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The effect of metal ions on *Staphylococcus aureus* revealed by biochemical and mass spectrometric analyses

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ABSTRACT

In this study, we focused on the effect of heavy metal ions in resistant strains of gram-positive bacteria *Staphylococcus aureus* using biochemical methods and mass spectrometry. Five nitrate solutions of heavy metals (Ag^+, Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}) were used to create *S. aureus* resistant strains. Biochemical changes of resistant strains in comparison with the non-resistant control strain of *S. aureus* were observed by microbiological (measuring - growth curves and inhibition zones) and spectrophotometric methods (antioxidant activity and alaninaminotransferase, aspartateaminotransferase, alkaline phosphatase, γ-glutamyltransferase activities). Mass spectrometry was employed for the qualitative analysis of the samples (changes in *S. aureus* protein composition) and for the identification of the strains database MALDI Biotyper was employed. Alterations, in terms of biochemical properties and protein composition, were observed in resistant strains compared to non-resistant control strain. Our results describe the possible option for the analysis of *S. aureus* resistant strains and may thus serve as a support for monitoring of changes in genetic information caused by the forming of resistance to heavy metals.

1. Introduction

The issue of emerging resistance is more frequent topic of research groups and their studies around the world. This topic is very well explored at the level of antibiotics resistance (Alm et al., 2014; Machado and Bordalo, 2014; Nedbalcova et al., 2014; Nguyen et al., 2014; Pesavento et al., 2014; Rodriguez et al., 2014). However, in the case of metal resistance there is not a sufficient amount of data for unambiguous understanding of cellular mechanisms due to the application of heavy metals. More interesting is also a problem of cross-resistance (Kaur et al., 2014; Kumari et al., 2014), a combination of resistance to antibiotics and heavy metals (Chudobova et al., 2014). To understand cross-resistance it is necessary to go from the basics and understand the sub-section of metal resistance.

Staphylococcus aureus is a gram-positive commensal bacterium causing nosocomial infections (Baker et al., 2011), and one of the main pathogens associated with skin infections, soft tissue, wound infections and more serious sequelae such as septicaemia, urinary tract infections, osteomyelitis and endocarditis (Duffy et al., 2013; Leucker et al., 2013; Taylor, 2013). Much of the dissimilarity between pathogenic *S. aureus* strains is dependent on the presence of virulence factors encoded mainly by mobile genetic elements, especially heavy metal resistance genes play an important role in virulence (Kahankova et al., 2010).

The mechanism of microorganism inhibition involves the entry of heavy metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}, Ag^+, etc.) to the metabolic system of an organism with consequent formation of secondary metabolites, which are toxic to the organism due to the presence of heavy metals (Lim et al., 2013). It has been shown that the heavy metal stress significantly contributes to the inhibition of bacterial growth (Seniya et al., 2012). However, most bacterial strains are able to create the resistance against the effect of heavy metal ions. Biological resistance is gained by the organisms against adverse effects of internal and external environment, such as the long-term effects of heavy metals from soil and water or widespread use of antibiotics (Ohlsen et al., 2003). In the response to exposition to toxic metals, metal resistance comes mostly in plasmid-encoded bacteria. Resistance genes encode genetic information of microorganisms that is changed by external or internal conditions. There

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are many mechanisms of resistance, as in the multiple-metal-resistant bacterium *S. aureus*, where Cd^{2+} (and probably Zn^{2+}) efflux is catalysed by the membrane-bound CadA protein and P-type ATPase. CadC protein is required for full resistance and CadR protein is hypothesized for regulation of the resistance determinant (Nies, 1992). The non-toxic metals are an important cofactor for many enzymes; however, they can show toxic effects at their high concentrations too. Therefore, bacteria must strictly control the intake of the metals into the cell for use as a cofactor and, more importantly, must limit free intracellular levels to prevent toxicity (Rouch et al., 1995).

Effects of heavy metals on bacterial cell are observed by changes in enzymes' activities, significant growth inhibition, inhibition of replication etc., whereas most of these mechanisms lead to cell lysis (Fig. 1A) (Silver and Ji, 1994). Baker *et al.* suggested that *S. aureus* has one main mechanism for adapting to high levels of environmental copper *via* increased oxidative stress resistance (Baker et al., 2010). Some microorganisms are able to resist the effects of heavy metals by formation of the antioxidant enzyme superoxide dismutase or by reduction of metal ions (Singh et al., 2013; Wiesemann et al., 2013). One of the most important target molecules for intracellular interaction with metals is cysteine-rich protein metallothionein. Its primary function is to detoxify the heavy metals in living organisms, which was the subject of many previous studies (Templeton and Cherian, 1991; Klaassen et al., 2009). Regulation of expression is probably caused by metal binding to the transcription factor MTF-1, although the information about expression is currently still insufficient (Babula et al., 2012).

Furthermore, some changes in genetic information can occur due to the heavy metal influence, like in the gene 16S rRNA. 16S ribosomal RNA, conferred by 16S rDNA, is one of the components of small subunit of prokaryotic ribosomes. This gene is about 1500 bp in length in *S. aureus* and it is often used in phylogenetic studies due to its hypervariable regions useful for identification of bacteria (species or genera). These variable regions result in numerous differences in endonuclease restriction site, which can be studied via restriction fragment length polymorphism (RFLP) and subsequently analysed by gel electrophoresis (Stomeo et al., 2013).

Based on the above mentioned facts, this work is focused on the studying of gram-positive bacteria *S. aureus* resistance to heavy metals at several levels. This issue was studied particularly through the changes of selected properties of bacterial strains, which were exposed to heavy metal ions. These properties were observed on the cellular and molecular levels. MALDI-TOF mass spectrometry was employed to identify non-resistant and resistant strains of *S. aureus* treated with silver, copper, cadmium, zinc and lead ions**.** Moreover, some biochemical assays including activities of alaninaminotransferase, aspartateaminotransferase, alkaline phosphatase, γ-glutamyltransferase, antioxidant activity and content of metallothionein closely connected to oxidative stress (Eckschlager et al., 2009; Babula et al., 2012; Krizkova et al., 2012) were used to confirm the observed phenomena.

2. Material and methods

2.1. Chemicals

Chemicals used in this study (Tryptone, Yeast Extract, NaCl, $AgNO_3$, $CuN_2O_6.3H_2O$, $Pb(NO_3)_2$, $Cd(NO_3)_2.4H_2O$, $Zn(NO_3)_2.6H_2O$) were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity unless noted otherwise. Heavy metals used for the preparation of *S. aureus* resistant strains were in the form of nitrates ($AgNO_3$, $CuN_2O_6{\cdot}3H_2O$, $Pb(NO_3)_2$, $Cd(NO_3)_2{\cdot}4H_2O$, $Zn(NO_3)_2{\cdot}6H_2O$) and dissolved in 100 ml MilliQ water in 2 mM concentration. Deionized water was prepared using reverse osmosis equipment Aqual 25 (Brno, Czech Republic). Deionized water was further purified using a MilliQ Direct QUV apparatus equipped with UV lamp. The resistance was 18 MΩ. The pH was measured using pH meter WTW inoLab (Weilheim, Germany).

2.2. Cultivation of S. aureus

S. aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms (Faculty of Science, Masaryk University, Brno, Czech Republic). Strains were stored in the form of a spore suspension in 20% (*v*/*v*) glycerol at -20 °C. Prior to use, the strains were thawed and the glycerol was removed by washing with distilled water. The composition of cultivation medium (LB medium) was prepared according to protocol from Sigma Aldrich. Composition was as follows: Tryptone 10 $g.l^{-1}$, NaCl 5 $g.l^{-1}$, Yeast Extract 5 $g.l^{-1}$ and sterilized MilliQ water with 18 MΩ. pH of the cultivation medium was adjusted to 7.4 before sterilization. Sterilization of media was carried out at 121 °C for 30 min in sterilizer (Tuttnauer 2450EL, Israel). The prepared cultivation media were inoculated with bacterial culture into 25 ml Erlenmeyer flasks. After the inoculation, bacterial cultures were cultivated for 24 h on a shaker at 600 rpm and 37 °C. Bacterial culture cultivated under these conditions was diluted by cultivation medium on Specord spectrophotometer 210 (Analytic, Jena, Germany) to $OD_{600} = 0.1$ and used in the following experiments.

2.3. Preparation of Resistant Strains of S. aureus

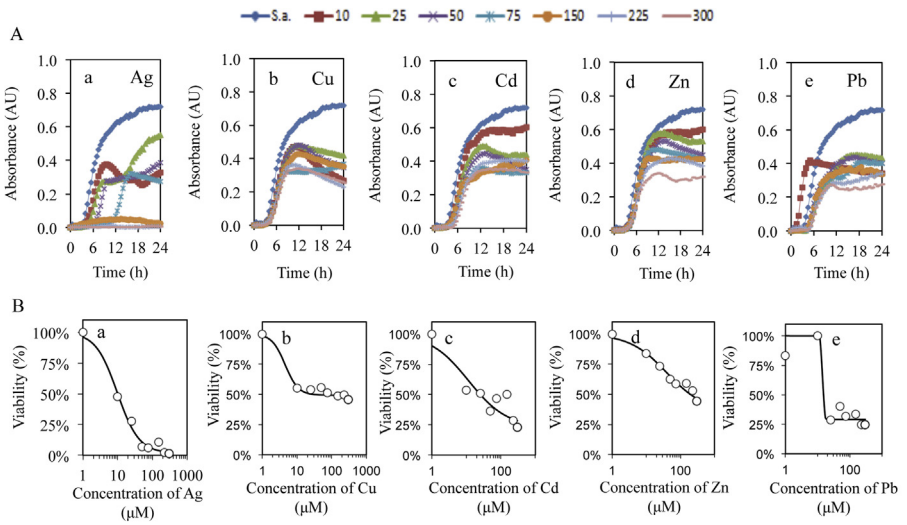
For this work, we developed a method for creating of resistant strains of *S. aureus* (NCTC 8511). To this bacterial culture 2 mM solutions of heavy metals (Ag, Cu, Cd, Zn and Pb) were added. Lowest resulting concentration of the metal in a medium inoculated with bacterial culture was found to be 50 μM, and then the metal concentration was gradually increased by 50 μM up to the maximum possible dose, in which *S. aureus* was still able to regenerate. It was always possible to revitalize resistant strains using pure medium without addition of metal.

2.4. Determination of Growth Curves

The procedure for the evaluation of an antimicrobial effect of tested heavy metals was based on the *S. aureus* bacterial culture. An apparatus Multiskan EX (Thermo Fisher Scientific, Germany) *via* Ascent Software for Multiskan was used with subsequent analysis in the form of growth curves. The bacterial culture growing overnight was diluted with LB medium to absorbance of 0.1 measured using a Specord spectrophotometer 210 (Analytik, Jena, Germany) at a wavelength of 600 nm. The diluted culture was pipetted into a microplate (total volume of 300 μl) alone as a control variant, or with various concentrations of tested heavy metals. The concentrations of these metals in the well were 0, 10, 25, 50, 75, 150, 225, and 300 μM. Measurements were carried out at time 0, then each half-hour for 24 h at 37 °C, at a wavelength of 600 nm. The measured absorbances were analysed in a graphic form as growth curves for each experimental group individually (Chudobova et al., 2013).

2.5. MALDI-TOF MS Identification of Resistant Strains of S. aureus

The following extraction protocol and sample preparation was based on MALDI Biotyper 3.0 User Manual Revision 2, similar extraction method was used also in (Sauer et al., 2008). A sample of 500 μl *S. aureus* (0.1 OD) culture, cultivated overnight, was centrifuged at 14.000 × g for 2 min. The supernatant was discarded and the pellet was resuspended in 300 μl of deionized water and



C

Heavy metal ions	Time of measurement	IC 50 (μM)
Ag	6 h	9.8
	12 h	18.0
	18 h	21.7
	24 h	33.3
Cu	6 h	4.5
	12 h	3.5
	18 h	2.1
	24 h	1.8
Cd	6 h	11.6
	12 h	12.3
	18 h	14.3
	24 h	14.1
Zn	6 h	3700.0
	12 h	404.9
	18 h	242.3
	24 h	40.7
Pb	6 h	15.3
	12 h	14.8
	18 h	6.0
	24 h	3.2

Figure 1. (**A**) Growth curves of *S.aureus* strains resistant to heavy metals (a) RAg, (b) RCu, (c) RCd, (d) RZn, (e) RPb. (**B**) Statistical evaluation of viability after the application of heavy metal ions (a) Ag^{+}, (b) Cu^{2+}, (c) Cd^{2+}, (d) Zn^{2+}, (e) Pb^{2+}. (**C**) Table of inhibition concentrations (IC_{50}) for each of different resistant strains at 6, 12, 18 and 24 hours of measurement.

900 μl of ethanol was added. After centrifugation at 14.000 × g for 2 min, the supernatant was discarded and the obtained pellet was air-dried. The pellet was then dissolved in 25 μl of 70% formic acid (*v*/*v*) and 25 μl of acetonitrile and mixed. The samples were centrifuged at 14.000 × g for 2 min and 1 μl of the clear supernatant was spotted in duplicate onto the MALDI target (MTP 384 target polished steel plate; Bruker Daltonik GmbH, Bremen, Germany) and air-dried at room temperature. Then, each spot was overlaid with 1 μl of saturated α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution in organic solvent (50% acetonitrile and 2.5% trifluoroacetic acid, both *v*/*v*) and air-dried completely prior to MALDI-TOF MS measurement on UltrafleXtreme MS (Bruker Daltonik GmbH, Bremen, Germany). Spectral data were taken in the *m*/*z* range of 2.000 Da to 20.000 Da, and each was a result of the accumulation of at least 1000 laser shots obtained from ten different regions of the same sample spot. These data were analysed with the Flex Analysis software (Version 3.4). Prior to analysis, the mass spectrometer was externally calibrated with a peptide mix of bombesin, angiotensin I, glu-fibrinopeptide B, adrenocorticotropic hormone (ACTH) (18-39), ubiquitin, and cytochrome c. Spectra with peaks outside the allowed average were not considered. Modified spectra were loaded into the MALDI BioTyper™ 3.1 Version (Bruker Daltonik GmbH, Bremen, Germany). Pictures of sample-matrix crystals were taken by built-in camera.

2.6. Spectrophotometric Measurements

Spectrophotometric measurements were carried out using an automated chemical analyzer BS 400 (Mindray, Shenzhen, China) under conditions described in previous publication (Gumulec et al., 2013). The transfer of samples and reagents into cuvettes was performed by a robotic arm equipped with a dosing needle. The cuvette contents were mixed by an automatic mixer. Determination of alaninaminotranspherase (ALT), aspartateaminotranspherase (AST) and alkaline phosphatase (ALP) activity was carried out according to the kit manufacturer's instruction (Greiner, Frickenhausen, Germany) as it is specified in previous publication (Sochor et al., 2012).

2.7. Determination of γ-glutamyltransferase (GMT) activity

150 μl of solution R1 (150 mM Glycylglycine) was pipetted into a plastic cuvette with subsequent addition of 7.5 μl of a sample. This solution was incubated for 270 seconds. Subsequently, 30 μl of solution R2 (6 mM L-Gamma-glutamyl-3-carboxy-4-nitroanilide) was added and the solution was incubated for 90 seconds. The absorbance measurement lasted for 180 seconds. The mean increase of absorbance per minute was calculated.

2.8. Determination of antioxidant activity by the FRAP method

The FRAP method (Ferric Reducing Antioxidant Power) is based on the reduction of complexes of 2,4,6-tripyridyl-*s*-triazine (TPTZ) with ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), which are almost colourless, and eventually slightly brownish. This chemical forms blue ferrous complexes after its reduction. Reagent preparation and analysis conducted by (Sochor et al., 2010). A 150 μl of reagent was pipetted into a plastic cuvette with subsequent addition of 30 μl of a sample. Absorbance was measured at 605 nm for 12 minutes. For the calculation of antioxidant activity, the values before decrease of absorbance (2^{nd} minute of measurement–A_2) and the last one (12^{th} minute of measurement–A_{12}) were used. Resulting value was calculated in accordance with following formula: Differential absorbance $A = A_{12} - A_2$. The calibration curve using gallic acid was prepared as described in previous paper (Sochor et al., 2010). Antioxidant activity was expressed as gallic acid equivalents (GAE).

2.9. Determination of Metallothionein Amount

The pellet of 1 ml of *S. aureus* incubated for 24 hours at 37 °C in LB medium was resuspended in 1 ml of phosphate buffer (pH 7), after that the sample was denaturated in thermoblock at 99 °C for 20 minutes and centrifuged for 20 minutes at 25 000 rpm. Supernatant from the sample was used for electrochemical detection.

Electrochemical detection was carried out using the method of differential pulse voltammetry (Raspor et al., 2001; Sevcikova et al., 2013) (three electrodes consisted of working mercury drop electrode (HMDE), reference silver-chloride electrode Ag/AgCl/3 M KCl and auxiliary carbon electrode). The analysed samples were deoxidized by argon for 120 seconds. As a supporting electrolyte, Brdicka solution, which contained 1 mM $[Co(NH_3)_6]Cl_3$ and 1 M ammonium buffer ($NH_3(aq) + NH_4Cl$, pH 9.6), was used as a supporting electrolyte. This electrolyte was changed after each analysis of sample. The parameters for measurements were following: initial potential -0.7 V, final potential -1.75 V, the time interval 0.2 s, step potential 2 mV, amplitude -250 mV. For the calibration curve of

metallothionein was used Metallothionein 95%, cat.no. MT_95_P_1 mg (IZKUS PROTEOMICS) in the concentration range 0.156 - 50 μM.

2.10. Isolation of DNA, Polymerase Chain Reaction and Restriction Fragment Length Polymorphism

2 ml of *S. aureus* culture was centrifuged at 5000 x g and 20 °C for 10 minutes. 400 μl of lysis solution (6 M guanidine hydrochloride + 0.1 M sodium acetate) was added to the pellet and the lysis was carried out at 20 °C and 600 rpm for 1 hour. Isolation of genomic DNA was performed using MagNA Pure Compact (Roche, Germany), Nucleic Acid Isolation Kit I, and protocol DNA Bacteria according to the manufacturer's instructions (Roche, Germany).

The *16S* gene was amplified using polymerase chain reaction (PCR) according to (Stomeo et al., 2013). The sequences of forward and reverse primers were 5′-GAGTTTGATCCTGGCTCAG-3′ and 5′-GGTTACCTTGTTACGACTT-3′ respectively. The volume of PCR reaction mixture was 100 μl containing 1× *Taq* reaction buffer (New England Biolabs, Ipswich, Great Britain), 0.2 mM dNTP, 0.5 mM each primer from Sigma-Aldrich (St. Louis, MO, USA) and 1.6 units of *Taq* DNA polymerase (New England Biolabs, Ipswich, Great Britain). The reaction profile was as follows: initial denaturation at 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min and a final extension for 7 min. The amplification generated a 1500 bp fragment. Restriction fragment length polymorphism (RFLP) was conducted according to (Stomeo et al., 2013) using 1.6 μg of isolated DNA, 10 units of HaeIII enzyme, 1× HaeIII buffer (New England Biolabs, Ipswich, Great Britain) and sterile water to the final volume of 50 μl, digested at 37 °C for 2 hours, with following enzyme inactivation at 80 °C for 20 min.

Agarose gel (2% *v*/*v*, high melt, Mercury, San Diego, CA, USA) was prepared with 1× TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM ethylenediaminetetraacetic acid) and ethidium bromide (5 μl per 100 ml of the gel) according to (Smerkova et al., 2013). 100 bp DNA ladder (New England BioLabs, Ipswich, MA, USA) within the size range from 100 to 1517 bp was used to monitor the size of the analysed fragment. The electrophoresis (Bio-Rad, Hercules, CA, USA) was running at 60 V and 6 °C for 160 minutes. The bands were visualized by UV transilluminator at 312 nm (Vilber-Lourmant, Marne-la-Vallée Cedex 1, France).

GenomeLab DTCS Quick Start kit (Beckman Coulter, USA) with 16 ng of amplified DNA was used for the sequencing reaction according to (Smerkova et al., 2013). The cycling conditions were as follows: 30 cycles of denaturation at 96 °C for 20 s; annealing at 50 °C for 20 s and 60 °C for 4 min. DNA fragments from this reaction were purified using magnetic particles CleanSEQ (Beckman Coulter, USA). DNA sequencing was performed on Genetic Analysis System CEQ 8000 (Beckman Coulter, USA). After denaturation at 90 °C for 2 min, the fluorescence-marked DNA fragments were separated in 33 cm capillary with 75 μm i.d. (Beckman Coulter, USA), which was filled with a linear polyacrylamide denaturing gel (Beckman Coulter, USA). The separation was performed at capillary temperature of 50 °C and voltage of 4.2 kV for 85 min.

2.11. Statistical Analyses

Mathematical analysis of the data and their graphical interpretation were realized by Microsoft Excel®, Microsoft Word® and Microsoft PowerPoint®. Software STATISTICA (data analysis software system) version 10.0 (Tulsa, Oklahoma, USA) was used for data processing. Half-maximal concentrations (IC_{50}) were calculated from logarithmic regression of sigmoidal dose-response curve.

3. Results

3.1. Growth characteristics

Heavy metal-resistant strains of *S. aureus* were prepared by prolonged cultivation in the increasing concentration of heavy metals (for further details see Experimental Procedures - Preparation of Resistant Strains of *S. aureus*). To characterize the resistant strains, the determination of minimal inhibition concentrations was performed by method of growth curves (Fig. 1A) (Bajpai et al., 2013). Data obtained from this method are transferred to the line graph and interleaved with the growth curve of *S. aureus* without any additives, for more illustrative evaluation of antimicrobial activity (Muller et al., 2013).

Results of microbiological determination showed differences in individual resistant strains after addition of the metal with the control strain of *S. aureus* (blue curve). The most resistant and therefore the least reacting to the addition of metal were the cadmium (RCd) and zinc (RZn) resistant strain. MIC (minimum inhibitory concentration) was determined in all resistant strains after the addition of 10 μM concentration of metal ions, but with different intensity. In the silver (RAg) and lead (RPb) resistant strains, the decrease (43%) of growth (absorbance values) was observed after the application of 10 μM metal ions after 24 hours of measurement (Figs. 1Aa-e, respectively). In copper resistant strain (RCu), the absorbance was decreased to 29% (Fig. 1Ab) and in RCd and RZn only by 14% (Figs. 1Ac and 1Ad, respectively). The relative standard deviation of growth curves determination was found to vary from 5.5 to 8.1% (calculated from three independent measurements). The total inhibition concentration after the addition of silver ions in concentrations of 150, 225 and 300 μM was achieved only in RAg strain (Fig. 1Aa).

These results were also supported by statistical evaluation, called viability of cells (Figs. 1Ba (silver), 1Bb (copper), 1Bc (cadmium), 1Bd (zinc) and 1Be (lead)), its values can be statistically calculated to the IC_{50} (the half maximal inhibitory concentration) value (Fig. 1 C). The IC_{50} showed the highest concentrations for inhibition of the growth of bacterial culture in the RZn strain. IC_{50} values were determined at 6, 12, 18 and 24 hours of measurement (Fig. 1C).

3.2. Spectrometric assays

In our study, ferric reducing ability power (FRAP) assay was used for the assessment of oxidative stress in non-resistant and resistant *S. aureus* (Fig. 2). Besides the oxidative stress, activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transferase (GMT) and alkaline phosphatase (ALP) were determined too (Fig. 2). The obtained results were subtracted from those measured in the case of control strain and were divided according to applied metal ion as silver (Fig. 2A), copper (Fig. 2B), lead (Fig. 2C), zinc (Fig. 2D) and cadmium (Fig. 2E).

3.3. Metabolic changes revealed by MALDI-TOF MS

The MALDI-TOF mass spectra of heavy metal resistant *S. aureus* strains are shown in Fig. 3. Moreover; there are shown the pictures corresponding to sample-matrix crystals to illustrate that crystals were formed randomly in relatively homogenous layer. The obtained spectra were analysed in Biotyper™ 3.1 Version software, which identified the samples within the genus *Staphylococcus*, but in different species compared to the control (without addition of metal). The control strain *S. aureus* was identified as a *S. aureus* ssp. *aureus* DSM 4910 (Fig. 3A), *S. aureus* with addition of 950 μM of Cu^{2+} as a *Staphylococcus saprophyticus* ssp. *saprophyticus* CCM 2682 (Fig. 3B), *S. aureus* with addition of 950 μM of Zn^{2+} as a *Staphylococcus felis* DSM 7377 T (Fig. 3C), *S. aureus* with addition of

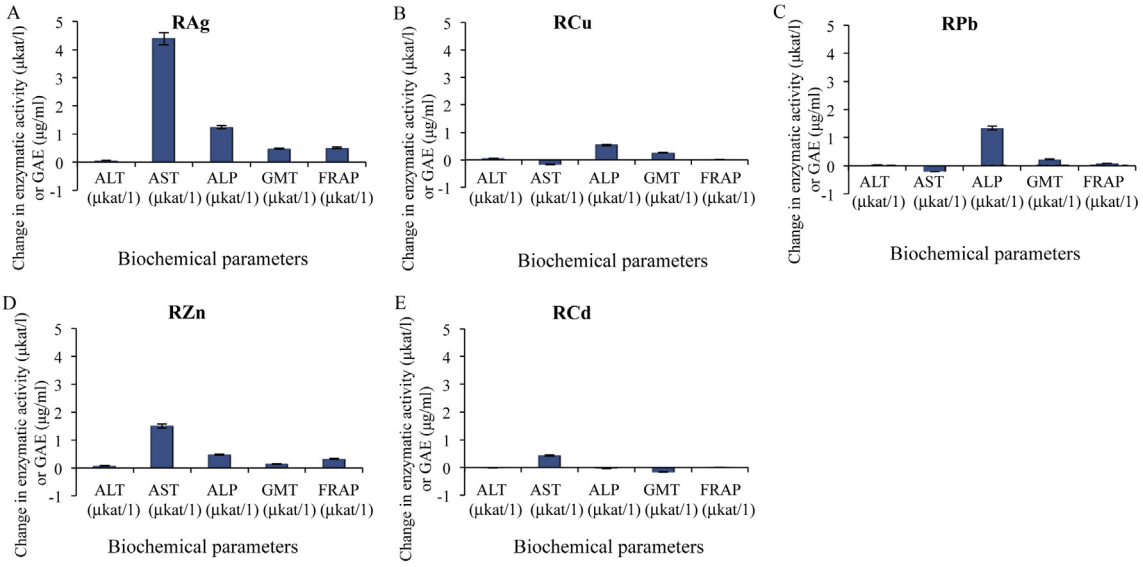


Figure 2. Spectrophotometric analysis of the non-resistant *S. aureus* or *S. aureus* resistant strains to different heavy metals. Determination of enzymatic activity by the ALT, AST, ALP and GMT and antioxidant activity by the FRAP. Results from each analysis were obtained three times and always subtracted from the value of the control measurements non-resistant *S. aureus* (A) *Staphylococcus aureus.* (B) RAg. (C) Resistant strain of *S. aureus* to copper ions. (D) Resistant strain of *S. aureus* to lead ions. (E) Resistant strain of *S. aureus* to zinc ions. (F) Resistant strain of *S. aureus* to Cd.

950 μM of Pb^{2+} as a *Staphylococcus capitis* ssp. *capitis* DSM 20326T DSM (Fig. 3D), *S. aureus* with addition of 950 μM of Cd^{2+} as a *S. aureus* ssp. *aureus* DSM 20491 (Fig. 3E) and *S. aureus* with addition of 350 μM of Ag^{+} a *Staphylococcus condimenti* DSM 11674T DSM (Fig. 3F). It is obvious that the resistant *S. aureus* strains exhibit distinct changes in protein profiles, which can be monitored by MALDI-TOF.

3.4. Metallothionein

Concentrations of thermostable protein metallothionein were higher in heavy metal resistant strains (mainly RCu and RPb) when compared with the control strain (Fig. 4A), whereas voltammograms obtained by the differential pulse voltammetry are shown in Fig. 4B. The high narrow peak (called RS_2Co) detected at potential of -1.25 ± 0.05 V is the specific response of the supporting electrolyte and serves as a control of the proper conditions for catalytic reaction. For evaluation of metallothionein content we determined a height of Cat2 peak (-1.55 ± 0.05 V) as we previously described in our study (Adam et al., 2008).

3.5. Ribosomal RNA

16S ribosomal RNA, conferred by 16S rDNA, is one of the components of small subunit of prokaryotic ribosomes. This sequence is considered as conservative and is used for molecular taxonomy. A significant change in the sequence shows the high level of mutations induced by heavy metals despite increased levels of antioxidants and heavy metals chelators, as it is shown by FRAP and MT analysis. It is interesting that in all cases this is the same mutation as shown by sequence and RFLP. At the same time, after prolonged exposure to heavy metals there were changes in the

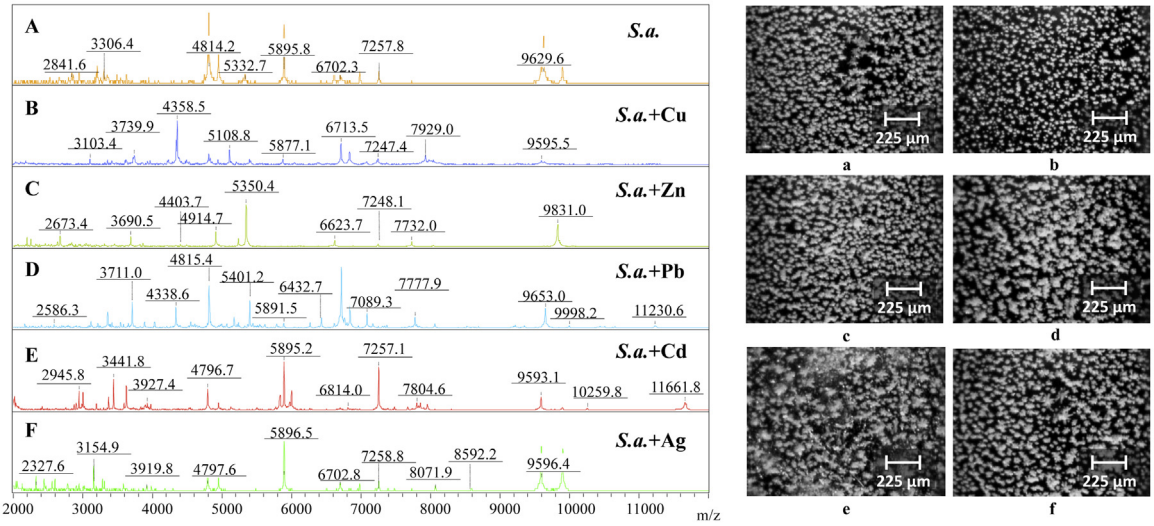


Figure 3. MALDI-TOF mass spectra of protein fingerprints for the identification of non-resistant strains of *S. aureus* and resistant strains of *S. aureus*. Data were collected in the m/z 2000-20000 range after processing 1 ml of *S. aureus*. (**A**) Control strain of *S. aureus* identified. *S. aureus* with addition of (**B**) 950 μM Cu^{2+}. (**C**) 950 μM of Zn^{2+}. (**D**) 950 μM Pb^{2+}. (**E**) 950 μM Cd^{2+}. (**F**) 350 μM Ag^{+}. The results were compared with the library software MALDI BioTyper™ 3.1 Version and were completed with photos of crystals.

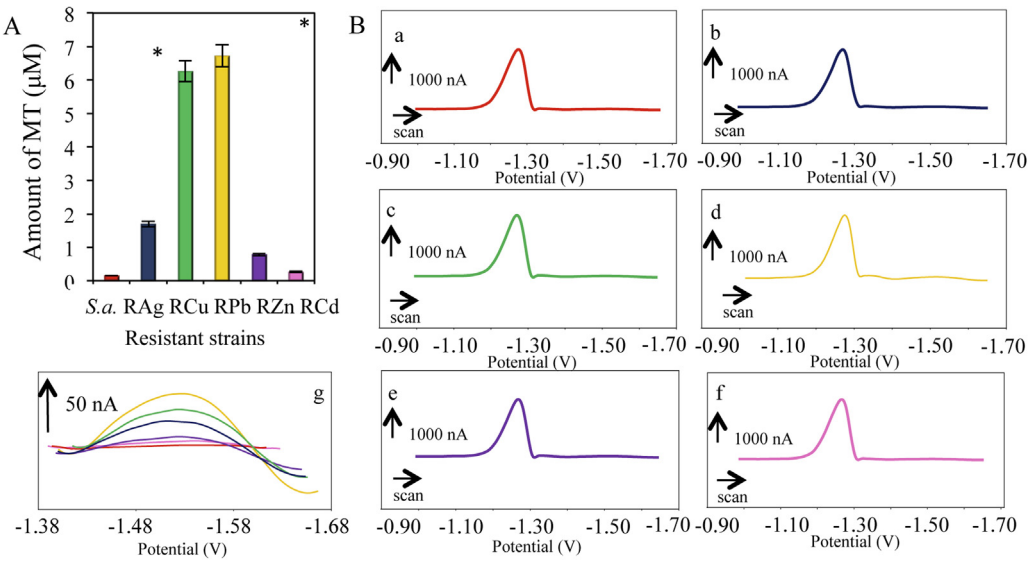


Figure 4. Electrochemical analysis of the non-resistant *S. aureus* or *S. aureus* resistant strains to different heavy metals. (A) Determination of amount of metallothionein (MT). (B) Comparison of DP voltammograms of extracts obtained from various resistant strains and non-resistant one: a) non-resistant strain of *S. aureus*, b) RAg, c) RCu, d) RPb, e) RZn, f) RCd, g) cut out of the range in potentials from -1.38 to -1.68 V, where the significant changes in the peak height were found.

biochemical profile of bacteria and changes were evident even by MALDI-TOF. This shows that the biochemical profile was not changed due to the inhibition or activation of the enzyme by heavy metals, but there was also a significant change in protein expression. For the digestion of this gene, endonuclease HaeIII, originally isolated from *Haemophilus aegypticus*, was used. The restriction site for this endonuclease is 5′-GG'CC-3′ (manufacturer's manual).

Fig. 5A showed the presence of 1500 bp PCR product of 16S rRNA gene amplification both in control and resistant strains. Results of RFLP analysis (Fig. 5B) showed two restriction fragments in control strain of *S. aureus* and additional fragments in all resistant strains compared to control sample. This is a result of the single base substitution in 16S rRNA gene sequence, probably caused by developed resistance. Sequencing data analysed by software BLAST show one restriction site at 326 bp in control strain and the exact lengths of restriction fragments are 326 and 1243 bp. Three transitions occurred in resistant strains at 349-bp the transition from thymine to cytosine, at 992-bp the transition from adenine to guanine and at 1461-bp the transition from thymine to cytosine. These transitions result in forming three additional restriction sites and the lengths of restriction fragments are 326, 22, 644, 469 and 108 bp (Fig. 5C). The 22-bp fragment is not visible on agarose gel due to its small size. These results support the theory that there are changes in genetic information of *S. aureus* resistant to metals.

4. Discussion

4.1. Metal detoxification mechanisms

Different types of organisms are able to intake contaminants limiting their adverse effects due to various types of detoxification mechanisms. Into these types we can also include bacterial strains. Various microorganisms have successfully adapted to the presence of several metals by use of chromosome, plasmid, or transposon-encoded, metal-resistance mechanisms. Most of these resistance mechanisms are plasmid-mediated and are highly specific to a particular cation or anion (Bruins et al., 2000). Inside the cell, metals may have various effects, according to their concentration and chemical properties (Bruins et al., 2000). When concentration levels are too elevated bacteria usually react by the expression of specific metal resistance systems such as P-type ATPases, metallothioneins, RND efflux pumps and/or CDF transporters (Nies, 2003). The resistance genes are located on chromosomes, plasmids or transposons and can therefore be transferred very efficiently to other community members (Bruins et al., 2000; Sorensen et al., 2005). An observation of an increased level of a specific metal resistance system in bacterial cell is good for monitoring of bioavailability of heavy metals from environment (Roosa et al., 2014).

From these facts, it is clear that the resistance can not only formed but also transferred inside the bacterial community. The emergence of resistance based on changes in morphology and biochemical properties can be observed using a number of basic microbiological and spectrophotometric methods. Spectrophotometry-based monitoring of the bacterial growth is suitable for studying of bacterial cultures influenced by various agents (Turner et al., 2012). Therefore, determination of growth dependences ("growth curves") (Rufian-Henares and Morales, 2008; Borneman et al., 2009; Fernandez-Saiz et al., 2010), where the 50% inhibitory concentration induced inhibition of growth - IC_{50} (Wong and Lee, 2014), was used. Our results show the highest resistance (Fig. 1A) and viability (Fig. 1B) was found at *S. aureus* strains treated with Cd^{2+} and Zn^{2+}. Resistance to Cd^{2+} is probably caused by the efflux pumps, the most prominent metal system (Bruins et al., 2000). *S. aureus* carried out cadmium resistance through efflux mechanism consisted from the P-type ATPase transport system (Nies and Silver, 1995). $Zn^{2+}/Cd^{2+}/Pb^{2+}$-translocating ATPase in *S. aureus (cadA)* is determined with location on plasmid, pI258 (Nies and Silver, 1995; Naik et al., 2013). The mechanism for zinc resistance utilizes on the one hand the same CadA system as for Cd^{2+} efflux and on the other hand zinc resistance is based on numerous other processes. One of them is the ability of *S. aureus* to build an effective shield on the bacteria surface thereby to damage antibacterial action of ZnO (Ann et al., 2014). Next is Zn^{2+} transportation by CorA system, which was found in many bacteria and archaea. Another potential chemiosmotically driven mechanism is by the MgtE proteins (Smith et al., 1995), that also probably transports Zn^{2+}. Next transport mechanism is MgtA, a P-type ATPase (Townsend et al., 1995). MgtA is regulated by magnesium starvation (Tao et al., 1995) and zinc may interfere with this process. However, this ATPase is not high-specific for zinc. In *Streptococcus pneumonia* (Dintilhac et al., 1997) and *gordonii* (Kolenbrander et al., 1998) ABC transporter was found to take place in metal resistance.

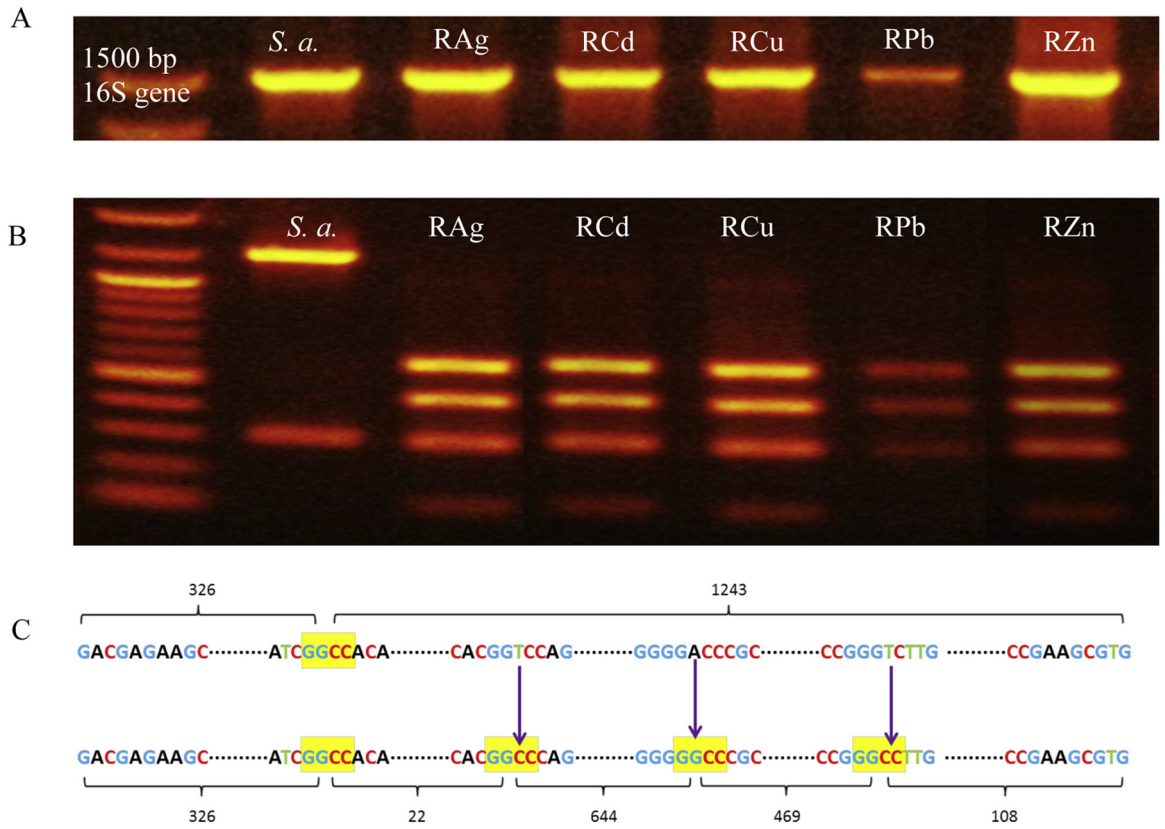


Figure 5. (A) Amplification of 16S rRNA gene in *S. aureus* resistant to 950 μM of different metals. Samples were as follows: control strain of *S. aureus*, RAg, RCd, RCu, RPb and RZn. (B) Amplification of 16S rRNA gene in *S. aureus* resistant to 950 μM of different metals using RFLP *via* HaeIII. Samples were as follows: control strain of *S. aureus*, RAg, RCd, RCu, RPb and RZn. (C) Genetic changes demonstrated by RFLP *via* HaeIII in control strain of *S. aureus* and generally in resistant strains.

These synergic facts, including fact that zinc has great biological importance and is present in all organisms, could explain high viability and resistance (Fig. 1Bd, 1Ad) of bacterial strain treated with Zn^{2+}.

Moreover, *S. aureus* shows lower tolerance to lead (Fig. 1Ae, 1Be) presumably caused by the same gene *cadA* like the resistance to cadmium. This gene encodes multipurpose P-type ATPase (Bruins et al., 2000; Blaszak and Bienkowska, 2009). In addition to membrane transport pumps intra- and extracellular binding of lead ions evade toxicity of free lead ions by precipitating lead as a phosphate salt (Levinson et al., 1996). The lowest resistance and viability was detected for *S. aureus* treated with Ag^{+} and Cu^{2+} (Fig. 1Aa, 1Ba, 1Ab, 1Bb). Copper is probably very toxic because of easy interaction with radicals. Its radical character makes copper very toxic (Nies, 1999). The explanation of toxicity mechanism could be based on the experiment on gram-positive bacterium strain *Enterococcus hirae* (Odermatt et al., 1993; Odermatt et al., 1994). *E. hirae* contains a *cop* operon with two structural genes that encode P-type ATPase. It is assumed that CopA is responsible for copper uptake and copper nutrition, CopB is responsible for copper efflux and detoxification. Both operons can probably transport copper as well as silver. *Gupta et al.* demonstrated that resistance of silver is encoded and is determined by plasmid pMG101 (Gupta et al., 1999). Silver also can be removed from bacteria through metabolic efflux (Schreurs and Rosenberg, 1982) and its toxic effect is caused by interfering with DNA. All results show that studied metals could be divided to four groups with different resistant mechanism, (i) zinc like biogenic element receiving help of blocking membranes, (ii) cadmium, lead and copper are mainly detoxified by ATPases that are unspecific and bind metals according to affinity of individual structures, (iii) copper has Cad and Cop transporters (this fact is probable reason of different viability and resistance as a comparison with cadmium and lead), (iv) silver, which is probably mainly influenced by the efflux mechanism.

4.2. Oxidative stress

Spectrophotometric methods like FRAP, determination of antioxidant enzymes like ALT, AST, ALP or GMT are the most common methods for determination of the oxidative stress in cells and whole tissues extracts (He et al., 2013; Kaiser et al., 2013). Spectrophotometric methods are necessary for determination of antioxidant capacity and oxidative stress in bacterial lysates (Barriere et al., 2001; Bir et al., 2012).

The most synthesized enzymes are AST, ALP and GMT (Figs. 2A - 2E). Only low change in enzymatic activity was observed in the case of ALT. The highest enzymatic activity was found in RAg. The high enzymatic activity was observed also in RZn, RPb and RCu strain. The lowest enzymatic activity was found in RCd strain. On the other hand regarding to antioxidant activity measured by FRAP method RAg and RZn only had the increased antioxidant activity, which was not observed in the case of RPb, RCu and RCd. This phenomenon could be connected with low resistance to Ag^{+} or large amount of Zn^{2+} necessary to form resistance of *S. aureus* strain. Regarding to the antioxidant activity of RCd measured by FRAP, showing a higher antioxidant activity, because level of all measured stressed enzyme is the lowest. Strains RAg, RPb and RZn showed the highest increases in enzymatic or antioxidant activity. An increase in enzymatic or antioxidant activity likely helps to cope better with oxidative stress, which is caused by the presence of heavy metals. Generally it could be suggested a theory that ROS generation by metals is responsible for the strong antibacterial activity. This was observed on *S. aureus* exposed to Ag nanoparticles (Kim et al., 2007).

4.3. Protein profile

Modifications at the protein level, thus continuing changes in metabolism in response to the effect of heavy metal ions onto a cell, can be monitored using MALDI-TOF MS. The changes in protein profiles were investigated using mass spectrometry method for its ability to achieve high sensitivity, fast analysis and precise results (Elased et al., 2005; Elased et al., 2006; Ahsan et al., 2009; Song et al., 2013). MALDI-TOF MS-based identification was shown to be a fast and accurate technology in the identification of a variety of *S. aureus* strains (Szabados et al., 2010; van Veen et al., 2010; Bohme et al., 2012; Charyulu et al., 2012). MALDI spectra of *S. aureus* and metal resistance strains are shown in Fig. 3A. *S. aureus* has four typical peaks (4814, 5896, 6702 and 9630), which are also presented in spectra of resistance strains. Other peaks in spectra are probably connected with the changes in protein morphology due to the metal influence. During bacteria identification control sample of *S. aureus* had the value of log(score) higher than 2 only. This value determines high conformity with the reference spectrum in the database. Other samples (metal resistant *S. aureus*) reached log(score) lower than 2 and probably conformity was defined only in the bacteria genus, however we were not able to identify the specific specie. Gopal *et al.* observed changes in MALDI spectra after the exposure to Pt nanoparticles (PtNPs) in gram-negative *Pseudomonas aeruginosa* (Gopal et al., 2013). They studied the bacterial toxicity of different sizes of PtNPs and the results show that PtNPs 1 - 3 nm sized have bacterio-toxic properties while PtNPs higher (4–21 nm) show bacterio-compatible properties. Pictures in the Fig. 3a–f are finger prints of each *S. aureus* strain evaporated on the target for MALDI analysis.

4.4. Metallothionein

Several genetic mechanisms are known in bacteria maintaining intracellular homeostasis of essential metals and regulating resistance against toxic metals. Metal efflux, intracellular sequestration by metal binding proteins, extracellular sequestration by exopolysaccharides, cell surface biosorption by negative groups, bioprecipitation and redox reactions are the resistance mechanisms, which are present in microorganisms to counteract heavy metal stress (Naik et al., 2012). Intracellular sequestration is mostly based on the presence of metallothionein. Metallothionein is an intracellular low molecular mass protein containing the amino acids rich in sulphur (Adam et al., 2005; Vasak, 2005; Krizkova et al., 2007), which is synthesized in the response to the presence of metal ions. Occurrence of metallothionein was observed in bacteria (Coyle et al., 2002; Henkel and Krebs, 2004; Vasak, 2005), plants (Coyle et al., 2002; Henkel and Krebs, 2004; Vasak, 2005), invertebrates (Ivankovic et al., 2005; Erk et al., 2008) and also in vertebrates (Coyle et al., 2002; Henkel and Krebs, 2004; Vasak, 2005; Dragun et al., 2013).

Metallothionein is involved in many cellular functions, particularly in the transport, storage and detoxification of metals, metabolism of essential metals and uptake of the free radicals (Babula et al., 2010; Krizkova et al., 2012; Ryvolova et al., 2012; Pekarik et al., 2013). Formation of metallothionein-metal complexes protects the organism mainly against acute toxic effects of metals (Waalkes et al., 1984). Therefore, an increased concentration of metallothionein can be important for homeostasis and detoxification of metals to acquire resistance to heavy metal in *S. aureus* strains. Therefore, the ability of *S. aureus* express metallothionein (MT) due to the expose of heavy metals compared to control *S. aureus* was tested (Fig. 4A). Figs. 4Ba-g show voltammograms of resistant strains measured in Brdicka electrolyte. The highest level of MT was detected in RCu > RPb » RAg > RZn > RCd. The explanation is connected with mechanism of metal ability to enter to bacteria (Lebrun et al., 1994) and with different MT affinity to metals (Toriumi et al., 2005). Lebrun *et al.* studied *Listeria monocytogenes*, which is similar to *S. aureus*. *L. monocytogenes* has cadmium resistance gene (*pLm74*) and this gene is like plasmid pI258 in *S. aureus* and prevents accumulation of Cd^{2+} in the bacteria by an ATPase efflux mechanism. Results showed cadmium-inducible synthesis of *L. monocytogenes cadAC* RNA (Lebrun et al., 1994). Moreover, the relationship between metallothionein-3 and metals was observed on the organism *E. coli* and it was indicated as follows Cu > Cd > Zn, same as metallothionein-1 and 2. The affinity of metallothionein-3 to Cu was much higher compared with other isoforms (Toriumi et al., 2005). According to Silver *et al.* the MT synthesis is regulated by gene *smtB* in gram-positive bacteria *Enterococcus hirae* during metal exposure. In addition to rapid response to toxic metals by growth depression, two long-term mechanisms of increasing *smtA* MT gene expression was found. After exposure by metal ions three levels of gene regulation can occur (a) by depression of the SmtB repressor, (b) by amplification of the gene, and (c) by deletion of the repressor gene between fixed chromosomal points (Silver and Phung, 1996).

4.5. Gene expression

The 70S ribosome consists of a small 30S and a larger 50S subunit. 16S ribosomal RNA and 21 proteins form the 30S subunit, while 23S RNA and 34 proteins form the 50S subunit. In order to create the 70S bacterial ribosome both subunits must be present and both are essential for translation, which uses specific activity centres in each subunit (Beach and Champney, 2014). There is a lot of information about ribosomal RNA in relation to antibiotics. Antibiotics targeting the 30S and 50S subunits inhibit both bacterial protein synthesis and subunit assembly, indicating that inhibition of ribosomal subunit assembly may be a synergistic process with translational inhibition (Poehlsgaard and Douthwaite, 2005). Antibiotics as well as heavy metals have a bacteriostatic activity and have had success in treating staphylococcal infections, however, resistance formation has continued to plague advances made in the field (Beach and Champney, 2014).

16S rRNA gene sequence is used for bacterial identification and molecular taxonomy. This sequence is highly conservative within single species and the level of similarity of 16S rRNA gene sequence is used as indicator of the phylogenetic distance within the species (Fig. 5A). The RFLP profile (Fig. 5B) shows formation of three new restriction sites for HaeIII at all resistants. Fig. 5C shows the sequences of *S. aureus* and heavy metal resistant strains with observed mutations. The point mutations formed (Fig. 5C, marked by arrows) are likely to be created by thymine tautomerization as a consequence of direct interaction with heavy metals or adenine oxidative deamination. In our work we show that heavy metal-resistant strains of *S. aureus* differ not only in metabolic and protein profiles, but also in influence of genetic information. Three new restriction sites for HaeIII, minimally three point mutations in 1500 bp long PCR product, indicate a high level of mutations induced by heavy metals despite the increased level of antioxidants and heavy metals chelators, as shown by FRAP, glutathione and MT analysis. Changes both in biochemical and protein profiles of heavy metal-resistant *S. aureus* indicate that the observed changes in biochemical profiles are caused mainly by change in protein expression and minor by heavy metals-caused enzymes inhibition or activation. The obtained data manifest the speed of change of genetic information and therefore protein and metabolic profiles of *S. aureus* even after cultivation in pure culture without the possibility of horizontal gene transfer, which is given by

both high mutagenity of heavy metals and their lethality and high selection pressure.

5. Conclusions

Resistance of bacterial strains to various types of environmental factors, mainly their formation and consequences, are still under investigations of numerous scientists worldwide. Therefore, some new concepts and methodologies are still looked for. In this study, we did an overview of the effects of some metal ions on *S. aureus* with the emphasis on the occurrence of the resistance to the presence of these ions. Our results describe the possible option for the analysis of *S. aureus* resistant strains and may thus serve as a support for monitoring of changes in genetic information caused by the forming of resistance to heavy metals. The analysis was done by resistance curve and viability, enzyme activity, metallothionein (as metal binding protein), protein profile and detection of mutants by RFLP. Resistance and viability results show that studied metals could be divided to three groups with different resistant mechanism, (i) zinc, (ii) cadmium, lead and copper and (iii) silver. Changes both in biochemical and protein profiles of heavy metal-resistant *S. aureus* indicate that the observed changes in biochemical profiles are caused mainly by modification in protein expression and minor by heavy metals-caused enzymes inhibition or activation. Changes in protein expression are caused by the great presence of point mutations by heavy metal-resistant *S. aureus*.

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References

Adam V, Baloun J, Fabrik I, Trnkova L, Kizek R. An electrochemical detection of metallothioneins at the zeptomole level in nanolitre volumes. Sensors 2008;8:2293–305.
Adam V, Petrlova J, Potesil D, Zehnalek J, Sures B, Trnkova L, et al. Study of metallothionein modified electrode surface behaviour in the presence of heavy metal ions-biosensor. Electroanalysis 2005;17:1649–57.
Ahsan N, Renaut J, Komatsu S. Recent developments in the application of proteomics to the analysis of plant responses to heavy metals. Proteomics 2009;9:2602–21.
Alm EW, Zimbler D, Callahan E, Plomaritis E. Patterns and persistence of antibiotic resistance in faecal indicator bacteria from freshwater recreational beaches. J Appl Microbiol 2014;117:273–85.
Ann LC, Mahmud S, Bakhori SKM, Sirelkhatim A, Mohamad D, Hasan H, et al. Antibacterial responses of zinc oxide structures against *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*. Ceram Int 2014;40:2993–3001.
Babula P, Kohoutkova V, Opatrilova R, Dankova I, Masarik M, Kizek R. Pharmaceutical importance of zinc and metallothionein in cell signalling. Chim Oggi-Chem Today 2010;28:18–21.
Babula P, Masarik M, Adam V, Eckschlager T, Stiborova M, Trnkova L, et al. Mammalians' metallothioneins and their properties and functions. Metallomics 2012;4:739–50.
Bajpai VK, Sharma A, Baek KH. Antibacterial mode of action of *Cudrania tricuspidata* fruit essential oil, affecting membrane permeability and surface characteristics of food-borne pathogens. Food Control 2013;32:582–90.
Baker J, Sengupta M, Jayaswal RK, Morrissey JA. The *Staphylococcus aureus* CsoR regulates both chromosomal and plasmid-encoded copper resistance mechanisms. Environ Microbiol 2011;13:2495–507.
Baker J, Sitthisak S, Sengupta M, Johnson M, Jayaswal RK, Morrissey JA. Copper Stress Induces a Global Stress Response in Staphylococcus aureus and Represses sae and agr Expression and Biofilm Formation. Appl Environ Microbiol 2010;76:150–60.
Barriere C, Leroy-Setrin S, Talon R. Characterization of catalase and superoxide dismutase in *Staphylococcus carnosus* 833 strain. J Appl Microbiol 2001;91:514–9.
Beach JM, Champney WS. An examination of the inhibitory effects of three antibiotics in combination on ribosome biosynthesis in *Staphylococcus aureus*. Arch Microbiol 2014;196:249–60.
Bir F, Khireddine H, Touati A, Sidane D, Yala S, Oudadesse H. Electrochemical depositions of fluorohydroxyapatite doped by Cu2+, Zn2+, Ag+ on stainless steel substrates. Appl Surf Sci 2012;258:7021–30.
Blaszak M, Bienkowska D. Effect of soil pollution on bacterial resistance to lead ions: an experimental approach. Pol J Ecol 2009;57:555–60.
Bohme K, Morandi S, Cremonesi P, No ICF, Barros-Velazquez J, Castiglioni B, et al. Characterization of *Staphylococcus aureus* strains isolated from Italian dairy products by MALDI-TOF mass fingerprinting. Electrophoresis 2012;33:2355–64.
Borneman DL, Ingham SC, Ane C. Mathematical Approaches To Estimating Lag-Phase Duration and Growth Rate for Predicting Growth of *Salmonella* Serovars, *Escherichia coli* O157:H7, and *Staphylococcus aureus* in Raw Beef, Bratwurst, and Poultry. J Food Prot 2009;72:1190–200.
Bruins MR, Kapil S, Oehme FW. Microbial resistance to metals in the environment. Ecotox Environ Safe 2000;45:198–207.
Charyulu EM, Gnanamani A, Mandal AB. Identification and Discrimination of Methicillin Resistant *Staphylococcus aureus* Strains Isolated from Burn Wound Sites Using PCR and Authentication with MALDI-TOF-MS. Indian J Microbiol 2012;52:337–45.
Chudobova D, Nejdl L, Gumulec J, Krystofova O, Rodrigo MAM, Kynicky J, et al. Complexes of Silver(I) Ions and Silver Phosphate Nanoparticles with Hyaluronic Acid and/or Chitosan as Promising Antimicrobial Agents for Vascular Grafts. Int J Mol Sci 2013;14:13592–614.
Chudobova D, Dostalova S, Blazkova I, Michalek P, Ruttkay-Nedecky B, Sklenar M, et al. Effect of Ampicillin, Streptomycin, Penicillin and Tetracycline on Metal Resistant and Non-Resistant *Staphylococcus aureus*. Int J Environ Res Public Health 2014;11:3233–55.
Coyle P, Philcox JC, Carey LC, Rofe AM. Metallothionein: The multipurpose protein. Cell Mol Life Sci 2002;59:627–47.
Dintilhac A, Alloing G, Granadel C, Claverys JP. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases. Mol Microbiol 1997;25:727–39.
Dragun Z, Marijic VF, Kapetanovic D, Valic D, Smrzlic IV, Krasnici N, et al. Assessment of general condition of fish inhabiting a moderately contaminated aquatic environment. Environ Sci Pollut Res 2013;20:4954–68.
Duffy J, Dumyati G, Bulens S, Namburi S, Gellert A, Fridkin SK, Lessa FC. Community-onset invasive methicillin-resistant *Staphylococcus aureus* infections following hospital discharge. Am J Infect Control 2013;41:782–6.
Eckschlager T, Adam V, Hrabeta J, Figova K, Kizek R. Metallothioneins and cancer. Curr Protein Pept Sci 2009;10:360–75.
Elased KM, Cool DR, Morris M. Novel mass spectrometric methods for evaluation of plasma angiotensin converting enzyme 1 and renin activity. Hypertension 2005;46:953–9.
Elased KM, Cunha TS, Gurley SB, Coffman TM, Morris M. New mass spectrometric assay for angiotensin-converting enzyme 2 activity. Hypertension 2006;47:1010–7.
Erk M, Muyssen BTA, Ghekiere A, Janssen CR. Metallothioneins and cytosolic metals in Neomysis integer exposed to cadmium at different salinities. Mar Environ Res 2008;65:437–44.
Fernandez-Saiz P, Soler C, Lagaron JM, Ocio MJ. Effects of chitosan films on the growth of *Listeria monocytogenes*, *Staphylococcus aureus* and *Salmonella* spp. in laboratory media and in fish soup. Int J Food Microbiol 2010;137:287–94.
Gopal J, Hasan N, Manikandan M, Wu HF. Bacterial toxicity/compatibility of platinum nanospheres, nanocuboids and nanoflowers. Sci Rep 2013;3:1–8.
Gumulec J, Raudenska M, Hlavna M, Stracina T, Sztalmachova M, Tanhauserova V, et al. Determination of oxidative stress and activities of antioxidant enzymes in guinea pigs treated with haloperidol. Exp Ther Med 2013;5:479–84.
Gupta A, Matsui K, Lo JF, Silver S. Molecular basis for resistance to silver cations in Salmonella. Nat Med 1999;5:183–8.
He RJ, Zou BQ, Fang H, Zhang Y, Wu YR. Free Radical Scavenging Activities of beta-Sitosterol Extracted from *Abies yuanbaoshanensis*. Asian J Chem 2013;25:3507–8.
Henkel G, Krebs B. Metallothioneins: Zinc, cadmium, mercury, and copper thiolates and selenolates mimicking protein active site features - Structural aspects and biological implications. Chem Rev 2004;104:801–24.
Ivankovic D, Pavicic J, Erk M, Filipovic-Marijic V, Raspor B. Evaluation of the *Mytilus galloprovincialis* Lam. digestive gland metallothionein as a biomarker in a long-term field study: Seasonal and spatial variability. Mar Pollut Bull 2005;50:1303–13.
Kahankova J, Pantucek R, Goerke C, Ruzickova V, Holochova P, Doskar J. Multilocus PCR typing strategy for differentiation of *Staphylococcus aureus* siphoviruses reflecting their modular genome structure. Environ Microbiol 2010;12:2527–38.
Kaiser A, Kammerer DR, Carle R. Impact of blanching on polyphenol stability and antioxidant capacity of innovative coriander (*Coriandrum sativum* L.) pastes. Food Chem 2013;140:332–9.
Kaur G, Singh TP, Malik RK, Bhardwaj A, De S. Antibacterial efficacy of nisin, pediocin 34 and enterocin FH99 against *L. monocytogenes*, *E. faecium* and *E. faecalis* and bacteriocin cross resistance and antibiotic susceptibility of their bacteriocin resistant variants. J Food Sci Technol-Mysore 2014;51:233–44.
Kim JS, Kuk E, Yu KN, Kim JH, Park SJ, Lee HJ, et al. Antimicrobial effects of silver nanoparticles. Nanomed-Nanotechnol Biol Med 2007;3:95–101.
Klaassen CD, Liu J, Diwan BA. Metallothionein protection of cadmium toxicity. Toxicol Appl Pharmacol 2009;238:215–20.
Kolenbrander PE, Andersen RN, Baker RA, Jenkinson HF. The adhesion-associated sca operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn2+ uptake. J Bacteriol 1998;180:290–5.
Krizkova S, Ryvolova M, Hrabeta J, Adam V, Stiborova M, Eckschlager T, Kizek R. Metallothioneins and zinc in cancer diagnosis and therapy. Drug Metab Rev 2012;44:287–301.

Krizkova S, Adam V, Petrlova J, Zitka O, Stejskal K, Zehnalek J, et al. A suggestion of electrochemical biosensor for study of platinum(II)-DNA interactions. Electroanalysis 2007;19:331–8.
Kumari H, Balasubramanian D, Zincke D, Mathee K. Role of Pseudomonas aeruginosa AmpR on beta-lactam and non-beta-lactam transient cross-resistance upon pre-exposure to subinhibitory concentrations of antibiotics. J Med Microbiol 2014;63:544–55.
Lebrun M, Audurier A, Cossart P. Plasmid-borne cadmium resitance genes in *Listeria-monocytogenes* are similar to Cada and Cadc of *Staphylococcus aureus* and are induced by cadmium. J Bacteriol 1994;176:3040–8.
Leucker TM, Reddy SV, Moffett BK. Methicillin-resistant *Staphylococcus aureus* Induced Reactive Arthritis Treated With Systemic Corticosteroid. Am J Med Sci 2013;346:172–3.
Levinson HS, Mahler I, Blackwelder P, Hood T. Lead resistance and sensitivity in *Staphylococcus aureus*. FEMS Microbiol Lett 1996;145:421–5.
Lim CK, Hassan KA, Penesyan A, Loper JE, Paulsen IT. The effect of zinc limitation on the transcriptome of *Pseudomonas protegens* Pf-5. Environ Microbiol 2013;15:702–15.
Machado A, Bordalo AA. Prevalence of antibiotic resistance in bacteria isolated from drinking well water available in Guinea-Bissau (West Africa). Ecotox Environ Safe 2014;106:188–94.
Muller P, Alber DG, Turnbull L, Schlothauer RC, Carter DA, Whitchurch CB, Harry EJ. Synergism between Medihoney and Rifampicin against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Plos One 2013;8:1–9.
Naik MM, Shamim K, Dubey SK. Biological characterization of lead-resistant bacteria to explore role of bacterial metallothionein in lead resistance. Curr Sci 2012;103:426–9.
Naik MM, Dubey SK, Khanolkar D, D'Costa B. P-type ATPase and MdrL efflux pump-mediated lead and multi-drug resistance in estuarine bacterial isolates. Curr Sci 2013;105:1366–72.
Nedbalcova K, Nechvatalova K, Pokludova L, Bures J, Kucerova Z, Koutecka L, Hera A. Resistance to selected beta-lactam antibiotics. Vet Microbiol 2014;171:328–36.
Nguyen HNK, Van TTH, Nguyen HT, Smooker PM, Shimeta J, Coloe PJ. Molecular characterization of antibiotic resistance in *Pseudomonas* and *Aeromonas* isolates from catfish of the Mekong Delta, Vietnam. Vet Microbiol 2014;171:397–405.
Nies DH. Resistance to cadmium, cobalt, zinc, and nickel in microbes. Plasmid 1992;27:17–28.
Nies DH. Microbial heavy-metal resistance. Appl Microbiol Biotechnol 1999;51:730–50.
Nies DH. Efflux-mediated heavy metal resistance in prokaryotes. FEMS Microbiol Rev 2003;27:313–39.
Nies DH, Silver S. Ion efflux systems involved in bacteria metal resistances. J Indust Microbiol 1995;14:186–99.
Odermatt A, Krapf R, Solioz M. Induction of putative copper ATPases, CopA and CopB, of *Enterococcus-hirae* by Ag+ and Cu2+, and Ag+ extrusion by CopB. Biochem Biophys Res Commun 1994;202:44–8.
Odermatt A, Suter H, Krapf R, Solioz M. Primary structure of 2-P-type ATPases involved in copper homeostasis in *Enterococcus-hirae*. J Biol Chem 1993;268:12775–9.
Ohlsen K, Ternes T, Werner G, Wallner U, Loffler D, Ziebuhr W, et al. Impact of antibiotics on conjugational resistance gene transfer in *Staphylococcus aureus* in sewage. Environ Microbiol 2003;5:711–6.
Pekarik V, Gumulec J, Masarik M, Kizek R, Adam V. Prostate cancer, miRNAs, metallothioneins and resistance to cytostatic drugs. Curr Med Chem 2013;20:534–44.
Pesavento G, Calonico C, Ducci B, Magnanini A, Lo Nostro A. Prevalence and antibiotic resistance of *Enterococcus* spp. isolated from retail cheese, ready-to-eat salads, ham, and raw meat. Food Microbiol 2014;41:1–7.
Poehlsgaard J, Douthwaite S. The bacterial ribosome as a target for antibiotics. Nat Rev Microbiol 2005;3:870–81.
Raspor B, Paic M, Erk M. Analysis of metallothioneins by the modified Brdicka procedure. Talanta 2001;55:109–15.
Rodriguez C, Taminiau B, Avesani V, Van Broeck J, Delmee M, Daube G. Multilocus sequence typing analysis and antibiotic resistance of *Clostridium difficile* strains isolated from retail meat and humans inBelgium. Food Microbiol 2014;42:166–71.
Roosa S, Wattiez R, Prygiel E, Lesven L, Billon G, Gillan DC. Bacterial metal resistance genes and metal bioavailability in contaminated sediments. Environ Pollut 2014;189:143–51.
Rouch DA, Lee BTO, Morby AP. Undestanding cellular-responses to toxic agents - a model for mechanism - choice in bacteria metal resistance. J Indust Microbiol 1995;14:132–41.
Rufian-Henares JA, Morales FJ. Microtiter plate-based assay for screening antimicrobial activity of melanoidins against *E-coli* and *S-aureus*. Food Chem 2008;111:1069–74.
Ryvolova M, Adam V, Kizek R. Analysis of metallothionein by capillary electrophoresis. J Chromatogr A 2012;1226:31–42.
Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R, Kostrzewa M, Geider K. Classification and Identification of Bacteria by Mass Spectrometry and Computational Analysis. Plos One 2008;3:1–10.
Seniya C, Verma SK, Trivedia SS, Verma R, Vijayarti HS, Vyas S. Metal Stress and Antibiotic Susceptibility Profile of Some Bacterial and Fungal Strains. J Pure Appl Microbiol 2012;6:1727–34.
Sevcikova M, Modra H, Kruzikova K, Zitka O, Hynek D, Adam V, et al. Effect of Metals on Metallothionein Content in Fish from Skalka and Zelivka Reservoirs. Int J Electrochem Sci 2013;8:1650–63.
Schreurs WJA, Rosenberg H. Effect of silver ions on transport and retention of phosphate by *Escherichia coli*. J Bacteriol 1982;152:7–13.
Silver S, Ji GG. Newer system for bacterial resistances to toxic heavy-metals. Environ Health Perspect 1994;102:107–13.
Silver S, Phung LT. Bacterial heavy metal resistance: New surprises. Annu Rev Microbiol 1996;50:753–89.
Singh P, Raghukumar C, Parvatkar RR, Mascarenhas-Pereira MBL. Heavy metal tolerance in the psychrotolerant *Cryptococcus* sp isolated from deep-sea sediments of the Central Indian Basin. Yeast 2013;30:93–101.
Smerkova K, Dostalova S, Skutkova H, Ryvolova M, Adam V, Provaznik I, Kizek R. Isolation of Xis Gen Fragment of lambda Phage from Agarose Gel Using Magnetic Particles for Subsequent Enzymatic DNA Sequencing. Chromatographia 2013;76:329–34.
Smith RL, Thompson LJ, Maguire ME. Cloning and characterization of MgTe, a putative new class of Mg^{2+} transporter from *Bacillus-firmus* OF4. J Bacteriol 1995;177:1233–8.
Sochor J, Pohanka M, Ruttkay-Nedecky B, Zitka O, Hynek D, Mares P, et al. Effect of selenium in organic and inorganic form on liver, kidney, brain and muscle of Wistar rats. Cent Eur J Chem 2012;10:1442–51.
Sochor J, Ryvolova M, Krystofova O, Salas P, Hubalek J, Adam V, et al. Fully Automated Spectrometric Protocols for Determination of Antioxidant Activity: Advantages and Disadvantages. Molecules 2010;15:8618–40.
Song YF, Cui J, Zhang HX, Wang GP, Zhao FJ, Shen ZG. Proteomic analysis of copper stress responses in the roots of two rice (*Oryza sativa* L.) varieties differing in Cu tolerance. Plant Soil 2013;366:647–58.
Sorensen SJ, Bailey M, Hansen LH, Kroer N, Wuertz S. Studying plasmid horizontal transfer in situ: A critical review. Nat Rev Microbiol 2005;3:700–10.
Stomeo F, Valverde A, Pointing SB, McKay CP, Warren-Rhodes KA, Tuffin MI, et al. Hypolithic and soil microbial community assembly along an aridity gradient in the Namib Desert. Extremophiles 2013;17:329–37.
Szabados F, Woloszyn J, Richter C, Kaase M, Gatermann S. Identification of molecularly defined *Staphylococcus aureus* strains using matrix-assisted laser desorption/ionization time of flight mass spectrometry and the Biotyper 2.0 database. J Med Microbiol 2010;59:787–90.
Tao T, Snavely MD, Farr SG, Maguire ME. Magnesium transport in *Salmonella-typhimurium* - MgtA encodes a P-type ATPase and is regulated by Mg2+ in a manner similar to that of MgtB P-type ATPase. J Bacteriol 1995;177:2654–62.
Taylor AR. Methicillin-Resistant *Staphylococcus Aureus* Infections. Primary Care 2013;40:637–54.
Templeton DM, Cherian MG. Toxicological significance of metallothionein. Method Enzymol 1991;205:11–24.
Toriumi S, Saito T, Hosokawa T, Takahashi Y, Numata T, Kurasaki M. Metal binding ability of metallothionein-3 expressed in Escherichia coli. Basic Clin Pharmacol Toxicol 2005;96:295–301.
Townsend DE, Esenwine AJ, George J, Bross D, Maguire ME, Smith RL. Cloning of the MgtE Mg^{2+} transporter from *Providencia-stuartii* and the distribution of MgtE in gram-negative and gram-positive bacteria. J Bacteriol 1995;177:5350–4.
Turner PE, Draghi JA, Wilpiszeski R. High-throughput analysis of growth differences among phage strains. J Microbiol Methods 2012;88:117–21.
van Veen SQ, Claas ECJ, Kuijper EJ. High-Throughput Identification of Bacteria and Yeast by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry in Conventional Medical Microbiology Laboratories. J Clin Microbiol 2010;48:900–7.
Vasak M. Advances in metallothionein structure and functions. J Trace Elem Med Biol 2005;19:13–7.
Waalkes MP, Harvey MJ, Klaassen CD. Relative in vitro affinity of hepatic metallothionein for metals. Toxicol Lett 1984;20:33–9.
Wiesemann N, Mohr J, Grosse C, Herzberg M, Hause G, Reith F, Nies DH. Influence of Copper Resistance Determinants on Gold Transformation by *Cupriavidus metallidurans* Strain CH34. J Bacteriol 2013;195:2298–308.
Wong SG, Lee MY. Comparison of the IC50-shift and % inhibition assay to evaluate time-dependent inhibition. Drug Metab Rev 2014;45:115.