Scientific Papers. Series E. Land Reclamation, Earth Observation & Surveying, Environmental Engineering. Vol. VIII, 2019
Print ISSN 2285-6064, CD-ROM ISSN 2285-6072, Online ISSN 2393-5138, ISSN-L 2285-6064

THE LIMITATION OF ANTIBACTERIAL USE OF SILVER IONS: ACTING AS SELECTIVE AGENTS FOR PROKARYOTE GENETIC RESOURCES

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Abstract

Silver cations are the building blocks of silver based antimicrobial traditional therapies, which are known and used by man for centuries. However, when facing heavy metals, bacterial genetic plasticity can induce the development of adaptive mechanisms required for survival under such major microbe-associated stressors. Whether they act as part of the natural environment, polluted industrial sites or medical antimicrobial protocols, silver ions are systematically exerting a selection pressure on bacterial communities. Thus, sublethal doses of silver ions stimulate inter-bacterial genetic material exchange and the expression of chromosome or plasmid located genes, which encode proteins with biochemical capabilities to counteract their harmful effects. Depending on Gram stain corresponding traits, species and strains, bacteria interact with silver ions at both cellular envelope and internal structure levels. The continued spread of using silver-based materials in the recent years offered opportunities to discover novel antimicrobial strategies but equally induced an increase in bacterial resistance and adaptation against this chemical element.

Key words: *bacteria, gene, Gram stain, silver ions, resistance.*

INTRODUCTION

Silver has been used for centuries as a hygiene catalyst in human society all over the world (Hobman & Crossman, 2014; Randall et al., 2015). Silver ions were the building blocks of traditional antimicrobial employment of silver even before any biochemical trait would have been found to explain their action. The heavy metal ions, as major microbial stressors, gave the opportunity to many bacterial species to develop adaptive mechanisms necessary for their survival in environments containing elements such as Cd^{2+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} or Ag^{+} (Babu et al., 2011). These evolutionary traits could be considered valuable since they constitute a resource for bioremediation in case of the far too many contaminated industrial, urban and health care associated sites occurring nowadays all around the globe.

As would be the case in other antibacterial agents, a major drawback in the extensive use of silver proved to be the increased microbial resistance encountered in various species and strains. The popularity of silver associated materials in human activity induced nowadays a significant environmental pollution (Kedziora et al., 2018).

Silver based compounds are increasingly used in various fields such as clinical healthcare and general hygiene sectors) agriculture and industry (Gupta et al., 2001). The growing popularity of silver antibacterial use in products such as wound dressings, textiles, catheters, or washing machines, gave way to significantly increased chances for selection of adapted bacterial populations than would have been the case several decades ago (Sutterlin et al., 2014). Thus, silver's clinical utility can be compromised in a manner very similar to nowadays occurrence of antibiotic resistance (Randall et al., 2015). Constant bacterial adaptation against antibiotics is still rising in recent years. Thus, in 2007 alone about 25000 patients died in Europe due to antibacterial "therapeutic failure", about two thirds of the cases involving Gram-negative species (Sutterlin et al., 2014). Bacterial enzymes such as extended-spectrum beta-lactamases (ESBLs) have been spread not only in hospitals or human communities but in ecological niches sometimes even lacking human activities (Sutterlin et al., 2014). As a defence against harmful compounds, silver resistance genes were found even to be placed on antibiotics associated plasmids, thus giving the possibility for this chemical element to exert a selective

pressure for antibiotics resistance as well (Sutterlin et al., 2014). The recent years recoderd an increasing number of reports signaling the isolation of silver resistant bacterial strains in silver contaminated environments or healthcare associated locations (Chabert et al., 2017).

In modern and recent times, the progress in life science confirmed the antibacterial role of silver, further spreading its use in activities requiring sterile environments. Sources report silver as being effective against more than 650 pathogens (Salomoni et al., 2017). Both environmental and clinical sources made possible the isolation of silver resistant strains from various species such as: *Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas stutzeri*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii* (Li et al., 1997), *Pseudomonas aeruginosa* (Muller & Merrett, 2014), *Staphylococcus aureus* (Loh et al., 2009; Sobisch et al., 2019), *Bacillus subtilis*, *Bacillus cereus* (Kroeger et al., 2015) or *Enterococcus* sp. (Sobisch et al., 2019).

However, silver remains a major antibacterial actor, including due to its plausible enhancing effect of antibiotics (Barras et al., 2018). In order to preserve its remarkable potential against pathogens a sustained worldwide monitoring strategy should be employed in order to quantify bacterial silver resistance traits and their perspective to spread among microbial communities.

MECHANISMS OF IONIC SILVER ANTIBACTERIAL ACTIVITY

Despite its importance in health care and other scientific or practical domains, the complete spectrum of interactions between ionic silver and bacterial biochemistry is yet to be fully understood (Saulou-Berion et al., 2015). Among the proposed models, most likely the basis of silver action relies on its affinity towards electron-donating groups such as thiols, imidazoles, indoles, amides, carboxylates, and hydroxyls (Saulou-Berion et al., 2015), in a similar manner to other heavy metals such as copper ions, engaged in amino acid binding via imidazole, thiolate or thioether functional groups (Rubino et al., 2011).

Most data suggest that at low concentration, silver ions act at the membrane level, which is the main entrance gate and the place of several fundamental processes such as the respiratory chain and transmembrane transport system (Holt & Bard, 2005). Additionally, at higher concentration silver penetrates further into the bacterial cell acting at cytoplasm level as well (Holt & Bard, 2005). Experimental procedures showed that silver ions enter bacterial cell within 30 minutes after exposure and subsequently bind to intracellular components (Yamanaka et al., 2005; Kedziora et al., 2018). Data gathered by Yamanaka et al. (2005) suggested that at least in *E. coli*, silver toxicity is primarily exerted at cytoplasm level, before the onset of membrane damage. Thus, silver ions were found to pass via ion channels without disrupting the membrane, subsequently inducing ribosome denaturation, with negative effects on proteins engaged in ATP synthesis. Compromising the ATP associated pathways was hypothesized to result in subsequent cellular failure to maintain the membrane structural integrity with the final cellular disruption as a possible outcome.

Under certain Ag+ treatment circumstances, the successful maintenance of cellular metabolism and physiology has been reported, but at the expense of the growth and viability loss, condition that suggested an “active but non-culturable state” under silver stress condition (Kedziora et al., 2018).

Several mechanisms have been identified in case of silver induced antibacterial activity (Saulou-Berion et al., 2015; Kedziora et al., 2018): (1) structural damage in the cell envelope; (2) interactions with intracellular molecules such as proteins and nucleic acids; (3) generation of reactive oxygen species.

EFFECTS OF SILVER IONS ON BACTERIAL CELL ENVELOPE

Bacterial plasma membrane is fundamental to procaryote metabolism as a location of essential reactions (e.g. respiratory chain events, protein transport and secretion, biosynthesis of lipids) (Bondarenko et al., 2018). Under ionic silver treatment, membrane disruption leading to cellular lysis, cytoplasm leakage and separation of plasma membrane from the cell

wall has been observed in both Gram-positive and Gram-negative species (Kedziora et al., 2018). The destabilization of the bacterial phospholipid bilayer is mostly induced via K^+ ion loss and ATP level decrease (Saulou-Berion et al., 2015), the latter being a consequence of the impairment of respiratory chain enzymes, thus decoupling ATP synthesis from respiration. Experimental studies confirmed an increased permeability of the cell envelope structures under silver ion stress (Barras et al., 2018). As revealed by transmission electron microscopy the cell envelope seems to be affected by silver ions via expansion of the periplasmic space, probably due to the detachment of the inner membrane from the peptidoglycan layers (Barras et al., 2018). The thicker cell wall and its negative charge seem to confer to Gram-positive bacteria a passive advantage by blocking and attracting the silver ions, thus acting like a filter and limiting their access into the cytoplasm (Dakal et al., 2016).

In Gram-negative species such as *Vibrio cholerae* changes in the pH gradient occurred on inside-out membrane vesicles under ionic silver treatment, by a presumable proton leakage, induced by changes in membrane proteins or phospholipid bilayer (Barras et al., 2018; Bondarenko et al., 2018). Silver was found to induce the shortening of cell membrane fatty-acyl chains, process that has been correlated with the down-regulation of *fadL* gene, engaged in fatty acid transport and lipid A synthesis/modification-associated *lpxA/arnA* genes (Saulou-Berion et al., 2015).

The increasingly altered secondary structure of proteins in the presence of ionic silver was correlated with *hfg* peptidase and *dnaJ* chaperone upregulation, as well as the regulation of transpeptidase genes *ycfS* and *ycbB* (Saulou-Berion et al., 2015). Both YcfS and YcbB are L, D-transpeptidases which are known in *E. coli* to have the following functions: YcbB forms the peptidoglycan crosslinks and YcfS has the role of anchoring the Braun's lipoprotein to peptidoglycan (Hugonnet et al., 2016). As enzymes engaged in the structural integrity of the cell wall, their regulation under these conditions identify also the cell membrane as being a key target for Ag^+ (Saulou-Berion et al., 2015).

INTERACTIONS BETWEEN IONIC SILVER AND PROTEINS

Whether part of cell envelope or cytoplasm, the proteins, through their specific amino acid sequence are actively engaged in interactions with heavy metals, including silver cations. Ionic silver was found to inhibit several fundamental cellular processes involving proteins, such as cellular respiration and proton motive force (Hobman and Crossman, 2014). At high concentration, the inhibitory activity of silver ions was targeted against proteins by compromising their α-helix conformation (Saulou-Berion et al., 2015). Recent studies found that this would not be always the case, at least in biochemical pathways engaged in silver toxicity control. Thus, silver ions interact positively with proteins engaged in their binding, as starting and consolidating mediators in their function-associated α-helix sequences (Asiani et al., 2016; Chabert et al., 2017).

The Ag^+ interactions with the thiol groups were also found to be often engaged in ionic silver antibacterial toxicity (Kedziora et al., 2018). The sulfhydryl group, as part of cysteine residues, links the silver ions to metalloprotein activity alteration, recent studies confirming silver as acting on the-S cluster containing proteins, which can be members of the respiratory chain, or could belong to the dehydratase group such as would be the case with fumarase A (Barras et al., 2018). The most common Fe-S clusters are mostly coordinated to proteins by cysteine residues (Ayala-Castro et al., 2008). Fumarase A can be reactivated under incubation with Fe^{2+} and thiols (Reaney et al., 1993). As previously mentioned, besides thiol, other electron-donating groups interacting with Ag^+ are carboxylates, hydroxyls, amides, imidazoles, or indoles, all being involved in membrane or cytoplasmic protein-associated reactions as well (Saulou-Berion et al., 2015).

Silver ions were found to have an affinity towards the S2 ribosomal protein, by compromising its structure and inhibiting subsequent protein biosynthesis (Kedziora et al., 2018). Yamanaka et al. (2005) found with high probability a significant decrease in the 30S ribosomal subunit protein S2, together with succinyl-CoA synthetase and MalK

maltose transporter under ionic silver treatment. Both succini-CoA synthetase and MalK are engaged in ATP associated functions, such as synthesis (Phillips et al., 2009) and binding/ transport (Schneider et al., 1995), their reduced titer reflecting an inhibition in ATP activity.

INTERACTION OF IONIC SILVER WITH NUCLEIC ACIDS

Although sometimes mentioned as a major target for silver toxicity (Greulich et al., 2012; Finley et al., 2015), the nucleic acids could play a secondary role in this process. Thus, due to the localization of bacterial genome in the cell core, usually surrounded by proteins, nucleic acids seem not to get often into contact with silver ions as previously considered (McQuillan and Shaw, 2014). Since cytoplasmic proteins interact more frequently with the heavy metal ions, experimental results at sub-inhibitory silver concentrations showed no evidence for significant nucleic acid damage (McQuillan and Shaw, 2014).

When interaction with nucleic acids does occur, silver binds mainly to pyrimidine bases, inducing DNA condensation and subsequent inhibition of replication (Kedziora et al., 2018). Indeed, microscopic studies showed DNA condensation in silver treated bacteria and at high concentration silver ions seem to interact with adenine and guanine, usually having a higher affinity for the latter and generally leading to an increasing risk of pyrimidine dimerization (Barras et al., 2018). Thus, DNA lesions can affect the bacterial chromosome replication (Saulou-Berion et al., 2015) under specific concentrations.

REACTIVE OXYGEN SPECIES (ROS) GENERATION

Silver cations associated increase in ROS is mainly induced by the inhibition of main respiratory chain proteins such as cytochrome b (Kedziora et al., 2018). Accumulation of reactive oxygen species induces itself protein damage or nucleic acid breakage, in addition to the more direct interaction of silver ions with these molecular structures. In silver treated *E. coli*, oxidative stress-related genes, together with metal ion/general stress genes were up-regulated on the expense of generally growth associated heredity units, which were down-regulated under ionic silver challenge (Saulou-Berion et al., 2015).

Experimental data showed that a significant number of silver induced *E. coli* and *Staphylococcus aureus* bacterial deaths in reporter strains expressing a specific response to super oxide radicals, were due to ROS-mediated activity (Park et al., 2009). These studies revealed a major generation of some of the superoxide radicals, but without the induction of H_2O_2. The limiting effect of ionic silver on hydrogen peroxide was also observed in multi-species co-culture experiments engaging pyocyanin synthesized by *Pseudomonas aeruginosa*. Thus, by adding Ag^+ to the medium, microbial induced hydrogen peroxide accumulation has been significantly inhibited (Muller, 2018).

SILVER ASSOCIATED RESISTANCE MECHANISMS

Many bacterial species and strains can withstand against toxic concentrations of heavy metal ions in polluted environments. This resistance is often encoded on plasmid genes, but their location on bacterial chromosome is not uncommon either (Li et al., 1997; Gupta et al., 2001; Randall et al., 2015).

The bacteriostatic or bactericidal effect of silver ions is often species and strain-dependent. Thus, by testing several tens of clinical isolates from several bacterial species, the minimal inhibitory concentrations were found to be around 200-300 μM Ag^+ for most of them excepting one *Enterobacter cloacae* and one *Klebsiella pneumoniae* strain which required significantly higher MIC values, of 5,500 μM Ag^+ (Finley et al., 2015).

In most cases environmental bacterial communities are not represented by single species, which means a constant biological interaction with complex and often unpredictable consequences. This fact constitutes a more elaborate level of dealing with environmental conditions, by enabling sensitive species or strains to benefit from better adapted populations in order to overcome adverse conditions. *Pseudomonas aeruginosa* has the ability to reduce the Ag^+ ion to nontoxic Ag^0

through pyocyanin biosynthesis, a redox-active toxin with broad-spectrum antimicrobial activity (Muller, 2018) via super oxide and hydrogen peroxide synthesis (Hall et al., 2016). Pyocyanin is a metabolite usually engaged in reduction of molecular oxygen and Fe^{3+} ions (Muller and Merrett, 2014) and was also found to be a reductant of Ag^{+} to Ag^{0} form. *E coli* and *S aureus* strains, usually unable to reduce ionic silver were capable to do it in the presence of pyocyanin, if cultured in the presence of this toxin-producing *Pseudomonas aeruginosa* strain. This result was considered to suggest that both oxygen and silver compete as electron acceptors for pyocyanin-associated reactions (Muller, 2018).

Often, bacterial silver resistance requires heavy metal exposure to induce gene expression. Sutterlin et al. (2014) found that none of the confirmed *sil* gene carrier bacteria were showing silver resistance phenotype before the Ag^{+} treatment, but *in vitro* resistance has been easily subsequently induced. By using sub-inhibitory concentrations of silver ions, a selection of silver resistant *E. coli* strains was possible after only 6 days of treatment (Randall et al., 2015; Kedziora et al., 2018).

The antibacterial effect of silver ions can be dependent on cellular or environmental conditions. Thus, silver cations can be neutralized following interactions with proteins such as albumin or by turning them into insoluble precipitates such as AgCl (Kedziora et al., 2018).

Bacteria can deal with ionic silver by reducing it via nanoparticle synthesis. *Bacillus* strain CS11 exposed to $AgNO_3$ at room temperature induced various sized silver particles within 24 hours (Siddiqi et al., 2018). Pyocyanin producing *Pseudomonas aeruginosa* strain neutralized 95% of the silver ions in a matter of minutes by reducing them to Ag^{0} based nanoparticles (Muller & Merrett, 2014).

Mechanisms regarding silver ion uptake into the bacterial cell differ between Gram-positive and Gram-negative species, due to the structural differences in the bacterial envelope (Kezdiora et al., 2018). The relative differences in silver resistance between Gram-positive and Gram-negative species are sustained by contradictory information available in the literature. Thus, according to some authors, *S aureus* still can be regarded as a target of the biocidal effects of silver due to its inner membrane high vulnerability (Randall et al., 2015), while others reveal at least a slightly higher staphylococcal resistance than *E coli* during a comparative study between the two species. The higher resistance of *S. aureus* was hypothesized as being induced by its thicker cell wall structure, at least in case of using silver acetate as ion source (Greulich et al., 2012; Saulou-Berion et al., 2015).

Generally, two mechanisms are identified in bacterial defence against heavy metal challenge, one endogenous and one exogenous (Randall et al., 2015; Kedziora et al., 2018): the endogenous mechanism (via mutations in the own genome) involve features such as the loss of uptake-associated protein functions of the outer membrane porins (ex. OmpF or OmpF/C). In *E. coli*, endogenous resistance against silver was induced by two-point mutations in *ompR* and *cus*S genes which resulted in both loss of OmpC/F porins and depression of the CusCFBA efflux transporter system (Randall et al., 2015). The final effect of both mutations was a reduced intracellular accumulation of silver.

The exogenous mechanism (induced by horizontally acquired genetic material) involves the presence or the increase of plasmid genetic activity such as that related to *sil* operon associated efflux mechanisms, by both periplasmic sequestration and active efflux of the silver ions in the extracellular environment (Randall et al., 2015).

RESISTANCE AGAINST SILVER IONS IN GRAM-NEGATIVE BACTERIA

In Gram-negative bacteria such as *E. coli*, the main gateway for ionic silver uptake are the major outer membrane proteins (OMPs) (McQuillan and Shaw, 2014) such as OmpF porin and OmpC (its homolog) (Kezdiora et al., 2018). OmpF is a trimeric beta-barrel transmembranar protein, each monomer consisting in sixteen hydrogen bonded antiparalel beta strands (Kezdiora et al, 2018). The strands form a cylindrical channel engaged in ion and molecule (such as drugs) transporting activity through the cellular outer membrane (Kedziora et al., 2018). As it has

been previously specified, mutations at porin genes group level can induce silver resistance. Experimental data showed that bacterial strains lacking expression of *ompF* or *ompF/ompC* genes were significantly more resistant against silver treatment (Randall et al., 2015; Kezdiora et al., 2018) by impaired outer membrane permeability. Additionally, the exposure to silver in species such as *E. coli* induced itself the loss of function of the OmpF/OmpC transcription factor OmpR (Kedziora et al, 2018). A reduced expression of *ompF* gene under silver treatment was observed in *Salmonella typhimurium* as well (Kedziora et al., 2018). These results show not only the mechanisms involved in bacterial silver uptake, but also the selective pressure made on the microbial high plasticity in genetic response by this chemical element.

A genotype-dependent response is suggested by similar studies, but showing no differences in culture reaction to silver regardless of the OmpF/OmpC status in the bacterial outer membrane (Kedziora et al., 2018) which could be explained by alternative pathways engaged in overcoming silver toxicity.

The *Salmonella* associated plasmid pMG101 is the most representative example of horizontally acquired silver resistance in Gram-negative bacteria. It contains the 14.2 kb *sil* operon, which gathers 9 ORFs (open reading frames) divided in three transcription units (Silver, 2003; Gupta et al., 2001; Randall et al., 2015), identified as *sil-PGABFCRSE* (Asiani et al., 2016). Out of these, seven seem to be structural genes (*silE*, *C, F, B, A, ORF105(G)* and *silP*) and the other two (*silR* and *silS*) are considered to encode a membrane sensor and responder grouped as a transcriptional regulatory system (Silver, 2003; Asiani et al., 2016).

The SilE protein has been found to have a silver binding role at periplasm level (Silver, 2003; Randall et al., 2015; Asiani et al., 2016) together with SilF protein. The two active efflux systems are the resistance-nodulation-division (RND)-type efflux transporter SilCFBA and the presumed P-type ATPase inner membrane transporter SilP (Silver, 2003; Randall et al., 2015; Asiani et al., 2016). The same genes were identified on at least 5 plasmids belonging to the 19 incompatibility group H (Silver, 2003; Finley et al., 2015). Several species/taxonomic groups have been identified until now to carry *sil* genes, including *Salmonella* sp., *E. coli*, *Enterobacter* sp., *Acinetobacter* sp., *Pseudomonas* sp., *Klebsiella* sp. and methicillin-resistant coagulase-negative staphylococci (Finley et al., 2015).

Close homologues for *silA, B, C, F* and *silR*, *S* from the *Salmonella* plasmid were found to be clustered on the *E. coli* K-12 and O157: H7 chromosomes (Gupta et al., 2001; Silver, 2003). These *E. coli* genome-associated genes constitute the former *agr* group, currently named *cus* (from copper and silver resistance), a 6 gene cluster grouped into CusCFBA and CusR/S systems (Silver, 2003; Holt and Bard, 2005; Randall et al., 2015). The original NCTC 86 *E. coli* strain, described by Theodor Escherich in 1885, has been recently sequenced and found to contain a nine ORF chromosomal 36 kb *sil* locus (ROD36) (Dunne et al., 2017).

The CusCFBA transporter was described first as a copper efflux system and later shown to be involved in silver binding and externalization as well. The *cus* operon, similarly to its *sil* plasmid counterpart includes genes coding for a sensor kinase (CusS), a periplasmic efflux system (CusBCA) and a periplasmic silver binding protein (CusF) (Finley et al., 2015). The amino acid sequence of the two systems has an 80% identity (Finley et al., 2015; Chabert et al., 2017).

SilE has no homologue ORF in the copper eflux pump (Chabert et al., 2017) and according to Gupta et al. (2001), this is an indication of a later addition of the gene to the plasmid system. Interestingly, in a study regarding the screening for 3 *sil* genes in *S. aureus*, *silE* was the only one identified, in a relatively small fraction (6%) of the analyzed strains (Loh et al., 2009), its presence together with its particular feature in Gram-negative bacteria suggesting a special evolutionary and selection pathway of this gene among microorganisms.

SilE protein has a primary sequence of 143 amino acids, including an N-terminal short (20 aa) signal peptide that is cleaved after reaching the periplasm (Chabert et al., 2017). Preious studies involving various experimental conditions reported the SilE protein as being able to bind between 5 and 38 Ag^{+} (Asiani et

al., 2016). Experiments of Asiani et al. (2016) on SilE binding activity showed the protein as shifting between two forms: a disordered protein as apo-SilE, which, upon binding Ag^+ ions, turns into a holo-SilE with a higly α-helical secondary structure. SilE-metal complexes showed a binding efficiency for up to 8 Ag^+ ions but also an affinity for divalent cations, bonded in a lower number of atoms per molecule: Cu^{2+} (up to six ions), Zn^{2+} (up to five) and Ni^{2+} (up to two). The silver binding capacity of the SilE protein confirmed the decisive role of histidine and methionine residues within specific sequence motifs in Ag^+ binding. Single and double mutations made on histidine (to alanine) and methionine (to leucine) confirmed the two amino acid dependent silver binding capacity of SilE and also the silver binding dependence of the holo-SilE structure. Thus, the study of Asiani et al. (2016) suggest that during subsequent binding/accumulation of silver ions, the SilE molecule increase its folding status to a maximum, reached after binding six Ag^+. Additional two ions were found to be possibly attached to the fully folded form of the protein în some cases. An additional indication that the binding and folding processes are linked was that in the case of Cu^{2+}, Zn^{2+} and Ni^{2+} treatments, lower binding yields induced a lower folding degree of the molecule as well. This mechanism of gradual molecular conformation during the cation accumulation process was suggested by the authors as a clear indicator of SilE protein as firstly being dependent on Ag^+ binding before subsequent resitance mechanisms against heavy metal toxicity to be initiated.

Based on SilE amino acid sequence, Chabert et al. (2017) tested the binding activity of the histidine and methionine associated sequences, grouped in nine MX_2H or HX_nM (n = 1, 2) motifs. Their results showed that all of them can coordinate a silver cation, thus suggesting an up to 9 Ag^+ ion binding efficiency by the protein. If these shorter peptides are grouped and looked at as larger motifs, the authors identified two, namely H80-M90 and M108-M121 sequences as essential for the successful silver coordinated α-helix folding.

Besides these resistance gene mediated responses, several other pathways are influenced by the presence of ionic silver in the proximity of bacteria. In *E coli*, 258 genes, representing 5.8% of the genome were differentially expressed under silver nitrate treatment, with a total of 220 up-regulated and 38 down-regulated (McQuillan & Shaw, 2014). A full transcriptome profile made by the authors showed a significant activity of the Heat Shock Response (HSR) gene group under these conditions. Since the main reaction of heat shock proteins against environmental pressure is to facilitate protein folding and to be engaged in protein rescue or degradation of denatured/misfolded polipeptides (Roncarati & Scarlato, 2017), a massive protein degradation/impairment must have been induced by the silver nitrate treatment.

From the Heat Shock Response gene group, several families were strongly up-regulated under silver stress, especially those related to polypeptide binding and stabilization, protein folding, synthesis of proteolytic enzymes (especially those engaged in breakdown of insoluble protein aggregates or denatured polypeptides), heat shock locus proteins and heat stress associated genes. Essential protein folding chaperone genes such as *groL* and *dnaK* were up-regulated by more than 9 and 19 times respectively and *ipaA*, engaged in denatured polypeptide binding and stabilization, had an expression ratio of over 180 (McQuillan & Shaw, 2014). Other gene groups, mostly iron and copper associated, such as Fur regulon, iron-sulphur cluster assembly complex, suplhate transport/ assimilation and copper homeostasis were significantly up-regulated as well. Among the Fur regulon associated genes, generally essential in iron acquisition (Tsolis et al., 1995), positively regulated were those engaged in enterobactin siderophore synthesis, Fe^{3+} associated membrane ligand and uptake, and those associated to ferric citrate and ferrichrome uptake. The sulphate transport and assimilation associated genes included those engaged in cysteine biosynthesis.

RESISTANCE AGAINST SILVER IONS IN GRAM-POSITIVE BACTERIA

As already mentioned, resistance against silver ions in Gram-positive species often takes place differently than in their Gram-negative

counterparts, mainly due to their cell envelope structural distinction (Kedziora et al., 2018; Greulich et al., 2012; Saulou-Berion et al., 2015). The major differences in the bacterial envelope structures between the two groups already suggest differences in influx/efflux mechanisms occurring at silver intake and its subsequent expulsion outside the cell. The lack of outer membrane, the less defined periplasmic space in Gram-positive bacteria, along with the significantly ticker peptidoglycan layer in the cell envelope suggests a more passive way to overcome environmental toxicity. The differences between Gram-positive and Gram-negative species fluctuate from slightly higher resistance (Greulich et al., 2012) to 32 times higher minimal bactericidal concentration for Gram-positive bacteria (Kedziora et al., 2018).

More than one and half decade ago in *Enterococus hirae* genes for both copper and silver resistance were identified coding for either uptake or efflux associated mechanisms (Holt and Bard, 2005). At least one of them proved to be a copper efflux ATPase, found to pump outside the cell Ag^{+} as well (Li et al, 1997). Some data suggest that in Gram-positive species, some of the most common silver ion binding sites seem to be the phosphate groups in teichoic acid and carboxyl groups in the glutamic acid (Kedziora et al., 2018).

The occurrence of *sil* genes seem to be signify-cantly lower in species such as *Staphylococcus aureus*, though being present and expressed in some strains. Thus, studies regarding *S. aureus* revealed no evidence of silver resistance in 876 strains taken from clinical isolates (Randall et al., 2015). Also, unlike in Gram-negative species such as *E coli*, *S. aureus* showed no reduction in silver susceptibility even during extended culture maintenance of 42 days under *in vitro* silver treatment.

On the other hand, studies performed on methicillin resistant *S. aureus* and MR coagulase negative staphylococci isolated from humans and other mammals revealed the presence of *silE* gene in 2 out of 33 tested MRSA strains and in one out of 8 MR-CNS isolates (*Staphylococcus sciuri*) (Loh et al., 2009). These findings show that even bacterial species with statistically low occurrence of *sil* genes can carry resistance against silver treatment via this pathway. Gram-positive species such as *S. aureus* and *Bacillus subtilis* were already shown to possess a periplasmic space between the peptidoglycan layer and the plasma membrane (Zuber et al., 2006). Interestingly, the presence of a gene encoding a periplasm associated protein in a Gram-positive species suggest that additional details could be revealed in the future about the role of this binding molecule in microbes, with or without the engagement of the other members of the *sil* gene group. Extra-chromosomal transfer between Gram-negative and Gram-positive bacteria do occur (Dakal et al., 2016) and, if accurate, the finding of *silE* in *S. aureus* could reveal new aspects regarding the functional features of this protein in bacterial biochemistry.

Usually encoded by the *sil* operon in Gram-negative species, the SilE protein is still considered to have unconfirmed details regarding its exact function of silver resistance excepting its binding activity (Chabert et al., 2017). Unlike the other members of the group *silRS* and *silCFBAGP*, *silE* is a separate transcriptional unit, controlled by its own promoter (Asiani et al., 2016). As mentioned before, SilE has the ability to bind not only silver ions, but copper, zinc and nickel as well. Matias and Beveridge, cited by Zuber et al. (2006) showed that *B. subtillis* and *S. aureus* have a periplasmic space, defined as the inner wall zone, which they suggested as containing low density compounds with high affinity for heavy metal stains, which in fact could constitute a fundamental defense environment against silver ions and other similarly toxic metals. According to some authors (Randall et al., cited by Kedziora et al., 2018) in staphylococci, the most important harmful effect of Ag^{+} ions seems to be located in the inner membrane.

In the literature, extensive studies regarding Gram-negative silver resistance describe a highly specialized capacity to overcome silver biocidal effect. However, experimental data suggest that at least in some cases, Gram-positive species have a higher potential to withstand silver contaminated environments (Kedziora et al., 2018; Sobisch et al., 2019). Among the surviving bacteria on stainless steel control versus silver or silver/ruthenium coated

surfaces on the International Space Station, Gram-positive species belonging to genera such as *Staphylococcus*, *Bacillus* and *Enterococcus* proved to be the most successful (Sobisch et al., 2019).

A *Bacillus cereus* transcriptome analysis made by Babu et al. (2011) showed a strong bacterial genetic response against silver treatment. Thus, high concentration (1 mM) of silver nitrate induced the up-regulation of about 10% (524) out of the 5234 genes in *Bacillus cereus* ATCC 14579. Most of them were related to DNA replication, nutrient transporter, chaperones and membrane protein groups. Genes linked to proteins engaged in carbohydrates, aminoacids, drugs and antibiotics synthesis, transcription, DNA replication, repair and recombination, inorganic ions, cell envelope and membrane synthesis were upgraded under silver stress also. Among the transport and membrane associated processes, mostly efflux and drug resistance and osmoprotectant transporter protein synthesis genes were preferentially activated as well. Other genes, such as those associated with chemotaxis and flagellar proteins were down-regulated by the silver ions, suggesting a significant inhibition of the bacterial motility under silver nitrate treatment.

Gram-positive bacteria such as *Bacillus cereus* have a large number of drug transporter coding genes, which possibly compensate for the lower occurrence of silver associated specific resistance genes and/or plasmids (Kroeger et al., 2015). Thus, the ATCC 14579 strain was found to contain 93 drug transporter annotated genes, representing about 1.7% of its the genome, which is significantly higher than 32 and 37 corresponding genes from *B. subtilis* and *E. coli*, respectively (totalizing 0.8 and 0.9% of their protein associated genes) (Kroeger et al., 2015). The high number of transporters associated genes in *Bacillus cereus* group was explained by its soil associated ecological niche, an environment characterized by a high variability regarding nutrients and sustained exposure to harmful chemicals (Hassan et al., 2017).

CONCLUSIONS

Gram-negative bacteria were found to acquire by horizontal gene transfer or by mutation highly effective genetic traits which enable them to survive an ionic silver treated/contaminated environment. These gene response mechanisms are made via cation binding and sequestration in the periplasm or by expulsion of the toxic metal outside the bacterial cell by efflux pump systems. Generally, Gram-positive species show a higher passive resistance against toxic silver due to their specific cell envelope and are engaged in a significant metabolic and repair, transporter and overall molecular protective systems traceable by transcriptome analysis.

The increased employment of silver in current modern society, visible mostly in industrial, agricultural, general hygiene or healthcare associated sectors induced a major selection pressure on microbial communities. The response is today obvious, species and strains proved to possess the resources to overcome silver toxicity in a manner similar to antibiotics resistance. This process is to be taken into consideration, especially because these genetic traits can be transferred horizontally between species that share the same ecological niche or can be selected by the bacterial remarkably high mutation rate. Last but not least silver associated resistant features are already available without being phenotypically proven until silver stressors act as catalysts to induce subsequent resistance pathways.

ACKNOWLEDGEMENTS

Financial support for this study was granted by the Ministry of National Education (MEN-UEFISCDI), project PN-III-P4-ID-PCE-2016-0637, no. 162/2017.

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