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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