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Effect of arsenic (III and V) on oxidative stress parameters in resistant and susceptible *Staphylococcus aureus*

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ABSTRACT

The presented study deals with the observation of properties of methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) in the toxic arsenic environment and influence of arsenic on antioxidant capacity. Two forms of arsenic (As(III), As(V)) with different concentrations were used for induction of the oxidative stress in tested strains. Microbiological methods showed that the growth inhibition of MSSA was higher than that of MRSA in presence of both arsenic ions. As(III) showed 24% and 33% higher anti-microbial effects than As(V) against MSSA and MRSA respectively. A similar result was found also in the experiment of reduction of biofilm-formation. By using spectrophotometry, it was revealed that As(III) induced higher antioxidant production in both bacterial cultures. Methicillin-susceptible *S. aureus* produced an app. 50 mg equivalent of gallic acid (GAE/1 mg of protein) and MRSA produced an app. 15 mg of GAE/1 mg of protein. The productions of metallothionein in MSSA and MRSA were decreased up to 62.41% and 55.84% respectively in presence of As ions. Reduction of As(III) and As(V) concentrations leads to a decrease in antioxidant production and increased the formation of metallothionein. All of these changes in the results were found to be significant statistically. Taken together, these experiments proved that in comparison with MSSA, MRSA is less susceptible not only to the antimicrobial effects of antibiotics but also against effects caused by metalloids, as arsenic. Thus, it can be stated that MRSA abounds with complex defensive mechanisms, which may in the future constitute significant problem in the efficiency of antibiotics alternatives as metal ions or nanoparticles.

1. Introduction

Isolates of methicillin-resistant *Staphylococcus aureus* (MRSA), show phenotypic resistance towards the antibiotics namely methicillin, cefoxitin and oxacillin, which belongs to the group of β-lactam antibiotics. MRSA was discovered in England (Shore et al., 2011), and since then MRSA has been declared as a major nosocomial problem. During this time, MRSA is referred to as one of the most important resistant pathogenic bacteria against antibiotics in hospitals and around the world (Lewis et al., 2014). Among bacterial species, Staphylococci have genetic factors causing arsenic resistance (Silver and Phung, 2005). Arsenic is a ubiquitous toxic compound that exists naturally in groundwater, soil, and food, in which the vast majority forms are arsenite As(III) or arsenate As(V) (Silver, 1996). Differences in penetration between As(III) and As(V) into the cells cause a higher or lower toxic effect on bacteria, however, the exact mechanism of arsenic detoxification in humans remains unclear (Phan et al., 2014). Toxicity and mobility of As in the human body proceeds from an inorganic to organic form, As(III) > As(V) > Organo-arsenic (Sattar et al., 2016). According to National Center for Biotechnology Information, the doses of As (III) and As(V) which are toxic to human are 600 and 7200 nmol/L respectively (24570, 2018; 61460, 2018). On the other hand, there are some evidences of As transportation in prokaryotic cells. Arsenic transportation in prokaryotic cells is performed via aquaporins and aquaglyceroporins, integral membrane proteins forming pores for the transportation of water molecules together with small uncharged solutes such as glycerol, CO_2, and urea, in and out of the cell (Rosen and Tamas, 2010). Arsenic generates reactive oxygen species (ROS) in the

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bacterial cell and damage DNA or bring mutations in its structure. It has been found that the virulence and antibiotic resistance genes in MRSA are mostly encoded by mobile genetic elements, including transposons, plasmids, and staphylococcal cassette chromosome (SCC) elements. SCCmec elements comprise of *mec* gene (*mecA/mecC*) which enables the emergence of resistance to methicillin and other β-lactam antibiotics. An extensive genetic diversity of SCCmec elements has been revealed in *S. aureus* and other staphylococci (Garcia-Alvarez et al., 2011; Li et al., 2011; Shore and Coleman, 2013). SCCmec elements comprise regions containing both genes of resistance to antibiotics and metals. An operon, encoding metal resistance gene cluster (*arsC, arsB,* and *arsR*) for the resistance to arsenic, can be found in J1 regions of the SSCmec elements (between the ccr genes and DR-2) (Shore et al., 2011). The occurrence of many similar SCCmec elements in MRSA and coagulase-negative staphylococci strikingly suggests the horizontal transmission of SCCmec (Sabat et al., 2013).

Formation of oxidative stress in bacterial cultures is triggered by numerous factors, one of such factors is exposure to metals (Paez et al., 2013). The oxidative stress begins when the balance between the formation of radicals and their removal by antioxidants is violated. Free radicals are highly reactive particles, formed by normal intracellular metabolism and by external factors like environmental pollutants, ionizing radiations etc. (Dizdaroglu and Jaruga, 2012). The oxidative stress is an inevitable part of the metabolism of aerobic bacteria and is one of the indicators allowing monitoring of general toxic effects of heavy metals and their ions on microorganisms (Ding et al., 2005; Silver, 1996). With the lack of defense, this stress can damage DNA by altering the enzymes responsible for it. This further can lead to mutation, proteolysis and inhibition of protein synthesis (Chudobova et al., 2013; Nafisi et al., 2005; Zhou and Greenberg, 2014). Bacterial protection against the oxidative damage is based on the synthesis of antioxidants and triggering of maintaining mechanisms (Gorokhova, 2011). Superoxide dismutase (SOD) catalyzing the conversion of superoxide radical to H_2O_2 belongs to the well-known antioxidant enzymes (Mishra et al., 2014). The antioxidant parameters can be easily determined by using various spectrophotometric methods (Chudobova et al., 2013). The most commonly used methods are: i) ABTS radical method, ii) DPPH test, and iii) the FRAP method, based on the reduction of the complex with ferric chloride hexahydrate (Thaipong et al., 2006).

Besides these, there are others markers of the oxidative stress as the level of a protein called metallothionein. This protein is an important component which provides protection against the effects of toxic harmful metals and helps to regulate essential metal ions. The first family of bacterial Metallothionein (MT) discovered is the BmtA family and it was known since the 1980s. Another MT which is a Copper-binding gene product MymT, was identified in mycobacteria in 2008. The entire smt operon was initially characterized from the cyanobacterium *Synechococcus* PCC 7942 (the structure has been determined in 2001) containing zinc and cadmium-binding SmtA and it has a Zinc finger fold. Metallothioneins are intracellular, low molecular weight and cysteine-rich protein which is able to bind heavy metals and eliminate their redox properties to protect DNA and other important biomolecules against the oxidative damage (Coyle et al., 2002; Levadoux-Martin et al., 2001; Ruttkay-Nedecky et al., 2013). SmtA homologues are also present in cyanobacteria, gammaproteobacteria, firmicutes, pseudomonads and alphaproteobacteria (Blindauer, 2011). Zinc finger motif is common for the homologue but there is a variety of their identity and metal ligand position. The SmtA is regulated by a repressor known as SmtB and it depends on the presence of the ions concentration (Osman and Cavet, 2010).

The increasing resistance of human pathogenic bacteria against a significant number of antibiotics has been a great concern for the past decades which forces to find the compounds or metal ions which can be the alternative to antibiotics. Metals/metalloids such as arsenic, mercury, silver and copper are used in various forms as antimicrobials with little understanding of their mode of action (Hobman and Crossman, 2015). The purpose of the present experiment was to observe the properties of MSSA and MRSA in the toxic arsenic environment which is important to investigate the correlation between antibiotic and metal resistance. The present research will also tell us if dangerous antibiotic resistant bacteria like MRSA can be controlled by environmentally significant metalloids like arsenic. Two different forms of arsenic (As (III), As(V)) were used for the detailed study. Microbiological parameters, such as bacterial sensitivity or resistance can be evaluated by measuring the zones of inhibition and spectrophotometric determination of growth curves (24 h) (Chudobova et al., 2014b). Moreover, the determination of biofilm formation and real-time cell-based assay (RTCA) xCELLigence were employed (Junka et al., 2012). In this technique, the bacteria must be all the time adhered on the surface of the electrode (Besinis et al., 2014; Dowling et al., 2014). The influence of arsenic on bacterial antioxidant capacity was also studied. For this purpose, the monitoring of bacterial growth, oxidative stress and MT production were performed.

2. Material and methods

2.1. Chemicals

All standards and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) in ACS purity, unless noted otherwise. The microbiological experiments were performed in a laminar flow hood – an aseptic environment. The laminar flow hood was sterilized by UV light and EtOH prior to use (Sanders, 2012).

2.2. Cultivation of methicillin-susceptible Staphylococcus aureus and methicillin-resistant Staphylococcus aureus

In the present experiment the following bacterial strains were used: methicillin-susceptible *S. aureus* (NCTC 8511) and methicillin-resistant *S. aureus* (ST239:SCCmec IIIA). The bacterial strains were purchased from the Czech Collection of Microorganisms, Masaryk University in Brno, Czech Republic. The bacterial cultures were cultivated for growth in Erlenmayer flasks with 15 mL LB medium (LB = Luria Bertani) and were left on a shaker at 37 °C and 130 rpm for 24 h. Then the cultures were diluted with LB medium to optical density 0.5 MacFarland standard (0.1 Absorbance) at 600 nm and were used for subsequent experiments.

2.3. Measurements of the formation of inhibition zones

The inhibition zones were made for testing of the relevant antibacterial activity. The agar surface of Petri dish was covered with a mixture of 100 µl of growing bacterial cultures and 3 mL of LB broth, to get final density 0.5 McFarland standard. The excess volume of the mixture of the Petri dishes was aspirated. The sterile blank discs (diameter 6 mm, Hi-Media, Mumbai, India) were soaked with the solution of As(III) or As(V) at concentration of 1 mM by addition of 10 µl of the solutions (Valgas et al., 2007; Wang et al., 1972; Wright, 1975). In the case of negative control, sterile H_2O was used in place As solutions. Commercial cefoxitin discs (30 µg/disc, Hi-Media Mumbai, India) were used as positive control. The discs with As solutions and control discs were placed onto the same plate contained Mueller Hinton (MH) agar. The 4 mm depth of the agar plates were maintained in all the cases. We put 22.7 mL of the molten sterilized agar in each of the plates (diameter 85 mm) to have the depth 4 mm. The MH agar plates were protected from possible external contamination and placed in a thermostat (Tuttnauer 2450EL, Tuttnauer Ltd., Beit Shemesh, Israel) at 37 °C for 24 h. After 24 h of incubation, the inhibition zones were measured and photographed (Balouiri et al., 2016). The experiment was performed in an aseptic condition using only sterilized or autoclaved materials.