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METHYLMERCURY INHIBITS GROWTH AND INDUCES MEMBRANE CHANGES IN *Pseudomonas putida*

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Methylmercury inhibits growth and induces membrane changes in Pseudomonas putida

A bacterial model system *(Pseudomonas putida* DSM 50026) was used in this research to assess toxicity of the environmentally relevant concentrations of mercury species (MeHg and Hg(II)) that represent important pollutants of aquatic ecosystems at sites of industrial or mining activities. In addition to direct monitoring of bacterial growth, we also analyzed fatty acid profiles of exposed and non-exposed cultures to determine possible toxic effects manifested on membrane level. The results showed that exposure of P *putida* to Hg(II) in concentrations of 0.2-200 |ig/L did not have any significant effects on growth nor fatty acid composition of exposed bacterial culture. On the other hand, when bacteria were exposed to up to 1600-times lower concentrations of MeHg (0.12-12.5 |g/L), growth inhibition as well as significant changes in fatty acid composition were detected. Observed adaptive membrane changes due to MeHg exposure were similar to those associated with responses to organic solvents and some other membrane-disrupting compounds.

Key words: microbiology / environmental protection / bacteria / *Pseudomonas putida* / aquatic ecosystems / pollution / mercury / methylmercury / growth inhibition / membrane adaptation / *cis-trans* isomerization

Metil živo srebro inhibira rast in povzroča spremembe v membranah bakterije Pseudomonas putida

V raziskavi smo na bakterijskem modelu *(Pseudomonas putida* DSM 50026) analizirali strupenost okoljskih koncentracij anorganske (Hg(II)) in organske (MeHg) oblike živega srebra, ki predstavljata pomembna vira onesnaženja vodnih ekosistemov v bližini nekaterih industrijskih in rudarskih območij. Poleg neposrednega spremljanja bakterijske rasti smo analizirali tudi maščobnokislinske profile izpostavljenih bakterijskih kultur in jih primerjali s tistimi, ki živosrebrovima spojinama niso bili izpostavljeni. Rezultati so pokazali, da izpostavitev P *putida* Hg(II) v koncentracijah med 0,2 in 200 |ig/L ne inhibira rasti, niti ne vpliva na maščobnokislinsko sestavo bakterijskih membran. Nasprotno pa je izpostavitev celic do 1600-krat nižjim koncentracijam MeHg povzročila tako upočasnitev rasti kot tudi prilagoditvene spremembe na membranskem nivoju. Slednje so bile podobne kot tiste, opažene ob izpostavitvi bakterij organskim topilom in nekaterim drugim spojinam, ki motijo integriteto membran.

Ključne besede: mikrobiologija / varstvo okolja / bakterije / *Pseudomonas putida* / vodni ekosistemi / onesnaževanje / živo srebro / metil živo srebro / inhibicija rasti / membranska adaptacija / *cis-trans* izomerizacija

1 INTRODUCTION

In the past few decades, environmental pollution has become one of the world's major concerns. Heavy metals are a group of pollutants representing environmental problem in most parts of the world. One of the most toxic metals is certainly mercury, which represents a significant concern especially in aquatic ecosystems at sites with industrial or mining activities. Mining operations in areas rich in cinnabar ore may represent strong sources of Hg for many years even after mining has been discontinued (Benoit *et al.,* 1994). One of mercury (Hg) affected sites also lies in Western part of Slovenia where

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lies the second largest Hg mine in the world. The Idrija Mine operated for 500 years until its closure in 1994, but mercury laden tailings still line the banks and the system is a threat to the Idrija River and water bodies downstream, including the Soča / Isonzo River and the Gulf of Trieste in the northern Adriatic Sea, which are therefore subjects to continuous environmental monitoring (Hines *et al.,* 2000; Faganeli *et al.,* 2003).

Mercury can cause acute as well as chronic poisoning in animals and humans (Sweet & Zelnikoff, 2001; Crespo-Lopez *et al.,* 2007; Han *et al.,* 2008). The most toxic forms of mercury are usually considered to be organic compounds, such as methylmercury (MeHg) and dimethylmercury (Me_2Hg), which have the tendency to accumulate in hydrophobic environments such as cell membranes (Mason *et al,* 1996; Girault *et al.,* 1997). Bioaccumulation on different trophic levels leads to biomagnification effect in natural ecosystems, which means that even low levels of organic mercury compounds in the environment may have detrimental effect on organisms at the end of the food chain (Mason *et al.,* 1996). However, not all the mercury that is present in natural ecosystems is bioavailable and therefore harmful to the living organisms (Golding *et al.,* 2007). Standard chemical analytical methods do not have the power to discriminate between bioavailable and fixed forms of mercury in environmental samples and therefore need to be complemented with methods, based on responses of living (micro)organisms for proper risk assessment (Farre *et al.* 2005). Living cells (organisms) can be used as bioindicators, as well as *test-species* in bioassays. By recent establishment of modern 3R concept (reduction, replacement, refinement), the development and application of bioassays based on microbial cells is being promoted, due to their simple cultivation in axenic cultures and lack of ethical issues usually present when using higher organisms (Marinšek Logar and Vodovnik, 2007).

Cell membrane as the first barrier separating cellular interior from its environment represents a primary defense line against unfavorable environmental impacts and therefore appears to be a good target for ecophysiological as well as toxicological studies. Bacteria are known to react to several environmental triggers by modifying fatty acid composition of their membranes, predominantly by changing the ratio of saturated to unsaturated fatty acids (Cronan 2002). However, several strains of ubiquitous bacterium *Pseudomonas putida* have been shown to use at least three adaptation mechanisms at membrane level which apply to different types of environmental stressors: (1) changes in the overall degree of saturation of fatty acids (Loffhagen *et al.* 2004), (2) the formation of cyclopropane fatty acids (Hartig *et al.* 2005) and (3) *cis-trans* isomerization (von Wallbrun *et al.* 2003). Meanwhile the first two responses are mainly associated with temperature stress and starvation, *cis-trans* isomerization, appears to be involved in toxic stress defence (Heipieper *et al.* 1995; Heipieper *et al.* 1996). It has been shown in solvent-tolerant strains of *P. putida* that toxicity and concentration of organic solvents in their membrane correlate with increase in *trans/cis* fatty acid ratio (Heipieper *et al.* 1992; Heipiepper *et al.* 1994; Weber *et al.,* 1996). Moreover, several heavy metal ions, namely Zn^{2+}, Cd^{2+}, Cr^{3+}, Co^{2+}, Cu^{2+} and Ni^{2+} have also been shown to induce adaptive changes resulting in increased accumulation of *trans* fatty acids (Heipieper *et al.* 1996). However, there are so far no reports on effects of mercury (Hg) species on *P. putida* (or other bacterial) membranes, which is the objective of this article. Our hypothesis was that organic (MeHg), as well as inorganic (Hg(II)) form of mercury may influence the membrane (fatty acid profile) of *P. putida.* However, due to its hydrophobic nature, the degree to which membranes are affected was expected to be larger in case of MeHg.

2 MATERIALS AND METHODS

2.1 MICROORGANISM

P. putida DSM 50026 cells were purchased in freeze-dried form from Deutsche Sammlung von Mikroorganizmen und Zellkulturen GmbH (DSMZ, 1998).

2.2 CHEMICALS

General reagents dimethylsulfoxide (DMSO), methanol, n-hexane and glycerol as well as both mercury species: MeHg in the form of CH_3HgCl and Hg(II) in the form of $HgCl_2$ were purchased from Sigma (St. Luis, MO, USA) or Merck (Darmstadt, Germany). Bacteriological agar and peptone were purchased from Biolife (Milan, Italy) and meat extract from Becton Dickinson (New Jersey, NJ, USA). Standard calibration mixture of bacterial FAME in methyl-caproate (BAME standard) was purchased from Sigma (St. Luis, MO, USA) and standard calibration mixture of bacterial FAMEs in hexane (MIDI standard) was purchased from Hewlett Packard (USA).

2.3 CULTURE CONDITIONS

P. putida DSMZ 50026 was cultivated for 20 hours in medium described by DSMZ (1998) containing 3 g of meat extract and 5 g of bacteriological peptone per

Table 1: *Concentrations of tested mercury species*
Preglednica 1: *Koncentracije testiranih živosrebrovih spojin*

Tested compound	Concentrations (|ig/L)			
$HgCl_2$ (Hg (II))	200	20	2	0.2
CH_3HgCl (MeHg)	12.5	1.25	0.12	-

1000 ml of distilled water (dH_2O). Cells were grown in 10 ml test-tubes, at 27 °C (without shaking).

2.4 EXPOSURE CONDITIONS

After 20 hours incubation, selected environmentally relevant concentrations (Quiu *et al.,* 2006) of tested mercury species (Table 1) were added to the cultures. MeHg, which is water insoluble, was dissolved in 50% DMSO instead of distilled water before added to the culture medium. Negative controls for those samples were performed with the addition of an adequate amount of DMSO as well. Cells exposed to tested concentrations of mercury species were incubated for another 24 hours at 27 °C. During incubations, growth was followed by measuring optical density at 654 nm by Novaspec II Visible Spectrofotometer. Cells were harvested by centrifugation (3000 rpm, 4 °C, 10 min). Pellets were resuspended in sterile double distilled water (1 mL), frozen (-20 °C) and freeze-dried.

2.5 LIPID EXTRACTION AND TRANSESTERIFICATION

Bacterial lipids were extracted from freeze dried samples and transesterified using modified HCl/methanol procedure that has already been described before (Ivancic *et al.,* 2009).

2.6 GAS CHROMATOGRAPHY

Fatty acid methyl esters (FAMEs) extracts in hexane were analyzed on gas chromatograph Shimadzu GC-14A equipped with flame ionization detector (FID). Capillary column (Equity-1; Supelco, 28046-U) with non-polar stationary phase (100% poly-dimethyl-siloxane) was used. The analysis followed the temperature program: temperature gradient from 150 to 250 °C at 4 °C min^{-1}. The flow rate of carrier gas (He) was 30 ml min^{-1}. The injector temperature was held at 250 °C and detector at 280 °C. The results were registered on Chromatopac C-R6A integrator. Relative proportions of fatty acids between C10 and C20 were calculated from peak areas. Identification was done either directly by comparison of retention times of unknown peaks with standard fatty acid calibration mixtures (BAME, MIDI; SIGMA-Aldrich) or indirectly by equivalent chain length (ECL) factors calculation (Mj0s, 2003).

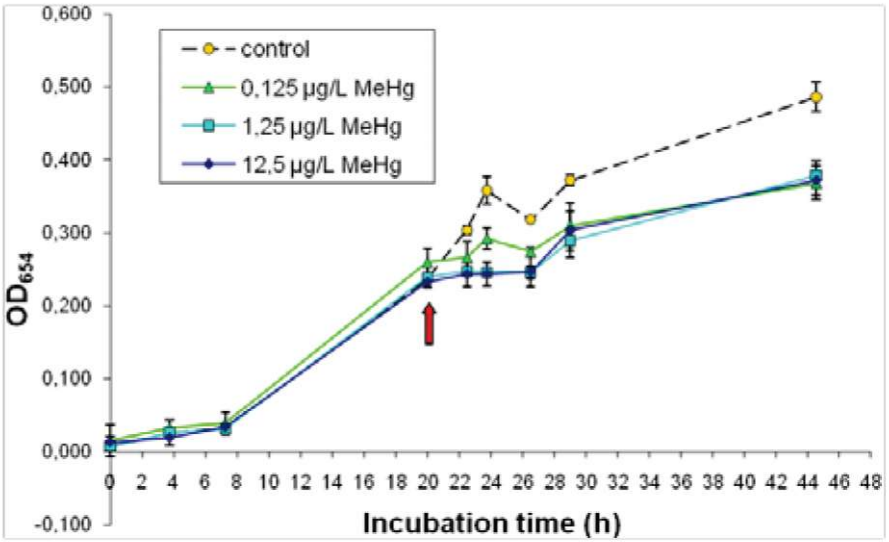

Figure 1: *Growth curves of P. putida DSM 50026 culture exposed to MeHg in concentrations from 0.12 to 12.5 pg/L in comparison to non-exposed cells (control). The time of MeHg addition is marked by arrow.*
Slika 1: *Rastne krivulje kulture P. putida DSM 50026 izpostavljene MeHg v koncentracijah od 0,12 do 12,5 pg/L v primerjavi s kontrolno (neizpostavljeno) kulturo. Začetni čas izpostavitve MeHg je označen s puščico.*

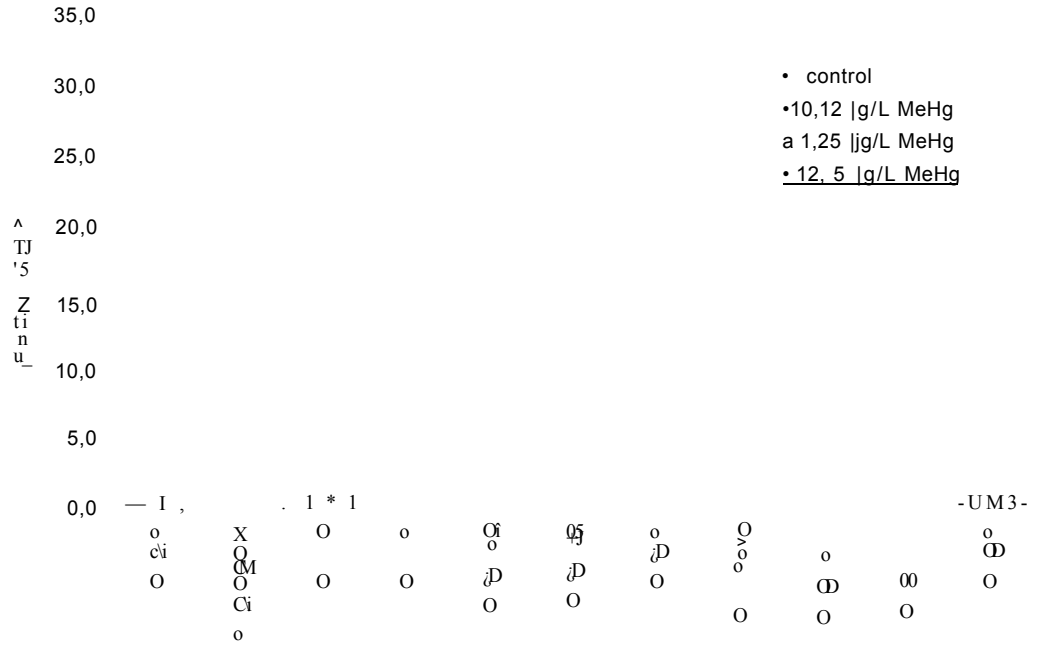

Figure 2: *Changes in fatty acid profile of P. putida DSM 50026 culture exposed to MeHg in concentrations from 0.12 to 12.5 pg/L.*
Slika 2: *Spremembe v maščobnokislinskem profilu kulture P. putida DSM 50026 ob izpostavitvi MeHg v koncentracijah od 0,12 pg/L do 12,5 pg/L.*

2.7 CALCULATIONS

Membrane fatty acids which were present in less than 0.5% of total fatty acids were signed as fatty acids in traces and were not considered for further interpretation.

Trans/cis ratio of unsaturated fatty acids was calculated according to Heipieper *et al.,* 1995.

2.8 STATISTICAL ANALYSIS

All the exposures were performed in 4 parallel samples. The data was statistically analyzed using Student's t-test with significance level of 0.05.

3 RESULTS

Our experiments showed that exposure *of P. putida* DSM 50026 to $HgCl_2$ in concentrations of 0.2-200 ^g/L did not result in any significant effects on growth nor fatty acid composition of exposed bacterial culture (results not shown). However, when bacteria were exposed to organic mercury in form of CH_3HgCl (from 0.12-12.5 ^g/L) growth inhibition as well as significant changes in fatty acid composition were observed (Figures 1, 2). Most significant (dose-effect) inhibition of cell growth occurred in the first 4-6 hours after exposure of cells to MeHg. After 8 hours of growth in MeHg supplemented medium, cell culture appeared to grow with approximately the same (attenuated) rate, regardless of mercury concentration. The behavior of growth curves that can be observed in Fig. 1 suggests the possibility of adaptive changes of microbial cells, enabling the culture to continue growing under the changed conditions. Since the membrane represents the primary barrier between cells and the environment and is responsible to regulate the flow of molecules into (and out of) the cell, we decided to focus our research on possible adaptive changes that may be detected on lipid level. Our results show that exposure of *P. putida* to MeHg in concentrations between 0.12-12.5 ^g/L significantly influences fatty acid profile of tested bacteria, resulting in increase of *trans/cis* fatty acid ratio from 0.35 ± 0.04 (in non-exposed cells) to 0.47 ± 0.02 (in cells exposed to 0.12 ^g/L or 1.25 ^g/L MeHg) or 0.48 ± 0.03 (in cells exposed to 12.5 ^g/L). The observed shift in *trans/cis* ratio is mainly associated with statistically significant decreases in C16:1cis9 and C18:1cis11 fatty acids, accompanied by increase in C16:1 *trans9.*

4 DISCUSSION

Little is known about the molecular mechanisms controlling (methyl)mercury uptake and toxicity so far. The primary targets of both, $CH_3Hg(II)$ as well as inorganic Hg(II), are considered sulfhydril-containing macromolecules (especially of various molecular weight thiol-containing proteins). Covalent binding of mercury compounds to proteins acting as antioxidants (i.e. glutathione) or components of electron transport chains appears to be associated with free radical accumulation, leading to oxidative damage of macromolecules and lipid peroxidation (Patrick, 2002; Han *et al.,* 2008). Despite the generally recognized common molecular targets, the levels of mercury species inducing toxicity usually differ. It is generally assumed that MeHg is the most toxic Hg species, which is often ascribed to its higher lipid solubility (Sweet, 2001). However, the octanol/water partition coefficients (K_{ow}) of uncharged $HgCl_2$ and CH_3HgCl species do not differ significantly (3.3 and 1.7 respectively) (Broniatowski, 2009). These data suggest that the actual interaction of the Hg species with the cell membranes is very much dependent on the environmental factors influencing their ionization as well as membrane charge (especially pH and the types, as well as concentrations of ions present in the solution) (Sweet, 2001).

Only few studies on methylmercury binding to biomembrane lipids have been reported. Early *"in vitro"* studies suggested a direct mechanism of $CH_3Hg(II)$ action on selected lipids. Segal & Wood (1974), for example, performed an NMR study which showed that MeHg can react both catalytically and directly with plasmalogens (a group of phospholipids which are important in a membrane structure for cells of the central nervous system of higher organisms). They showed that MeHg ion is soluble in phospholipids and catalyses rapid hydration and hydrolysis of the vinyl ether linkage to give a mixture of palmitic and stearic aldehydes plus the linolenic monoglyceride product (Segal and Wood, 1974). Furthermore, studies performed by LeBlanc *et al.* (1984) revealed a pH-dependent binding of MeHg to acidic phosphatidylserine (PS) and phosphatidylinositol (PI) phospholipids, but not to zwitterionic phosphatidilcholine (PC) and sphingomielin (SM). The most extensive study on MeHg interaction with phospholipid membranes is probably the one performed by Girault *et* al.(1997), in which the authors used three complementary approaches: (i) ^{199}Hg-NMR which quantitatively describes MeHg mobility and complexation, both in solution and at the membrane interface, (ii) fluorescence polarization which reveals dynamic changes of the hydrophobic interior and (iii) solid state ^{31}P-NMR which is indicative of the phosphate group structure and mobility and allows detection of non-bilayer phases. The study revealed that $CH_3Hg(II)$ interactions with membrane lipids are electrostatic in nature and primarily depend on the polar head groups negative charges (phosphate moiety), which is not the case with $HgCl_2$(Delnomdedieum *et al.,* 1992; Girault *et al.* 1997). Extensive metal binding (up to three MeHg molecules per lipid) induces limited membrane destabilization, which may, in some cases, be associated with loss of its integrity (Girault *et al.,* 1997).

Our results confirmed the hypothesis that effects of mercury compounds on *P. putida* cells essentially depend on their chemical structures. Meanwhile chosen concentrations of inorganic mercury in form of $HgCl_2$, did not inhibit growth nor induced any adaptive changes in bacterial membranes the opposite was the case with its methylated form. MeHg exhibited toxicity that reflected at both levels (culture growth as well as membrane changes) at concentrations up to 1600-times lower that the highest Hg(II) concentration tested. Most significant inhibition of cell growth occurred in the first 4-6 hours after exposure to MeHg. After 8 hours of growth in MeHg supplemented medium, cell culture appeared to grow with approximately the same (slightly attenuated) rate, regardless of methylmercury concentration. The lack of dose-effect inhibition at this stage may indicate that differences in chosen concentrations were too small to inhibit significantly different proportions of cells that would be observable by spectrophotometric measurements. However, the behavior of growth curves that can be observed in Fig. 1 suggests the possibility of adaptive changes in certain number of microbial cells that have survived the MeHg presence, enabling the cultures to continue growing under the changed conditions.

Observed membrane changes associated with MeHg exposure resulted in overall increase in *trans/cis* fatty acid ratio, indicating the prevalent isomerization of *cis-* to *trans-* unsaturated fatty acids. This adaptive response is known to be associated with decrease in membrane fluidity, enabling *Pseudomonas* strains to grow in the presence of membrane-disrupting compounds (Von Wallbrun *et al.,* 2003; Härtig *et al.,* 2005). The same type of response has already been described when selected *P. putida* strains were exposed to toxic concentrations of toluene (Weber *et al.,* 1994; Heipieper *et al.,* 1994), phenol (Heipieper *et al.,* 1992), ethanol (Heipieper *et al.,* 1994) and six different heavy metals, namely Zn^{2+}, Cd^{2+}, Cr^{3+}, Co^{2+}, Cu^{2+} and Ni^{2+} (Heipieper *et al.,* 1996). The degree of isomerization was shown to depend on the toxicity and the concentration of membrane-affecting agents. The described way of membrane adaptation is performed by *cis-trans-isomerase* (Cti), a constitutively expressed periplasmic enzyme that, to exert its action, necessitates neither ATP nor other cofactors, and consistently, is in-

dependent of de novo synthesis of lipids. Due to its direct correlation with toxicity, cis-trans-isomerization is a potential biomarker for recording solvent stress or changes of other environmental conditions (Bernal *et al.,* 2007; Heipieper *et al.,* 2010). The question that needs to be addressed at this point is how do *P. putida* cells detect the presence of membrane disrupting compounds like MeHg, which leads to activation of protective mechanism(s). In the presence of organice solvents, the detection and activation appears to be directly associated with detected increase in membrane microviscosity caused by changes of the acyl chain order (Killian *et al.,* 1992). According to Neumann *et al.,* the hydrophilic structure and periplasmic location of Cti supports the assumption that the enzyme can only reach its target (the double bonds of unsaturated fatty acids that are located at a certain depth of the membrane) when the membrane is destabilized (i.e. the fluidity at certain regions is increased) by environmental factors (Neumann *et al.,* 2003; Härtig *et al.,* 2005). Since direct effect on membrane fluidity has also been observed in the case of MeHg (Girault *et al.,* 1997; Schara *et al.,* 2001), the abovementioned mechanism may apply here as well.

5 CONCLUSIONS

In our research a bacterial model has been used to assess toxicity of two mercury species that represent important pollutants of aquatic ecosystems at sites of industrial or mining activities.

The results showed different toxicities of Hg(II) and MeHg to (bacterial) cells. Meanwhile inorganic form, Hg(II) did not influence the growth nor induce any significant changes in fatty acid profile of *P. putida,* exposure to methylated form of mercury resulted in partial growth inhibition, which appears to be balanced by adaptive membrane changes. We showed that changes in fatty acid profile of *P. putida* resulting from MeHg exposure are similar to those observed as a response to organic solvents, as well as some other membrane-disrupting compounds, and are associated with (adaptive) decrease in membrane fluidity.

Despite the fact that response of *P. putida* to MeHg is not specific, these bacteria might possibly be used to develop a bioassay, used to indicate the potential presence of toxic bioavailable concentrations of MeHg in environments where mercury represents the major pollutant (i.e. Idrijca river, where MeHg also accumulates in freshwater fish and crabs). Neverthless, more research needs to be done to assess the influence of different physico-chemical parameters (like pH, ionic strength etc.) as well as other potentially interfering compounds on the responsiveness of the system.

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