effect of cadmium on cytochrome biosynthesis

a) *P. denitrificans*: Figure 5 shows the effect of cadmium on cytochrome biosynthesis in this bacteria. It shows that there is not a significant change in the level of cytochromes *b,c* and oxidase up to a concentration of 0.025mM cadmium. Thereafter the amount of cytochromes *b* and *c* decreases sharply up to 0.25mM cadmium. For concentrations of 0.25 to 2.5mM cadmium a near plateau is reached. For cytochrome *c* oxidase a surprising effect was an increase in the amount of this protein up to 0.25mM cadmium, thereafter a plateau was reached (Fig. 5)

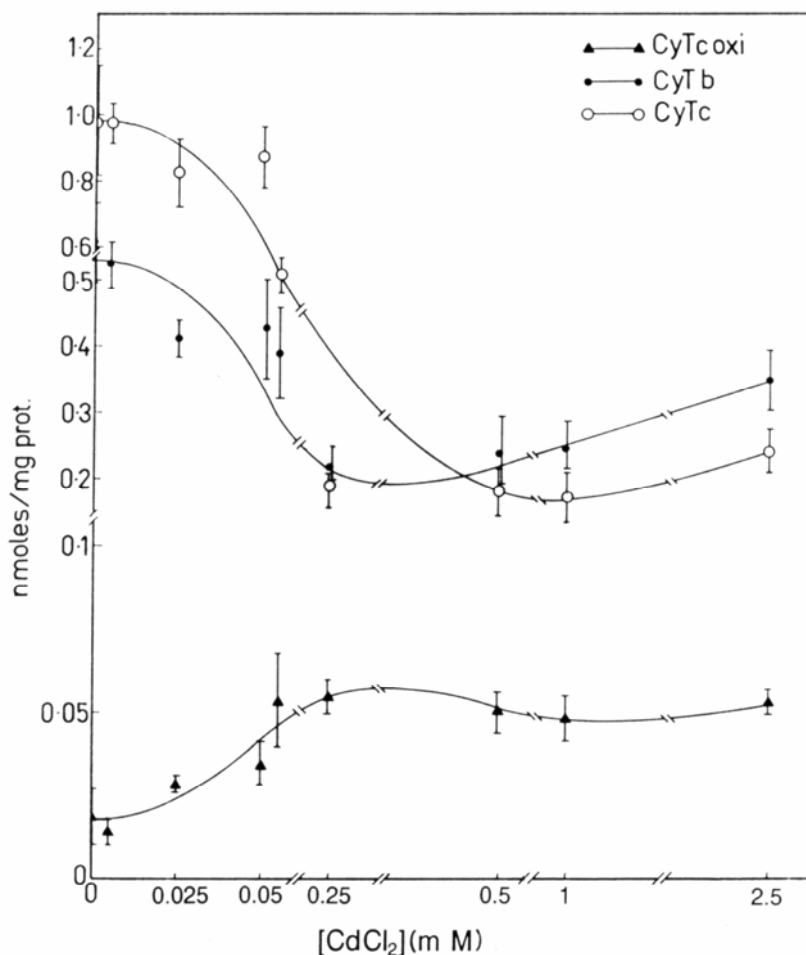


Figure 5 - The amount of cytochrome *c* oxidase and cytochromes *b* and *c* in *P. denitrificans* grown in the presence of increasing cadmium concentrations. Note that while the amount of cytochromes *b* and *c* were inhibited by cadmium, that of cytochrome *c* oxidase was increased.

b) *C. utilis*: Fig.6 shows the effect of cadmium on cytochromes biosynthesis in the yeast *C. utilis*. It shows that the amounts of all three cytochromes decrease rapidly as a function of cadmium concentrations up to 0.0625mM. Thereafter the amount of cytochrome *c* oxidase decreases progressively, slowly reaching zero for 1mM cadmium concentration. In contrast, the amount of cytochromes *b* and *c* remains roughly the same from 0.125 to 1mM cadmium.

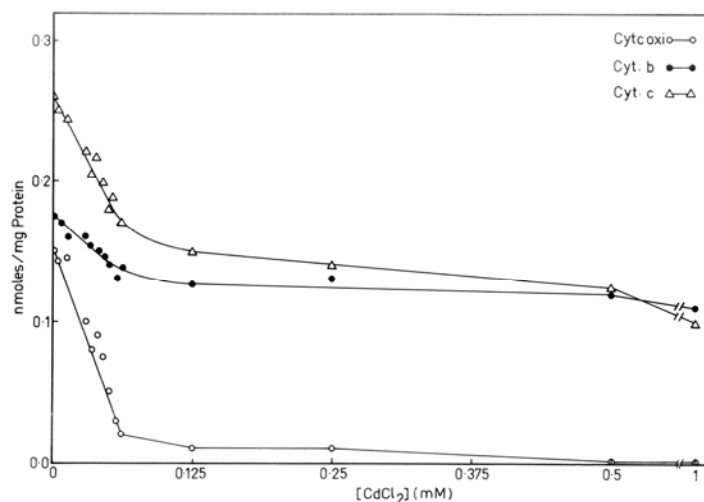


Figure 6 - The amount of cytochrome c oxidase and cytochromes *b* and *c* in *C. utilis* grown in the presence of increasing cadmium concentrations. Note the inhibition of all three cytochromes by cadmium. In the case of cytochrome c oxidase there was a total inhibition at 1mM cadmium. A significant decrease in cytochrome c oxidase was already observed at 0.05 mM cadmium.

Effect of cadmium on colony size and colony counting

Investigations on the effect of Cd^{2+} on colony count in *C. utilis* showed that as little as 0.012 mM Cd^{2+} induced a marked decrease in colony size (Fig. 7B), but had no effect on colony count (Fig. 8). Increasing concentrations of Cd^{2+} led to further decrease in colony size for *C. utilis* grown in up to 0.125 mM cadmium (Fig.7c) but had still no effect on colony count (Fig. 8). In 0.25 mM cadmium, the number of colonies decreased dramatically, reaching zero in 0.5 mM cadmium (Fig.8).

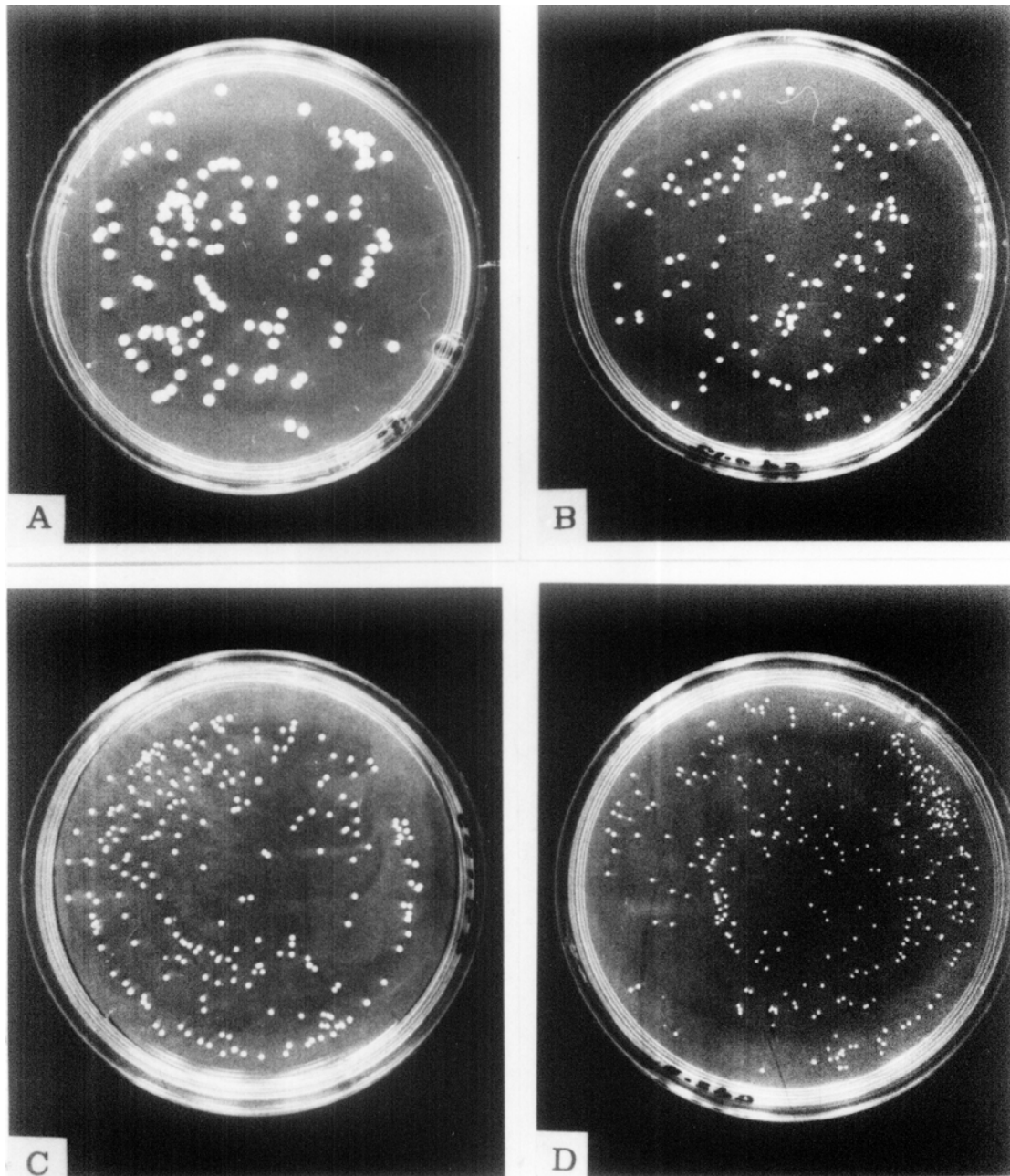


Figure 7- This figure shows the comparison of colony size of cadmium-treated cells and untreated cells. Note the small size of cadmium-treated cells (B: 0.012, C: 0.125 and D:0.25mM) compared to control (A).

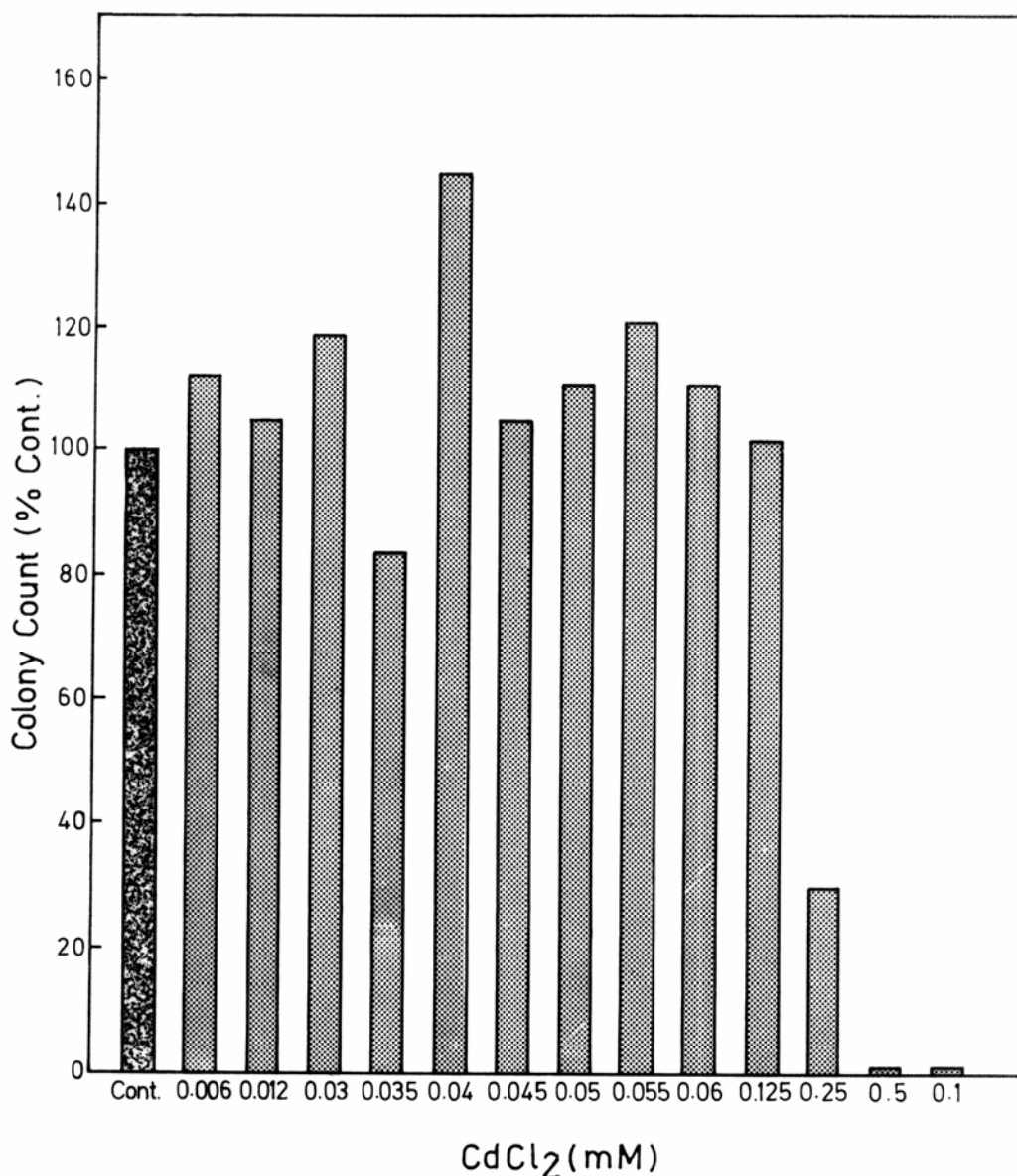


Figure 8- Colony counting of the cells treated with increasing concentrations of cadmium. Note the rapid decrease in the number of colony at 0.25 mM cadmium concentration.

Discussion

The cytochromes system is the key element of cellular metabolism and is involved in cellular respiration and oxidative phosphorylation. The energy generated by the respiratory chain is used for all metabolic purposes within the cell. Thus the study of the effect of metals on cytochromes provides the possibility of better use of microorganisms in bioremediation.

As shown in the present study, cytochrome biosynthesis was severely inhibited when *P. denitrificans*, a bacterium, and *C. utilis*, a yeast cell, were grown in the presence of cadmium chloride. The cytochrome *c* oxidase showed a distinct pattern of biosynthesis bacteria and in yeast cell, however. In *P. denitrificans*, cytochrome *c* oxidase biosynthesis was specifically enhanced. In both systems other metals such as Ni, Co and Cu inhibit the biosynthesis of all respiratory pigments (Golkhou, 1994). The amount of cytochrome *c* oxidase never reached zero concentration in either system, however (Fig.5). Some explanation can be provided about the distinctive effect of cadmium on the cytochrome *c* oxidase of *P. denitrificans* compared to *C. utilis*. One possible explanation is that in this bacterium, by stabilizing the membrane or by some unknown mechanism, cadmium inhibits cytochrome oxidase degradation so that this enzyme accumulates in the membrane. Denovo synthesis cannot be ruled out, however. On the other hand, in *C. utilis* cytochrome *c* oxidase is located in the mitochondria and the chemical composition of the mitochondrial membrane compared to that of *P. denitrificans* is perhaps responsible for the sensitivity to the inhibitory effect of cadmium. Furthermore bacterial cytochrome *c* oxidase comprises two subunits while the yeast enzyme comprises 7-8 subunits coded for by nuclear DNA for some of them and by mitochondrial DNA for others. The differential effect of cadmium on yeast and bacteria was also observed for Cu. A mutant of *P. denitrificans* was obtained exhibiting an unusual resistance to exogenous copper (Golkhou, et al., 1995; Sarchar, 1992), while efforts to obtain the same type of mutant in *C. utilis* was so far unsuccessful.

Another interesting point presented in this paper is the resistance of bacterial growth to cadmium when compared to the yeast cell. In *Candida utilis*, even low concentrations of cadmium inhibited the growth of the yeast cell. In contrast, in *Paracoccus denitrificans* up to 0.025mM cadmium did not affect the growth of the yeast cell. Both intracellular and extracellular sites should be considered as possible sites for metal-cell interactions. Many extracellular microbial polymers consist of neutral polysaccharides or other compounds to bind metal ions thus removing metal from the environment. consequently in the case of *P. denitrificans* one may argue that the

removal of cadmium by an extracellularly secreted compound might explain the differential effect on cytochrome oxidase level in this bacteria. Alternatively the difference in the chemical composition of the cell walls may have resulted in the observations reported in this paper. Several authors have reported (Beveridge *et al.*, 1980, Doyle *et al.*, 1980) strong evidence that the carboxyl groups (of glutamic acid) in the peptidoglycans present in the cell wall of Gram-Positive *Bacillus subtilis* are the primary sites of divalent metals complexation. In *Bacillus licheniformis*, teichoic acid and teichuronic acid are the primary site for metal deposition in the cell walls (Beveridge *et al.*, 1982). Polar heads of phospholipids have also been considered as primary sites of metal binding (Beveridge *et al.*, 1981).

The lethal effect of Cd^{2+} is also the subject of considerable interest. The frequency of cadmium-induced DNA strand breaks and chromosomal aberrations are reduced in cells treated with antioxidant, thus suggesting that cadmium is genotoxic by producing hydrogen peroxide, which can form hydroxyl radicals in the presence of iron and copper (Rossman *et al.*, 1992). Protein molecules will also be damaged by the hydroxyl radicals (Hunt *et al.*, 1988). Moreover, the hydroxyl radicals cause lipid peroxidation, leading to various alterations in cell structure and metabolism. Other changes in the cellular metabolism may be due to variations in Ca^{2+} concentration induced by Cd^{2+} . The ionic charge and radius of Cd^{2+} are comparable to that of Ca^{2+} . Therefore Cd^{2+} could conceivably replace Ca^{2+} at cellular Ca^{2+} binding sites and lead to disturbance in cellular calcium homeostasis (Verbost *et al.*, 1989).

Bioremediation of cadmium contamination of the environment is of high interest. The present research gives some information regarding the different sensitivities towards cadmium between prokaryotic and eukaryotic organisms and indicates the potential for the use of prokaryotes in removing cadmium contamination of the environment. In particular, *P. denitrificans* is more appropriate than *C. utilis* in decontamination of cadmium.

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