THE EFFECT OF HEAVY METAL IONS ON *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Our objective was to determine the effect of heavy metal ions on resistant strains of Gram-positive bacterial strain of *Staphylococcus aureus* using mass spectrometry. The resistant strains of *S. aureus* were prepared using the nitrate solutions of metals (Ag, Cu, Cd, Zn and Pb). MALDI-TOF mass spectrometry was used for observation the changes in the protein composition in the cell wall and also for the determination and identification of the strains using the database MALDI Biotyper. Results obtained from analysis with resistant strains were compared with sensitive control strain of *S. aureus*. We observed alterations in *S. aureus* protein composition pointing at resistance development under influence of heavy metals ions. Our results develop the possible option of analysis of resistant strains and may serve as a support for the monitoring of changes in genetic information in resistant strains.

Key words: *S. aureus*, resistance, heavy metals, mass spectrometry

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INTRODUCTION

S. aureus is a Gram-positive bacterium acting as one of the main pathogens associated with skin infections, soft tissue, wound infections and more serious sequelae such as septicaemia, urinary tract infections, osteomyelitis or endocarditis (Duffy, Dumyati et al. 2013; Leucker, Reddy et al. 2013; Taylor 2013; Zuniga and Nguyen 2013). Seniya *et al.* previously reported that exposure of heavy metal ions triggers oxidative stress significantly contributing to bacterial strains growth inhibition (Seniya, Verma et al. 2012).

The growth inhibition mechanism involves the entrance of heavy metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}, Ag^{+}, etc.) into the metabolic system of the organism. Further it leads to the formation of secondary metabolites, subsequently constituting the compounds toxic to the organism (Lim, Hassan et al. 2013). Most bacterial strains are able to form the resistance against the undesirable effects of heavy metal ions.

Metal resistance is in bacteria mostly plasmid-encoded (Nies 1992). Resistance genes are encoding genetic information, responsible to factors which influence original properties of microorganisms. In the multiple-metal-resistant bacterium *S. aureus*, Cd^{2+} (and probably Zn^{2+}) efflux is catalyzed by the membrane-bound CadA protein, a P-type ATPase, CadC protein is required for full resistance and a CadR protein is hypothesized for regulation of the resistance determinant (Nies 1992).The metals are important cofactors for many enzymes; however, high levels of metals are toxic. Therefore, bacteria must ensure that there are sufficient metal levels for utilization as cofactors but, more importantly, they must limit free intracellular metal levels to prevent toxicity. Baker *et al.*, suggested that *S. aureus* has one major mechanisms for adapting to high levels of environmental copper, through increased oxidative stress resistance (Baker, Sitthisak et al. 2010). Some microorganisms are able to form resistance to the effects of heavy metals by formation of the antioxidant enzyme superoxide dismutase or by reduction of metal ions (e. g. Ag^{3+} to Ag^{2+} and Ag^{0}) (Singh, Raghukumar et al. 2013; Wiesemann, Mohr et al. 2013). Changes of genetic information, biochemical properties or changes in the mass spectra of individual bacterial strains can be an indicator of the resistance.

In this study identification of the bacterial strains and their analysis by mass spectrometry MALDI-TOF/TOF was carried out. Using the identification by mass spectrometry technique has been shown that this method is rapid and precise technology for identification of different strains of *S. aureus* (Jordana-Lluch, Catala et al. 2012; Velstra, van der Burgt et al. 2012; Singh, Raghukumar et al. 2013; Wiesemann, Mohr et al. 2013). MALDI-TOF mass spectrometry is known as a sensitive analytical tool for the characterization of different types of biological substances (Velstra, van der Burgt et al. 2012). This technique is now commonly used for identification of bacteria in clinical samples (Jordana-Lluch, Catala et al. 2012; Lu, Tsai et al. 2012; Kok, Chen et al. 2013) or in issue of searching for the new potentional biomarkers (Ouedraogo, Daumas et al. 2012). MALDI-TOF mass spectrometry was used in this study to classify non-resistant and resistant strains of *S. aureus*. Moreover, it was found that the use of heavy metal ions on the bacterial culture led to the significant changes in the mass spectra of different fragments of proteins (Fig.1).

The aim of our study was to observe the alterations in *S. aureus* bacterial strains proteome developed due to exposure of these strains to the heavy metal ions.

MATERIAL AND METHODS

1. Cultivation of S. aureus

S. aureus (NCTC 8511) was obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. *S. aureus* was inoculated in LB medium for

24 h on a shaker at 40 x g and 37 °C. Bacterial culture was diluted to OD_{600} = 0.1 for the realization of all experiments.

2. Heavy Metals Ions Preparation

Heavy metals used for the preparation of resistant strains of *S. aureus* have always been in the form of nitrates of these metals ($AgNO_3$, CuN_2O_6.3H2O, Pb $(NO_3)_2$, Cd $(NO_3)_2.4H_2O$, $Zn(NO_3)_2.6H_2O$) dissolved in 100 ml MiliQ water and always in 2mM concentration.

3. Preparation of Resistant Strains of S. aureus

Resistant strains of *S. aureus* have been developed in the laboratory that to the bacterial culture *S. aureus* was added 2mM solutions of heavy metals (Ag, Cu, Cd, Zn and Pb). Low resulting concentration of the metal in a medium inoculated with bacterial culture was 50 µM, and then the metal was always increased by the concentration of 50 µM to the maximum possible dose for regeneration of *S.aureus*. Resistant strains were always possible to revitalize using pure medium without addition of metal.

4. Determination of protein MALDI-TOF mass spectra

500 µl (0.1 OD) of culture cultivated overnight was centrifuged at 14,000 × g for 2 min. Supernatant was discarded and the pellet was suspended in 300 µl of deionized water. Then, 900 µl of ethanol was added. After centrifugation at 14,000 × g for 2 min, supernatant was discarded and obtained pellet was air-dried. Then it was 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 Daltonics, Bremen, Germany) and air-dried at a room temperature. Each spot was overlaid with 1 µl of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution. Spectra were measured on MALDI-TOF/TOF Bruker in the m/z range of 2-20 kDa. Spectra 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), ubiquitine, and cytochrome c.

RESULTS AND DISCUSSION

The results obtained by mass spectrophotometry using MALDI-TOF leads to investigate of changes in metabolisms of *S. aureus* influenced by heavy metal ions. Using this method we have observed the changes in protein structure and enzyme activity for its ability to achieve a high sensitivity, fast analysis and precise results (Elased, Cool et al. 2005; Elased, Cunha et al. 2006). This method is influenced by many factors, such as matrix, its structure and the type of MALDI plates (Lee, Masuda et al. 2013). Number of analyzes such as MALDI-TOF, which showed the accuracy of proteomic analyzes, were performed (Ahsan, Renaut et al. 2009; Song, Cui et al. 2013).

In Fig. 1 can be seen identification of the control strain *S.aureus* as a *S. aureus ssp aureus* DSM 4910 (A), *S. aureus* with addition of 950 µM of Cu^{2+} as a *Staphylococcus saprophyticus ssp saprophyticus* CCM 2682 (B), *S. aureus* with addition of 950 µM of Zn^{2+} as a *Staphylococcus felis* DSM 7377T(C), *S. aureus* with addition of 950 µM of Pb^{2+} as a *Staphylococcus capitis ssp capitis* DSM 20326T DSM (D), *S. aureus* with addition of 950 µM of Cd^{2+} as a *S. aureus ssp aureus* DSM 20491(E) and *S. aureus* with addition of 350 µM of Ag^{+} a *Staphylococcus condimenti* DSM 11674T DSM (F). We confirmed that MALDI-TOF analysis of proteome alterations may serve as a tool for identification of *S. aureus* resistance development.

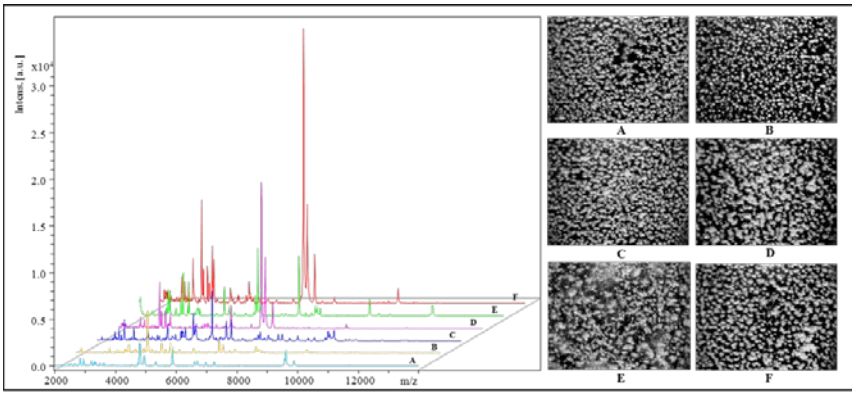

Fig. 1 *MALDI/TOF mass spectra protein fingerprints for the identification of non-resistant S. aureus and resistant strains of S. aureus. Data were collected in the m/z 2000-20000 range after processing 1 ml of S. aureus and the results were compared with a library of software MALDI-TOF.* ***(A)*** *Control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

CONCLUSIONS

In this experiment we have compared the resistant strains of *S. aureus* formed by the action of heavy metal ions (Ag, Cu, Cd, Pb and Zn) with non-resistant culture. Results pointed at the significant changes in biochemical properties between resistant and non-resistant strains of *S. aureus*. The obtained results can be used for understanding the changes closely associated with the formation of resistance under the influence of heavy metal ions. These results may serve as a base for further molecular biologic analyses following up a development of resistance in different bacterial cultures.

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