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Interaction of Ni^{2+} and Cd^{2+} with the envelopes of sensitive and resistant Gram-negative bacteria

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The bactericidal and membranotoxic effects of Ni^{2+} and Cd^{2+} on the selected strains of *E. coli* and *S. enterica* sv. Typhimurium have been investigated. The Ni^{2+}-resistant *E. coli* strain V38 showed also an enhanced resistance to Cd^{2+}. The wild type strain *S. enterica* sv. Typhimurium DS88 is more resistant to Ni^{2+} and Cd^{2+}, in comparison with the strain SL1102 which possesses a shorter polysaccharide chain in the bacterial lipopolysaccharide (LPS). Cd^{2+} exerted a stronger depolarising effect on *E. coli* and *S. enterica* sv. Typhimurium cells than Ni^{2+} did. At 2–10 mM concentrations Ni^{2+} stimulated the respiration of the bacterial cells. At concentrations higher than 20 mM it exerted an inhibition of the respiration and diminished the viability of the bacterial cells tested, except the *E. coli* V38 strain. 1 mM Cd^{2+} inhibited cell respiration and acted bactericidally on the cells of all the strains tested.

Key words: nickel, cadmium, bactericidity, membranotoxicity

INTRODUCTION

An elevated level of toxic heavy metals in the biosphere is a well-documented phenomenon having notable biological and environmental implications. Microorganisms, especially bacteria, play an important role in the fate of metals in the environment. Metal interaction with microbial cell occurs in several stages. The first sites of interaction are at the cell envelope. The aim of this study was to investigate the interactions of selected heavy metal ions with envelopes of Gram-negative bacteria. Ni^{2+} and Cd^{2+} were chosen for this investigation as representatives of essential and toxic heavy metals, respectively. Ni^{2+} represents the bivalent cations (like Fe, Mn, Mo, Wo, Co, Zn, Cu) that are required physiologically at trace amounts but usually are toxic at higher concentrations. Cd^{2+} has no known essential functions; it interferes with many cellular processes even at low concentrations and is classified as toxic (like Ag, Sn, Hg, Pb, Bi, Tl and Au) (Beveridge et al., 1997). Apart from toxicological criteria, Ni and Cd were chosen as cations of environmental importance, ubiquitous throughout the world.

We combined the measurements of membrane voltage ($\Delta\psi$) and respiratory activity of bacteria with the estimations of cell viability upon interaction with the metals with the purpose to elucidate the relationship between bactericidity and membranotoxicity of Ni^{2+} and Cd^{2+}.

GENERAL METHODS

Salmonella enterica serovar Typhimurium strains DS88 (SL5676 Sm^r pLM2) and SL1102 ($rfaE^b$) were kindly provided by Prof. D. H. Bamford (University of Helsinki, Finland). *E. coli* V38, a strain isolated from Vilnius city sewage sludge, and *E. coli* JM101 (F' traD36 proAB lac^q ΔlacZ M15lsupEI thi Δ(lacproAB) were the same strains used by J. Ru-

bikas et al. (1997) in their studies on Ni^{2+} uptake and efflux systems. The cells were grown, harvested and stored as described previously (Daugelavičius et al., 1997). Measurements of the lipophilic cation tetraphenylphosphonium (TPP^+) distribution and determinations of membrane voltage ($\Delta\psi$) were performed by a TPP^+ selective electrode as described earlier (Daugelavičius et al., 1997). The bacterial respiration rate was measured at 37° in a 2 ml chamber with a Clark-type oxygen electrode. To define the zero level of oxygen in the incubation chamber, sodium dithionite was used.

The tests of bacterial cell sensitivity to heavy metal ions based on the use of metal-salt-impregnated filter discs were performed as described in (Nies and Silver, 1989). Overnight cultures of the bacterial strains tested were diluted into the soft top agar and layered onto solid nutrient broth in Petri dishes. Filter discs (15 mm in diameter) impregnated with certain metal salt solution ($NiCl_2$ or $CdCl_2$) were placed on the top agar. After overnight incubation at 37 °C, the diameters of the bacterial growth inhibition zones around the disks were measured.

RESULTS AND DISCUSSION

The characterization of sensitivity of the selected *Escherichia coli* and *Salmonella enterica* serovar Typhimurium strains to heavy metals

In the present study, certain strains of *E. coli* and *S. enterica* sv. Typhimurium were examined as the representatives of the best characterized Gram-negative bacteria commonly used in laboratory investigations. The strains of *E. coli* used expressed different sensitivity to Ni^{2+} ions. As shown previously (Rubikas et al., 1997), the nickel resistance in *E. coli* V38 is determined mainly by the energy-dependent efflux mechanism. *E. coli* JM101 was used as a Ni^{2+} sensitive control strain. The strains of *S. enterica* Typhimurium used: DS88 (wild type) and SL1102 (heptoseless deep rough LPS mutant) differed in polysaccharide chain length of cellular lipopolysaccharide (LPS) (Vaara, 1992).

Preliminary tests of bacterial cells sensitivity to Ni^{2+} and Cd^{2+} ions were performed using filter discs impregnated with a metal salt solution. It is generally assumed that the diameters of bacterial growth inhibition zones around the disks reflect the metal's toxicity to bacterial cells (Nies and Silver, 1989). The results presented in Fig. 1 show different sensitivity to heavy metals of the bacterial cells tested. *E. coli* V38 and *S. enterica* Typhimurium DS88 appear to be more metal-tolerant than *E. coli* JM101 or *S. enterica* Typhimurium SL1102.

As reported (Rubikas et al., 1997), Ni^{2+}-resistant *E. coli* strain V38 was isolated from Vilnius city sewage sludge. The results obtained in our investigations show an enhanced resistance of *E. coli* V38 cells not only to Ni^{2+}, but also to Cd^{2+}. Contamination of wastewater with heavy metals is often responsible for the acquisition of co-resistance to metal ions by naturally occurring microorganisms (Rouch et al., 1995). The best-studied systems conferring concomitant resistance to some different metal cations in Gram-negative bacteria are the Czs system (determining resistances to cadmium, zinc and cobalt) and the closely homologous system Cnr (mediating resistance to nickel and cobalt) of *Ralstonia* sp. strain CH34 (Diels et al., 1995; Nies, Silver, 1995). It has been shown that these systems confer resistance through an active metal cation efflux. The energy-dependent mechanism of efflux has been also demonstrated to be the basis of Ni^{2+} resistance in *E. coli* strain V38 (Rubikas et al., 1997). However, it is not clear whether the same efflux system confers resistance of *E. coli* V38 to both heavy metals. It is well known that the determinants functioning by energy-dependent efflux of toxic ions constitute the largest group of metal-resistance systems. Some of efflux systems are ATP-ases and others chemiosmotic cation/proton antiporters (Silver, Phung, 1996). There is no evidence so far to distinguish the type of Ni^{2+} efflux system that operates in *E. coli* strain V38; the evaluation of its mechanism needs further investigation.

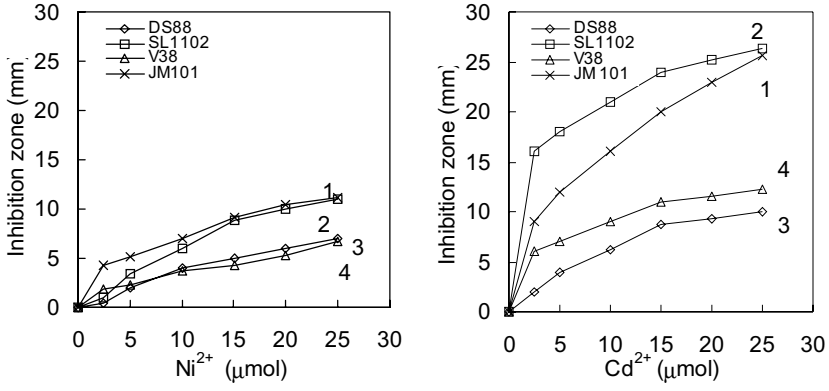

Fig. 1. Toxicity of $Ni^{2+}$and Cd^{2+} to bacterial cells
The metal-sensitivity of *E. coli* JM101 (curves 1), *S. enterica* sv. Typhimurium strains SL1102 (curves 2) or *S. enterica* sv. Typhimurium DS88 (curves 3) and *E. coli* V38 (curves 4) cells has been tested. Experimental details are presented in Materials and Methods

The reasons for a certain tolerance of *S. enterica* Typhimurium DS88 to heavy metals were not explored so far. The differences in sensitivity to Ni^{2+} and Cd^{2+} between the strains of *S. enterica* Typhimurium: DS88 possessing wild type LPS and SL1102, the deep rough LPS mutant (Re chemotype) (Vaara, 1992) suggest that sensitivity/tolerance to heavy metal ions, at least partially, could be determined by the nature of LPS. It is well known (Vaara, 1992) that LPS comprises the outer layer of the outer membrane (OM) of Gram-negative bacterial cell. The polyanionic nature of the entire LPS molecule determines its high affinity to multivalent cations. Mutations in LPS should have profound effects on the interactions of bacteria with metal ions. Hence, strain SL1102, which lacks the distal carbohydrate groups, *i.e.* possesses a reduced amount of anionic sites in LPS (Snyder, 1999), binds less Cd^{2+} and Ni^{2+} than do wild-type *S. enterica* Typhimurium and *E. coli* strains (Dervinytė et al., 2002), and this in turn could predetermine the differences of heavy metal–bacterial cell interactions at the later stages.

The membranotoxic action of Ni^{2+} and Cd^{2+}

Wide ranges of cell components are susceptible to metal-induced damage (Frausto da Silva and Williams, 1993). The cell envelope is the first site of metal–cell interaction and one of the targets for the deleterious action of heavy metals. Both membrane lipids and proteins may be affected. In order to elucidate the mode of action of Ni^{2+} and Cd^{2+} on the integrity and functions of the bacterial cell membranes, changes in membrane voltage ($\Delta\psi$) and respiratory activity of bacteria upon interaction with the metals were studied. The distribution of the lipophilic cation tetraphenylphosphonium (TPP^+) between the cells and external medium was monitored to assay the $\Delta\psi$. TPP^+ easily penetrates phospholipid bilayers, however, the OM of Gram-negative bacteria such as *E. coli* or *S. enterica* Typhimurium is not permeable to lipophilic compounds (Vaara, 1992). Therefore the cells of both *E. coli* strains tested and *S. enterica* Typhimurium DS88 were pretreated with EDTA (Daugelavičius, 1997) in order to enhance the permeability of their OM to TPP^+. *S. enterica* Typhimurium strain SL1102, lacking the terminal oligosaccharide chain in LPS, was permeable to TPP^+ without any treatment.

The experiments revealed that both Ni^{2+} and Cd^{2+} diminished the voltage of cell plasma membrane (PM) of all bacterial strains studied (Fig. 2, A), but Cd^{2+} exerted a stronger depolarizing effect than Ni^{2+}. Both the disturbance of PM permeability or/and the damage of $\Delta\psi$ generating systems, *e.g.*,

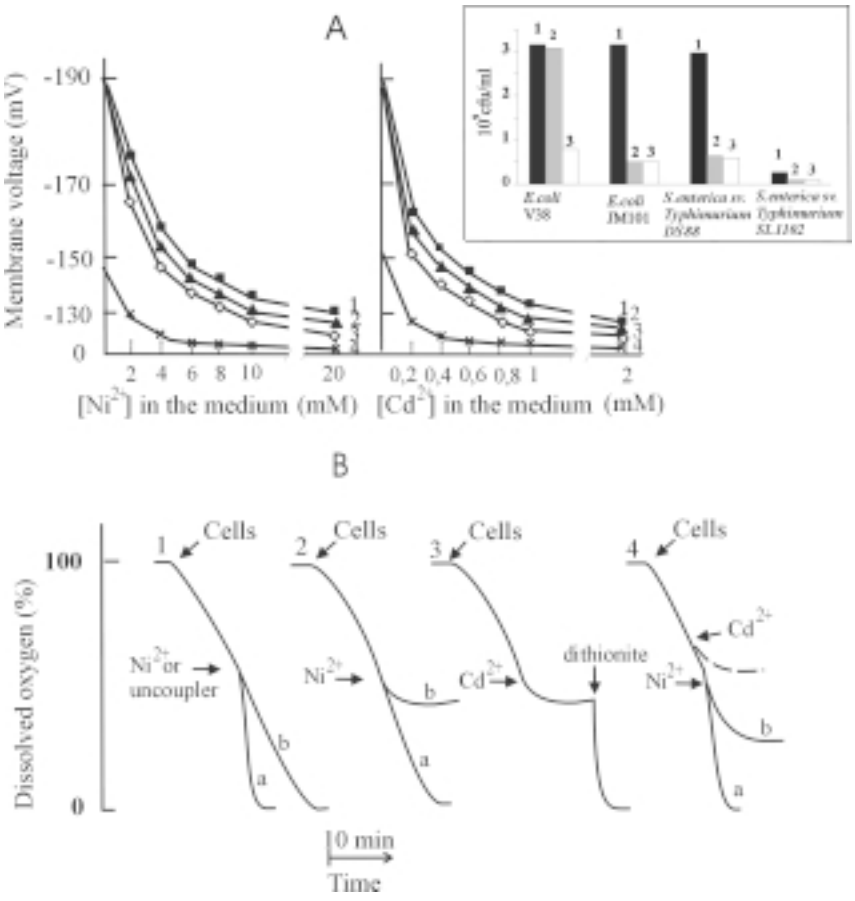

Fig. 2. Comparative effects of Ni^{2+} and Cd^{2+} on $\Delta\psi$, respiration rate and viability of bacterial cells

The experiments were carried out at 37 °C in 100 mM Tris/HCl buffer, pH 8.0. The concentrations of EDTA-treated *E. coli* V38 (A, curves 1 and B, curves 1, 2a and 3), *E. coli* JM101 (A, curves 2 and B, curves 1, 2b and 3) or *S. enterica* sv. Typhimurium DS88 (A, curves 3 and B, curves 1, 2b and 3) and native *S. enterica* sv. Typhimurium SL1102 (A, curves 4, and B, curve 4) cells were 3×10^9 cells/ml. Other additions: panel B, curve 1 – 5 mM Ni^{2+} or 1 mM 2,4-dinitrophenol (a) or 10 mM Ni^{2+}(b), curve 2 – 20 mM Ni^{2+}, curve 3 – 1 mM Cd^{2+}, a grain of sodium dithionite, curve 4 – 10 mM Ni^{2+} (a) or 20 mM Ni^{2+} (b), or 1 mM Cd^{2+}. For estimation of viability (insert), the cells were incubated with Ni^{2+} or Cd^{2+} for 20 min at 37 °C, pH 8.0, diluted and plated. (*1*) – control without heavy metals, (*2*) – 20 mM Ni^{2+}, (*3*) – 1 mM Cd^{2+}

the respiratory chain or H^+-ATPase, may account for the depolarization. To distinguish between these two possibilities the respiration of the cells was examined. The measurements of the respiration rate revealed differences in the membranotoxic action of Ni^{2+} compared to Cd^{2+} (Fig. 2, B). An increase in the respiration rate was observed upon addition of 2–10 mM of Ni^{2+} to a suspension of EDTA treated both with *E. coli* and *S. enterica* Typhimurium DS88 cells (Fig. 2 B, curve 1a) or native *S. enterica* Typhimurium SL1102 (Fig. 2 B, curve 4a). It seems reasonable to explain this finding by the Ni^{2+}-induced increase in PM permeability, because the respiration was also stimulated by a well-known membrane-active agent, uncoupler of oxidative phosphorylation 2,4–dinitrophenol (Fig. 2 B, curve 1a). At higher concentrations of Ni^{2+} the stimulatory effect was not observed any more (Fig. 2 B, curve 1b), and concentrations higher than 20 mM led to a noticeable inhibition of respiration (Fig. 2 B, curves 2b and 4b). However, no inhibition was observed in *E. coli* V38 cells (Fig. 2 B, curve 2a). The stimulatory effect of Ni^{2+} can be explained by its damaging action on membrane phospholipids or some membrane-stabilizing proteins, resulting in membrane leakage. An inhibition of respiration at higher concentrations of Ni^{2+} might be due to interference with activities of respiratory chain enzymes. Notably, the depolarizing activity of Ni^{2+} did not correlate with its bactericidal action (Fig. 2, insert): the cells of *E. coli* strain V38 survived a temporarily treatment with Ni^{2+} at concentrations up to 20 mM, in spite of alterations in PM permeability changes registered in Fig. 2 A and B. It is plausible to suggest that binding of Ni^{2+} to the sensitive sites in PM of *E. coli* V38 cell would be reversible and these cells, contrary to the cells of sensitive strains (Fig. 2, insert), are able to remove Ni^{2+} and restore PM barrier function during the subsequent plating on the solid nutrient broth without Ni^{2+}. This is in accordance with the active Ni^{2+}-efflux mechanism demonstrated previously in *E. coli* V38 cells (Rubikas et al., 1997), however, the efflux system capacity alone is insufficient to recover cells from the hazardous action of the high quantities of Ni^{2+} used in this study. It should be noted that the Ni^{2+} concentration used in the latter experiments several times exceeded the previously determined MIC (Nies and Silver, 1989) or the concentrations used in the experiments depicted in Fig. 1. However, it should be borne in mind that MIC was determined by growing the cells in the constant presence of Ni^{2+}, whereas in the experiments presented in Fig. 2 the cells were in contact with Ni^{2+} only for 20 min. Additional investigations are needed to elucidate the mechanisms that allow *E. coli* V38 to survive the toxic effect of high Ni^{2+} concentrations during a short treatment. It could be supposed that another type of resistance can act along with or/and in synergy with the resistance produced by the Ni^{2+} efflux pump.

Contrary to Ni^{2+}, Cd^{2+} caused a strong inhibition of the respiration of the cells starting from the concentration of 1 mM (Fig. 2 B, curves 3 and 4). The inhibitory action of Cd^{2+} is consistent with strong binding character of this metal. Cd^{2+} is known for its ability to form stable bonds with easily polarizable electronegative ligands such as sulphydryl groups (Frausto da Silva and Williams, 1993). Therefore it could exert an inhibitory action on cell respiration by binding to active centres and other essential sites in enzymes of the bacterial electron transport chain, thereby interfering with their function. The parallel studies on cell viability revealed a bactericidal action of Cd^{2+} on all strains tested. (As for *S. enterica* Typhimurium SL1102, it was leaky in the Tris/HCl medium used for the measurements (Fig. 2, A, curves 4) and hardly formed colonies after incubation in it even without heavy metals (Fig. 2, insert).

This ongoing research provides new information concerning the Ni^{2+} and Cd^{2+} toxicity and resistance mechanisms developed by certain Gram-negative bacteria. One of the most promising applications of this investigation could be the use of bacterial cells as the model system to assess membranotoxicity of heavy metals to living organisms.

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Ni^{2+} IR Cd^{2+} SĄVEIKA SU JAUTRIŲ IR ATSPARIŲ GRAMNEIGIAMŲ BAKTERIJŲ APVALKALĖLIAIS

S a n t r a u k a

Tirtas baktericidinis ir membranotoksinis Ni^{2+} ir Cd^{2+} poveikis tam tikrų *Escherichia coli* ir *S. enterica* sv. Typhimurium kamienų ląstelėms. Nustatyta, kad Ni^{2+} atsparios *E. coli* V38 ląstelės taip pat pasižymi didesniu atsparumu Cd^{2+}. Laukinio tipo *S. enterica* sv. Typhimurium DS88 ląstelės atsparesnės Ni^{2+} ir Cd^{2+} poveikiui, negu SL1102 ląstelės, turinčios trumpesnę lipopolisacharido grandinę išorinėje ląstelių membranoje. Cd^{2+} depoliarizuojantis poveikis *E. coli* ir *S. enterica* sv. Typhimurium ląstelėms yra stipresnis, negu Ni^{2+}. 2–10 mM Ni^{2+} skatina visų tirtų kamienų ląstelių kvėpavimą. Esant didesnėms nei 20 mM koncentracijoms, Ni^{2+} slopina visų ląstelių, išskyrus *E. coli* V38, kvėpavimą. Tirtose koncentracijose Ni^{2+} baktericidinis poveikis *E. coli* V38 ląstelėms, skirtingai negu visoms kitoms ląstelėms, nepasireiškė. Cd^{2+} koncentracijos, didesnės nei 1 mM, slopino bakterijų kvėpavimą ir veikė jas baktericidiškai.

Raktažodžiai: nikelis, kadmis, baktericidinis, membranotoksinis