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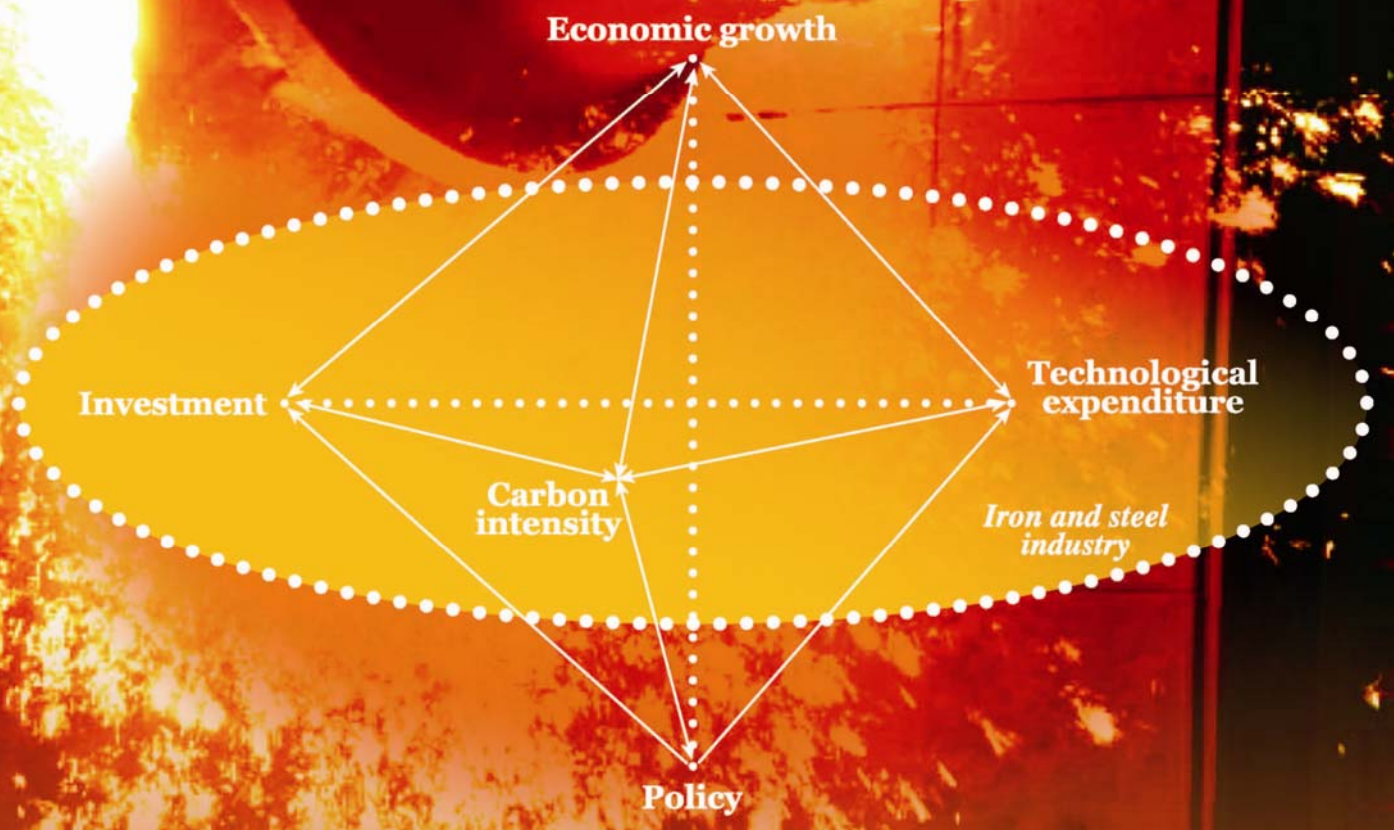

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Cu(II), Fe(III) and Mn(II) combinations as environmental stress factors have distinguishing effects on *Enterococcus hirae*

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ABSTRACT

Pollution by various heavy metals as environmental stress factors might affect bacteria. It was established that iron (Fe(III)), manganese (Mn(II)) and copper (Cu(II)) ion combinations caused effects on *Enterococcus hirae* that differed from the sum of the effects when the metals were added separately. It was shown that the Cu^{2+}–Fe^{3+} combination decreased the growth and ATPase activity of membrane vesicles of wild-type *E. hirae* ATCC9790 and *atpD* mutant (with defective F_OF_1-ATPase) MS116. Addition of Mn^{2+}–Fe^{3+} combinations within the same concentration range had no effects on growth compared to control (without heavy metals). ATPase activity was increased in the presence of Mn^{2+}–Fe^{3+}, while together with 0.2 mmol/L *N,N′*-dicyclohexylcarbodiimide (DCCD), ATPase activity was decreased compared to control (when only 0.2 mmol/L DCCD was present). These results indicate that heavy metals ion combinations probably affect the F_OF_1-ATPase, leading to conformational changes. Moreover the action may be direct or be mediated by environment redox potential. The effects observed when Fe^{3+} was added separately disappeared in both cases, which might be a result of competing processes between Fe^{3+} and other heavy metals. These findings are novel and improve the understanding of heavy metals ions effects on bacteria, and could be applied for regulation of stress response patterns in the environment.

Introduction

In recent decades, heavy metal pollution in the environment has become a serious problem for living organisms, and the amount of pollutants has increased many folds due to both natural and anthropogenic sources.

It is known that microorganisms interact with heavy metal ions in the environment and participate in biochemical cycling of these ions (Spain, 2003). Bacteria are exposed not only to one or two types of different heavy metals ions like Fe^{3+}, Mn^{2+} or Cu^{2+}, but also to different heavy metal ion combinations. Moreover, the effects of different heavy metals ion combinations can be unexpected and may differ from effects detected with single heavy metal ions. Some investigations have been carried out involving heavy metal ion combination effects on bacteria, but the effects and mechanisms are not yet clear (Gikas, 2007; Wyszkowska et al., 2008).

It is known that different heavy metal ions such as Fe^{3+}, Mn^{2+}, Ni^{2+} and Zn^{2+} are referred to as "essential" and are necessary for normal metabolism of bacteria (Nies, 1999). Some other heavy metals such as Pb^{2+} or Hg^{2+} are harmful for bacteria even in small quantities. In any case both the "essential" and "non-essential" heavy metal ions at high concentrations become toxic to microorganisms (Nies, 1999). The addition of heavy metal ions to the environment can lead to changes in the growth properties, morphology, biomass and fermentative activity of bacteria (Roane and Pepper, 2000). The possible targets of heavy metal ions in bacteria are cell membranes, enzymes and DNA (Bruins et al., 2000).

In our previous papers we have shown that Fe^{3+}, Fe^{2+}, Cu^{2+} and Mn^{2+} markedly affect the growth and membrane activity of

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Enterococcus hirae when they are added separately (Vardanyan and Trchounian, 2010, 2012, 2013). Enterococci are known as gastrointestinal organisms, and an important characteristic of this group is their resistance to different chemical factors, such as heavy metals and antibiotics (De Niederhäusern et al., 2013). These bacteria are used in the food industry and could be added as bio-preservatives (Foulquié Moreno et al., 2006; Iseppi et al., 2008). At the same time, among enterococci there are pathogenic species that can cause endocarditis and infections of the urinary tract and central nervous system (Foulquié Moreno et al., 2006). In this respect it is interesting to study the metabolism and behavior of enterococci in the presence of different heavy metals.

During *E. hirae* growth in anaerobic conditions at alkaline pH, changes in pH and environment oxidation–reduction potential (E_h) can be detected (Poladyan et al., 2006). As is known, different oxidizers and reducers can affect E_h, thus regulating bacterial growth, and different heavy metal ions have been used for this purpose. Oxidizers (Cu^{2+}, Fe^{3+}) and reducers (Mn^{2+}, Fe^{2+}) were used in previous studies (Vardanyan and Trchounian, 2010, 2012, 2013). All these ions are "essential" and required for bacteria in small quantities. They are mostly contained in the reaction centers of oxidation–reduction enzymes directly participating in appropriate reactions (Touati, 2000) and in cofactors of enzymes, as DNA and RNA polymerases, oxidases, dehydrogenases and kinases (Crowley et al., 1999). For all these ions there are specific transport systems in bacteria for entry to the cell. Cu^{2+} homeostasis in *E. hirae* is determined to be regulated by the *cop* operon (Solioz and Stoyanov, 2003). For Cu^{2+} transport, the *E. hirae* membrane P-type ATPases (CopA and CopB) are responsible. Fe^{2+} uptake is an ATP-driven process and the appropriate transport system is encoded by three *feoABC* genes (Kammler et al., 1993), while Fe^{3+} is taken up together with siderophores (Ouyang and Isaacson, 2006). The Fe^{3+}-siderophore complex passes through the plasma membrane together with specific proteins that are components of ABC transporters (Ouyang and Isaacson, 2006). For Mn^{2+} two types of transport systems are known: the first is considered to be a member of the P-type ATPase and the second one is suggested to be the protein-dependent ABC transporter system (Hao et al., 1999; Makui et al., 2000).

As is known, oxidizers inhibit bacterial growth by maintaining E_h at positive values, and it is expected that both Cu^{2+} and Fe^{3+} should suppress *E. hirae* growth (Vardanyan and Trchounian, 2010, 2012). Thus it was unexpected that growth inhibition was detected only in the case of Fe^{2+} and Cu^{2+}, while with Fe^{3+} and Mn^{2+} *E. hirae* growth was enhanced (Vardanyan and Trchounian, 2010, 2012, 2013). The effects were concentration dependent. The results indicate that heavy metal ions do not act only as oxidizers and reducers, but that specific heavy metal ions action mechanisms of can occur. All these ions markedly affect E_h changes during *E. hirae* growth as well as disturbing membrane proton-coupled processes (Vardanyan and Trchounian, 2010, 2012, 2013). These effects may be due to changes in E_h or by direct effects on membrane proteins.

Taking into consideration the limited knowledge on the effects of heavy metal mixtures on bacteria, the aim of this study was to study *E. hirae* growth and membrane-associated ATPase activity in the presence of different heavy metal ion combinations. The results were compared with the effects of single heavy metals ions.

1. Materials and methods

1.1. Bacterial strains and growth, membrane vesicles

The wild-type strain *E. hirae* ATCC9790 and the *atpD* mutant strain MS116 (lacking the β subunit in F_1) (Poladyan and Trchounian, 2006) were used in this study. MS116 mutant strain expresses F_oF_1 at the same level as wild-type, but it has a lowered ATPase activity (Arikado et al., 1999). The strains were kindly supplied by Prof. H. Kobayashi (Graduate School of Pharmaceutical Sciences, Chiba University, Chiba 263, Japan).

The bacterial culture was grown anaerobically at initial pH 8.0 in a medium containing 1% tryptone, 0.5% yeast extract, 1% K_2HPO_4 and 0.2% glucose at pH 8.0 as described earlier (Trchounian and Kobayashi, 1998; Poladyan and Trchounian, 2006). Bacteria were incubated at 37°C for 24 hr. The growth of bacteria was monitored by changes in the optical density (OD) of the bacterial suspension using a spectrophotometer (Spectro UV–VIS Auto, Labomed, Los Angeles, CA, USA) at the wavelength of 600 nm. The concentrations of 0.1 and 1 mmol/L (in both cases metals were added in equal quantities) metal ions, respectively, were added, when mentioned; control was without metal ion additions. Growth properties: lag phase duration was determined graphically as described before (Kirakosyan et al., 2004; Poladyan et al., 2006) and the specific growth rate was calculated by dividing 0.693 ($\log^2 = 0.693$) by the doubling time of OD in the ranges where changes in the logarithm of OD depended on time in a linear manner.

Membrane vesicles were isolated as described earlier (Kirakosyan et al., 2004) except that the buffers lacked K^+.

1.2. E_h and pH determination

The E_h of bacterial growth mediums was measured using a platinum electrode (EPB-1, Electrometer Equipment State Enterprise, Gomel, Belarus; GDEEE, Hanna Instruments, Amorim, Portugal) as described elsewhere (Poladyan et al., 2006; Kirakosyan et al., 2008). Note that the E_h value was changed 25–30 mV by a *ca.* 8-fold change of bacterial count, and was not changed more than on 20 mV by addition of metal ions within the concentration range used. So the changes of E_h during bacterial growth did not depend on changes of bacterial count or metal ions concentration.

The pH values were measured by a selective pH-electrode (HJ1131B, Hanna Instruments, Amorim, Portugal) and were adjusted by 0.1 mol/L NaOH or HCl.

1.3. ATPase assay and others

The ATPase activity of membrane vesicles was measured by the amount of liberated inorganic phosphate (P_i) after adding 5 mmol/L ATP by a spectrophotometric method (Blbulyan et al., 2011). The assay mixture was 50 mmol/L Tris–HCl (pH 8.0), containing 0.4 mmol/L $MgSO_4$ and 100 mmol/L KCl. When necessary, membrane vesicles were pre-incubated with heavy metal ions or N,N′-dicyclohexylcarbodiimide (DCCD) for 10 min. Corrections were made for blanks without ATP or membrane vesicles. Relative ATPase activity was expressed in nmol P_i per mg protein in 1 min.

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. All assays were routinely carried out under anaerobic conditions and all measurements were done at 37°C.

1.4. Data processing

The average data are presented from three independent measurements; standard errors were within 3% if not indicated.

The Student's validity criteria (p) were calculated to show the reliability of difference between changed values and control.

1.5. Reagents

Tryptone, yeast extract and Tris (aminomethan) were from Roth (Karlsruhe, Germany), agar, ATP (Tris salt) and DCCD were from Sigma (St. Louis, MO, USA), glucose was from Borisov Medical Preparations Plant (Borisov, Belarus), and other reagents used in the study were of analytical grade.

2. Results

2.1. Bacterial growth in the presence of heavy metals combinations and E_h changes

We studied the growth properties of *E. hirae* ATCC9790 wild-type strain and MS116 mutant strain when different heavy metal ion combinations were added to the growth medium. Four combinations of heavy metals ions (Cu^{2+}–Fe^{3+}, Cu^{+}–Fe^{3+}, Cu^{2+}–Fe^{2+}, Mn^{2+}–Fe^{3+}) were used in 0.1 and 1 mmol/L (heavy metal ions were added in equal quantities) concentrations. It was shown that growth lag phase was prolonged in the presence of Cu^{2+}–Fe^{2+} (not shown) and Cu^{2+}–Fe^{3+} (Fig. 1), and the effects were concentration dependent. In the case of Mn^{2+}–Fe^{3+}, no statistically reliable differences were observed (Fig. 1), even in the case of 1 mmol/L concentration ($p > 0.05$). Similar results were determined in the case of specific growth rate (Fig. 1).

The results for only two of the combination of metals ion are shown in Figs. 1–3, and these combinations were used in further experiments, as the effects for the other two combinations (Cu^{+}–Fe^{3+}; Cu^{2+}–Fe^{2+}, not shown) did not differ from the results observed with the single heavy metals ions. When Cu^{2+}–Fe^{2+} and Cu^{+}–Fe^{3+} were added together, bacterial growth was inhibited by the same extent as in the case when the separate heavy metals were present (no synergism, antagonism or additive interactions were detected) within the same concentration range. In any case, bacterial growth was inhibited in the presence of Cu^{2+}–Fe^{2+} and enhanced in the presence of Cu^{+}–Fe^{3+} (not shown). When Cu^{+} was added in bacterial growth medium separately there was no effect on *E. hirae* growth, while the addition of Fe^{3+} led to the increase in the growth of these bacteria. As in the case of simultaneous addition of these heavy metals within the same concentration range, we observed the enhancement of bacterial growth in the same manner, and moreover the values of lag phase duration and specific growth rate were the same as in the case of Fe^{3+}. These results enabled us to assume that the metals are stable in this system. In spite of this fact, oxidation and reduction of various ions cannot be ruled out. These results are in accordance with data observed previously (Vardanyan and Trchounian, 2010, 2012). In contrast to these results, it was interesting to discover that simultaneous addition of Mn^{2+} and Fe^{3+} had no marked effects on bacterial growth, while separate addition of these ions led to an increase of the specific growth rate (Vardanyan and Trchounian, 2012, 2013). Meanwhile when Cu^{2+} and Fe^{3+} were added together, the stimulatory effect of Fe^{3+} disappeared (Fig. 1). Such results indicate that heavy metal ion combinations may have different action mechanisms.

Interestingly, a similar pattern was found with *atp* mutant MS116 strain (see experimental procedures) (Fig. 1). As shown in Fig. 1, the growth lag phase duration with MS116 was 4-fold higher than in the case of wild type ATCC9790, while the specific growth rate was almost the same. These results indicate that F_OF_1 is not crucial for *E. hirae* growth at pH 8.0 as suggested previously (Trchounian and Kobayashi, 1998; Poladyan and Trchounian, 2006; Vardanyan and Trchounian, 2012).

Changes in E_h values were observed during *E. hirae* ATCC9790 and MS116 growth as well. As shown in Fig. 2, the initial positive E_h values dropped to negative ones during 8 hr of bacterial growth. Such results indicate that many redox reactions took place during bacterial anaerobic growth (Bagramyan et al., 2000; Poladyan et al., 2006). In the case of *E. hirae* ATCC9790 the initial value of E_h was (40 ± 10) mV, which dropped to (−180 ± 20) mV in the control sample where no metals were added. It was interesting to note that addition of 0.1 mmol/L (0.1 mmol/L $MnCl_2$+ 0.1 mmol/L $FeCl_3$) and 1 mmol/L (1 mmol/L $MnCl_2$+ 1 mmol/L $FeCl_3$) Mn^{2+}–Fe^{3+} to the growth medium did not cause any obvious changes in E_h ($p > 0.05$) (Fig. 2a). In contrast, in the presence of 1 mmol/L

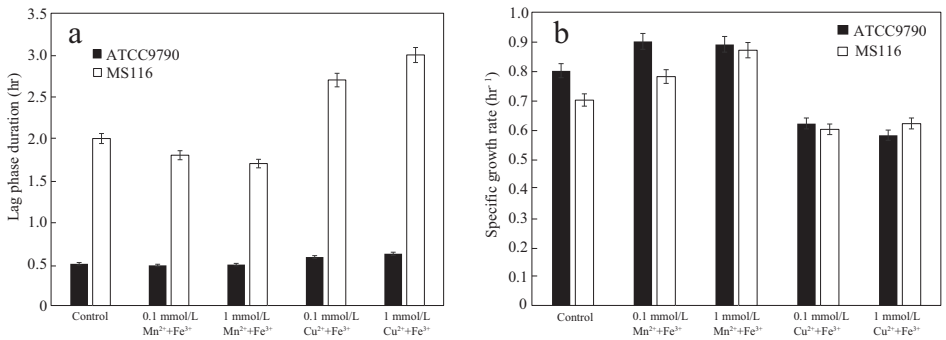

Fig. 1 – Effects of Mn^{2+}–Fe^{3+} and Cu^{2+}–Fe^{3+} on *E. hirae* wild type ATCC9790 and *atp* mutant MS116 cell growth. (a) Lag phase duration, (b) specific growth rate. Metal ions at 0.1 mmol/L and 1 mmol/L were added to the growth medium before inoculation with bacteria.

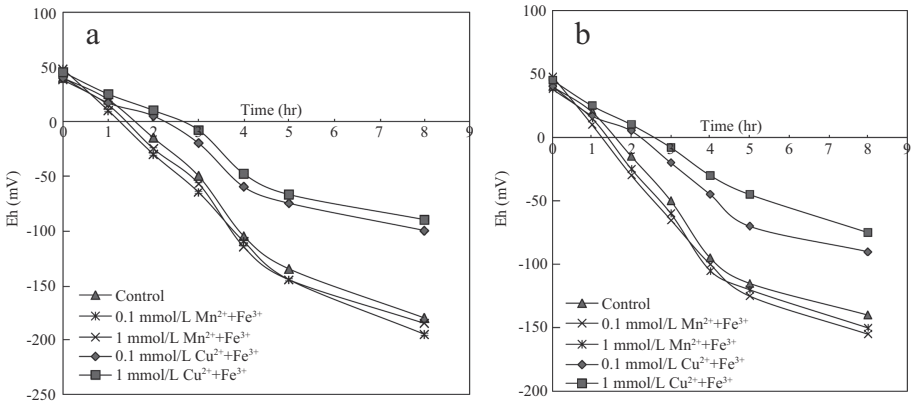

Fig. 2 – Changes in redox potential during growth of *E. hirae* ATCC9790 (a) and MS116 (b) in the presence of Mn^{2+}–Fe^{3+} and Cu^{2+}–Fe^{3+}.

Cu^{2+}–Fe^{3+}, E_h declined to (−90 ± 15) mV. As shown in Fig. 2b, similar effects were observed with MS116 mutant. In the control sample, E_h dropped to (−140 ± 15) mV, while with 1 mmol/L Cu^{2+}–Fe^{3+} it only declined to (−75 ± 10) mV.

2.2. Effects of heavy metals combinations on membrane-associated ATPase activity

To clarify the possible targets and mechanisms for heavy metal ion combinations, membrane-associated ATPase activity was determined. ATPase activity was measured in a medium containing 100 mmol/L K^+ in the presence or absence of DCCD, an inhibitor of the F_OF_1 ATPase (Trchounian and Kobayashi, 1998; Vardanyan and Trchounian, 2012). As shown in Fig. 3a, in the case of *E. hirae* ATCC9790, heavy metal ion combinations affected ATPase activity, but the strongest effects were found with Mn^{2+}–Fe^{3+}. These effects were concentration dependent. The addition of 0.2 mmol/L DCCD decreased the ATPase activity, moreover values with metals were lower compared to control samples. In the case of Cu^{2+}–Fe^{3+}, ATPase activity was decreased compared to control even without DCCD, but in the case when DCCD and metal ions were added together, the effects were strongest. A similar pattern was found with MS116 (Fig. 3b), but in this case the addition of DCCD did not cause marked effects.

3. Discussion

It is known that *E. hirae* growth is accompanied by changes in the environment E_h. Moreover, positive values of E_h inhibit bacterial growth (Riondet et al., 1999) while negative E_h values are essential for bacterial cell growth (Bagramyan and Trchounian, 1997). The bacterial growth can be regulated by oxidizers that maintain E_h at positive levels (Riondet et al., 1999; Bagramyan et al., 2000) and by reducers, which drop E_h to negative values. In our previous papers (Vardanyan and Trchounian, 2010, 2012) the effects of Fe^{3+} and Cu^{2+} as oxidizers on *E. hirae* growth and membrane-associated activity were studied. It was interesting to note that these ions had opposite effects on bacterial growth. Fe^{3+} enhanced the *E. hirae* growth and ATPase activity even in the presence of DCCD while the other oxidizer, Cu^{2+}, suppressed bacterial growth and ATPase activity without addition of DCCD (Vardanyan and Trchounian, 2010, 2012). It was thought that Fe^{2+} and Mn^{2+}, as reducers, should stimulate *E. hirae* growth, whereas experimental data show that Fe^{2+} inhibited *E. hirae* growth by increasing lag phase duration and decreasing the specific growth rate, while low concentrations of Mn^{2+} had opposite effects (Vardanyan and Trchounian, 2012, 2013). These results indicate that heavy metal ions may have

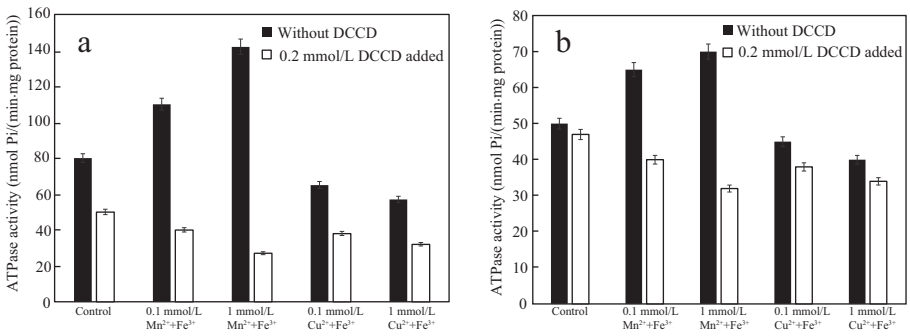

Fig. 3 – Changes in ATPase activity of membrane vesicles of *E. hirae* ATCC9790 (a) and MS116 (b) in the presence of Mn^{2+}–Fe^{3+} and Cu^{2+}–Fe^{3+}.

specific action mechanisms, and such effects might be explained by the action of these ions on membrane proteins, particularly on membrane-associated ATPase (Vardanyan and Trchounian, 2010, 2012, 2013).

As there is a mixture of different heavy metals in the environment, it is interesting to examine the effects of different metal combinations on bacterial growth. Moreover, the total effect might markedly differ from the sum of individual effects. The combined effect can be greater compared to the sum of individual effects (synergism) or contrariwise (antagonism) (Gikas, 2007, 2008). When effects are neither synergistic nor antagonistic and action is the sum of the effects when bacteria are exposed to each metal alone, these cases are called additive interactions.

There have been different findings concerning simultaneous addition of two or three heavy metal ions to bacterial media. Some authors reported that simultaneous addition of heavy metals did not increase the effects observed for the individual metals (Wyszkowska et al., 2008) but the mechanisms of the neutralization of effects are not yet clear. At the same time, Gikas (2007) showed that when low concentrations of Ni^{2+} and Co^{2+} were present in bacterial growth medium together, the growth stimulation was more effective compared to control. At higher concentrations they became more toxic compared to the individual metals (Gikas, 2007).

During this study, different combinations of heavy metal ions were chosen to examine the nature of interactions between the ions: whether synergism, antagonism or additive interactions take place. For this purpose different pairs were chosen: a pair where two enhancers were present (Mn^{2+}–Fe^{3+}); a pair where two inhibitors were present (Cu^{2+}–Fe^{2+}); and pairs with metals that have different effects on *E. hirae* growth (Cu^{2+}–Fe^{3+}; Cu^{+}–Fe^{3+}), to examine which heavy metal effect will be expressed.

Unexpected results were obtained with 0.1 and 1 mmol/L Cu^{2+}–Fe^{3+} (Figs. 1–3). As mentioned above, Fe^{3+} had a stimulatory effect on *E. hirae* growth, but when these oxidizer ions were added simultaneously, only the inhibitory effect of Cu^{2+} was detected. It seems that the influence of Fe^{3+} disappeared (Figs. 1–3). It was suggested that the effects of Cu^{2+} can be explained by direct action of these ions on the F_oF_1-ATPase (Vardanyan and Trchounian, 2010), while in the case of Fe^{3+} the existence of Fe-dependent ATPase is possible, which is active even in the presence of DCCD or in an *atp* mutant strain (Vardanyan and Trchounian, 2012). According to our results, it can be assumed that in conditions when these heavy metals are present in the medium together, the activity of Fe-dependent ATPase is not expressed. This idea can be proved by the ATPase activity results (Fig. 3a): it is clear that in the presence of Cu^{2+}–Fe^{3+} the results were lower compared to control even in the absence of DCCD. In such conditions the inhibition of not only the F_OF_1-ATPase but of Fe-dependent ATPase as well by copper ions is possible. Certain competitive processes between metal ions might be present here. Moreover, Cu^{2+} may directly affect membrane-associated proteins, particularly the F_oF_1-ATPase, but this action can be mediated by E_h too. In general, *E. hirae* membrane-associated ATPase activity is K^+-dependent (Trchounian and Kobayashi, 1998; Poladyan and Trchounian, 2011) and it is suggested that ATPase activity and H^+-coupled K^+ transport resulted from the F_oF_1-ATPase interaction with the K^+ transport system, KtrI (Trchounian, 2004). It is also known that bacterial ATPase is a redox-regulated enzyme (Bald et al., 2001). To ascertain the role of ATPase during bacterial growth, 0.2 mmol/L DCCD was added to the bacterial growth medium (not shown), and it was established that in the control sample and in samples where Cu^{2+}–Fe^{3+} was added, the growth was inhibited markedly, and the E_h value was positive even after 8 hr of growth. These findings confirm that the F_oF_1-ATPase might be a target for Cu^{2+} action in *E. hirae* cells.

In the case of the other heavy metal ion combination of Mn^{2+}–Fe^{3+}, interesting results were obtained. As mentioned in our previous paper (Vardanyan and Trchounian, 2013) high concentrations of $MnCl_2$ (0.1 and 1 mmol/L) had no effect on *E. hirae* growth. Simultaneous addition of Mn^{2+} and Fe^{3+} had similar effects to those found with Cu^{2+}–Fe^{3+}, and the stimulatory effects of Fe^{3+} on bacterial growth disappeared in this case. It has been established that separate addition of Mn^{2+} increased ATPase activity compared to control, while the addition of DCCD decreased activity many fold (not shown). Similar results were obtained when Mn^{2+} and Fe^{3+} were added simultaneously (Fig. 3a). In the presence of DCCD, ATPase activity of the samples with metals was lower compared to control, which provided evidence of the major role of F_oF_1 in the action of heavy metal ions (Vardanyan and Trchounian, 2010). At the same time, the growth of bacteria was decreased when together with Mn^{2+}–Fe^{3+}, 0.2 mmol/L DCCD was added to the growth medium (not shown). The specific growth rate was lower and E_h had positive values (not shown). These findings proved that in this case, the action of heavy metals is connected with F_oF_1 as well. This hypothesis was verified by results observed with mutant MS116 (Fig. 3b), as there was a similar pattern but to less extent. At the same time, mechanisms connected with the disappearance of the stimulatory effects of Fe^{3+} during bacterial growth and ATPase activity are not yet clear, and further investigations are required.

4. Conclusions

It was observed that the effects of heavy metal combinations markedly differed from the effects found for the individual heavy metals. Moreover in both cases studied (Mn^{2+}–Fe^{3+}; Cu^{2+}–Fe^{3+}), the stimulatory effect of Fe^{3+} disappeared, which can provide evidence of several competing processes between Fe(III) and the two other heavy metal ions. The effects of Fe^{3+} were not detected in the case of ATPase activity, while the addition of Fe^{3+} alone increased ATPase activity even in the presence of DCCD (Vardanyan and Trchounian, 2012). It is clear that Fe-dependent ATPase, which may be present in *E. hirae* membranes (Vardanyan and Trchounian, 2012), is not active in the presence of the other heavy metal. It is suggested that the target in bacterial cells for heavy metals ions' action may be membrane-associated F_OF_1, which can be regulated by direct action of heavy metals on enzymes or can be mediated by E_h.

These findings are novel and improve the understanding of heavy metal ion effects on bacteria, and could be applied for the regulation of stress response patterns in the environment.

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