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ORIGINAL RESEARCH

Consequences Of Long-Term Bacteria's Exposure To Silver Nanoformulations With Different PhysicoChemical Properties

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Purpose: Resistance to antibiotics is a major problem of public health. One of the alternative therapies is silver – more and more popular because of nanotechnology development and new possibilities of usage. As a component of colloid, powder, cream, bandages, etc., nanosilver is often recommended to treat the multidrug-resistant pathogens and we can observe its overuse also outside of the clinic where different physicochemical forms of silver nanoformulations (e.g. size, shape, compounds, surface area) are introduced. In this research, we described the consequences of long-term bacteria exposure to silver nanoformulations with different physicochemical properties, including changes in genome and changes of bacterial sensitivity to silver nanoformulations and/or antibiotics. Moreover, the prevalence of exogenous resistance to silver among multidrug-resistant bacteria was determined.

Materials and Methods: Gram-negative and Gram-positive bacteria strains are described as sensitive and multidrug-resistant strains. The sensitivity of the tested bacterial strains to antibiotics was carried out with disc diffusion methods. The sensitivity of bacteria to silver nanoformulations and development of bacterial resistance to silver nanoformulations has been verified via determination of the minimal inhibitory concentrations. The presence of *sil* genes was verified via PCR reaction and DNA electrophoresis. The genomic and phenotypic changes have been verified via genome sequencing and bioinformatics analysis.

Results: Bacteria after long-term exposure to silver nanoformulations may change their sensitivity to silver forms and/or antibiotics, depending on the physicochemical properties of silver nanoformulations, resulting from phenotypic or genetic changes in the bacterial cell. Finally, adaptants and mutants may become more sensitive or resistant to some antibiotics than wild types.

Conclusion: Application of silver nanoformulations in the case of multiple resistance or multidrug-resistant bacterial infection can enhance or decrease their resistance to antibiotics. The usage of nanosilver in a clinic and outside of the clinic should be determined and should be under strong control. Moreover, each silver nanomaterial should be considered as a separate agent with a potential different mode of antibacterial action.

Keywords: silver, antibiotics, resistance, *sil* genes, mutant, adaptant

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Introduction

The overuse of antibiotics has led to the increase of bacterial resistance.[1] Currently, one of the most popular alternatives and often a supporting way, recommended as an antibacterial factor in medicine and in most industrial branches, is silver. Silver, among other metals, possesses biological activity and its high efficacy against a broad range of microorganisms (including fungi, Gram-negative and Gram-positive

bacteria, or even viruses) is well described in the literature.[2–6] Development of nanotechnology gives us new possibilities of silver usage as a new generation of silver-based products, e.g. nanoparticles (AgNPs). It is a kind of structure (with at least one dimension lower than 100 nm) which obtains unique physicochemical and biological properties due to its nanoscale.[7,8] Nanotechnological modifications of silver are usually connected with the transfer of silver ions (Ag^+) to metallic silver (Ag^0). Despite the production of silver-based medical products (orthopedic implants, bandages, biomedical tools, or devices), the potential of antibacterial nanosilver is more exploited in industrial branches. Due to the popularity of silver nanoparticles, the commercial market is full of fabrics, clothes, cosmetics, or simple household items with AgNPs.[9–11] The similarities and differences between antibacterial activity of silver ions and silver nanoformulations were reviewed by the authors.[9]

Molecular mechanisms of bacterial resistance to silver ions are well known, especially in Gram-negative bacteria.[12,13] The first described bacterial silver resistance occurred in 1975, in Massachusetts General Hospital. Gram-negative *Salmonella* Typhimurium, which caused the death of several burn ward patients, was the host of the pMG101 plasmid, a 180 kb construct with specific regions involved in bacterial resistance to silver ions.[12,14] This type of resistance was called "exogenous silver resistance" by Randall et al.[13] The sil region covers an operon of nine *sil* genes, gathered in three transcriptional units (*silRS, silE*, and *silCFBAGP*), each controlled by a separate promoter.[13,15] Functions of proteins encoded by *sil* genes were determined after comparison of their structures to homolog components of the CusCFBA and PcoE system with a chromosome-encoded efflux pump involved in the transport of copper and silver ions.[13,16] Despite that, the function of the SilG protein remains unknown. The transcription of *sil* genes is carried out by the SilS/SilR two-component regulatory system, made of membrane histidine kinase and a response regulator.[12,17] Products of *silA* (inner membrane substrate-binding transporter), *silB* (membrane fusion protein), and *silC* (outer membrane protein) genes form a tripartite resistance–nodulation–division (RND) efflux pump, SilCFBA – a structure spanning both membranes, responsible for ejecting silver ions out of the cell (from cytoplasm or periplasmic space).[12,14] The protein encoded by the *SilP* gene is a P-type ATPase located in the inner membrane. It transports all of the toxic Ag^+ from cytoplasm to periplasmic space, where it can be captured and delivered to SilCFBA by periplasmic chaperone – SilE.[15] A similar role is performed by another protein, encoded by the *silF* gene. It stops the uptake of silver ions from the environment, by binding Ag^+ in periplasmic space and passing them to the efflux pump.[14,16] The distribution of *sil* genes in pMG101 plasmid is presented in Figure 1. The presence of pMG101 in bacteria is not a requirement for changing their phenotypes to silver-resistant. Randall et al[13] proved that silver ions exert selective pressure on bacterial cells treated during 6 days with silver ions (Ag^+), causing point mutations in the bacterial (*Escherichia coli*) genome, e.g. in *ompR* or *cusS* genes, leading to the loss of their function. OmpR is a transcription factor of OmpC and OmpF – two outer membrane proteins, porins with a β-barrel structure, responsible for the transport of drugs and cations such as silver ions. CusS is a histidine kinase, a component of the regulatory system of chromosome-encoded efflux pump CusCFBA.[12,17] As described by Randall et al,[13] the examined bacteria became 6 times more resistant to silver ions. Randall et al[13] proved that there were no changes in *Staphylococcus aureus* sensitivity to silver ions after 42 days of exposition to Ag^+. Despite that, the

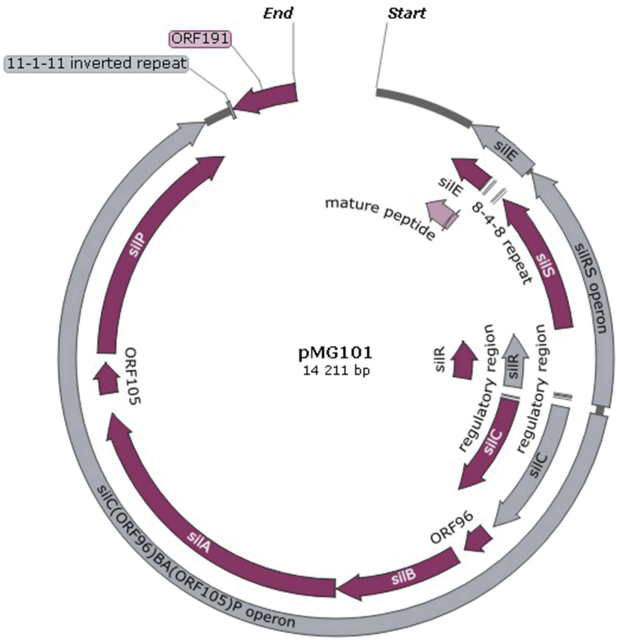


Figure 1 Map view. Distribution of *sil* genes in the plasmid pMG101 (created with SnapGene).

occurrence of a single *sil* gene (*sil*E) in staphylococci is possible but with an unknown function.[18] Besides silver, resistance to mercury, tellurite and a few antibiotics such as streptomycin, chloramphenicol, tetracycline, and ampicillin are encoded on the pMG101 plasmid.[12]

Resistance to antibiotics is a major and growing public health problem. It is estimated that every year about 25,000 patients in Europe die due to infections caused by bacterial strains resistant to antibiotics.[19] The increasing percentage of isolates of bacteria, both Gram-negative and Gram-positive, resistant to many antibiotics is indicated at the same time.[20]

The term "multiple resistance" (MR) refers to microorganisms resistant to more than one antibiotic used in the treatment of infection.[21] Bacteria described as "multidrug resistant" (MDR) are characterized by bacterial resistance to antibiotics that belong to 3 or more different classes.[21] MDR strains are a group of microorganisms that are especially dangerous due to the fact that therapeutic options are limited. Data on the phenomenon of antibiotic resistance pathogens in European countries were included in the European Centre for Disease Prevention and Control (ECDC) report (November 2017) summarizing data for 2013–2016 from 30 European countries.[20] According to the report, the percentage of antibiotic-resistant strains is higher in the countries of southern and south-eastern Europe in comparison to the countries of northern Europe. Over 1/3 of the isolates of *Klebsiella pneumoniae* in Europe are resistant to one therapeutic group, but resistance to 3 or more therapeutic groups was noticed more often. Among the *E. coli* in European countries, a statistically significant increase in resistance was observed in the case of using third-generation cephalosporins, fluoroquinolones, and aminoglycosides. In 2013–2016, the amount of carbapenem-resistant strains of *E. coli* and *K. pneumoniae* increased in several countries. In the years 2013–2016, the European trend of methicillin-resistant *S. aureus* gradually decreased, although in 10 countries the percentage of such strains still remains above 25%. In Poland, since 2013, it has remained at the level of 10–25%. As a result, standard treatments become ineffective, and infections persist and may spread.[19] Bacteria producing carbapenemases (usually belonging to *K. pneumoniae, Pseudomonas aeruginosa*, and *Acinetobacter*spp.) become a significant problem. The treatment of this infection is limited to one or two antibiotics.[22,23] The rapid spread of carbapenemase resistance (CPE) in *Enterobacteriaceae* is one of the most serious problems for the health and safety of patients. Prudent use of silver nanoformulations and widespread observance of the principles of control and prevention of infections in all health care sectors, e.g. hospitals and nursing homes, form the basis for effective interventions to prevent the selection and spread of antibiotic-resistant bacteria.[24,25] Otherwise, Europe may soon be faced with epidemic hospital diseases caused by *Enterobacteriaceae* with extended multiple resistance (extensively drug resistant, XDR), or even strains completely resistant (pandrug resistant, PDR).[26]

The main purpose of this study was to check the sensitivity of bacteria to silver nanoformulations after long-term exposure, to analyze the exogenous and endogenous resistance to silver, and, finally, to compare the antibiotic susceptibility of Gram-positive and Gram-negative bacteria strains (wild types and variants with changed sensitivity after repeated exposure to silver nanoformulations).

Materials And Methods

Bacteria Strains

The wild types (19 strains altogether) and their selected variants (signed in the text as S1 V, S2 V, S7 V strains altogether) of Gram-negative strains (*E. coli, K. pneumoniae, Enterobacter cloacae*) and Gram-positive bacteria strains (*S. aureus*) belonging to MR or MDR bacteria were tested. Bacterial strains (wild type) were purchased from ATCC or provided by Dialab Medical Laboratory. The variants were selected after repeated exposure to silver nanoformulations (S0–S7), described below.

Chemicals

The media (Luria Broth [LB], Mueller Hinton Agar [MHA], Mueller Hinton Broth [MHB]) were purchased from Biocorp. Antibiotics were obtained from Oxoid. The Plasmid Mini Kit, Genomic Mini Kit, and DNA Ladder marker (0.1 µg/mL. 100–1000 bp) were purchased from A&A Biotechnology. Midori Green Advance DNA Stain (Nippon Genetics), Phusion Hot Start II High Fidelity PCR Master Mix, and 50× Tris–acetate EDTA buffer (TAE, pH 8.3) were purchased

from Thermo Scientific. All of the primers were ordered in Genomed. The sequences of all used primers are summarized in Table 1 (based on the information described by Woods et al[27]). Silver nanoformulations S1–S6, described in detail in our previous study,[5,28] were prepared by A. Kedziora. The formulations S0 and S7 were commercially available. All silver nanoformulations are summarized in Table 2.

Long-Term Exposure Of Bacteria To Silver Nanoformulations

This task was performed with long-term exposure in gradual increases of minimal inhibitory concentration (MIC) of silver nanoformulations S0–S7 (0.5 MIC, 1 MIC, and 4 MIC). Initially, the sensitivity of bacteria to silver nanoformulations was determined via MIC measurement.[29] Bacteria strains were stored at −70 °C before each investigation and the standard protocol was performed to check their purity and revitalization. The inoculum was spread on the agar plate (MHA), incubated at 37 °C for 19 h, and after that used in the experiment. In the first step, the stock of silver samples was prepared (at concentrations between 2048 µg/mL and 0.03125 µg/mL, depending on the nanoformulation sample). 100 µL of each concentration was poured to hole. Next, the bacteria strains at the final concentration of $1.5x10^6$ cfu/mL were added and incubated at 37 °C. Minimal inhibitory concentration values were read within 16–19 h. The experiment was repeated 3 times. For 0.5 MIC and 1 MIC, broth (MHB) was used; hence, in the case of 4 MIC, an agar plate (MHA) was applied. The appropriate bacteria density (cfu/mL comparable with MIC determination) was poured into the 0.5 MIC and incubated for 24 h at 37 °C in a final volume of 10 mL. After that time, 0.5 mL of overnight culture was transferred to the new broth (containing 0.5 MIC concentration of the appropriate silver nanoformulations) and incubated under the same condition. Overnight culture of bacteria was transferred to the new broth (with 1 MIC), incubated at 37 °C forr 24 h, and the steps were repeated for 1 MIC as described for 0.5 MIC. The

Table 1 Sequences Of Forward And Reverse Primers

***sil* Genes**	**Primers**	
	Forward	**Reverse**
silE	AGGGGAAACGGTCTGACTTC	ATATCCATGAGCGGGTCAAC
silRS	GGCAATCGCAATCAGATTTT	GTGGAGGATACTGCGAGAGC
silCBA	CGGGAAACGCTGAAAAATTA	GTACGTTCCCAGCACCAGTT
silF	CGATATGAATGCTGCCAGTG	ATTGCCCTGCTGAATAAACG
silB	CAAAGAACAGCGCGTGATTA	GCTCAGACATTGCTGGCATA
silA	CTTGAGCATGCCAACAAGAA	CCTGCCAGTACAGGAACCAT
silP	CCTGGGTTTACAGCGTCATT	ATGGCACCTGAGGTTTGTTC

Note: Data from Woods EJ, Cochrane CA, Percival SL. Prevalence of silver resistance genes in bacteria isolated from human and horse wounds. *Vet Microbiol.* 2009;138:325–329.[27]

Table 2 Silver Nanoformulations, Which The Bacteria Were Treated With

Silver Nanoformulations	**Details**	**References/Origin**
S0	Silver nanoparticle powder without carrier and stabilizers; silver size <100 nm	Commercially available
S1	Silver ions immobilized on amorphous TiO_2 (TiO_2/Ag^+)	28
S2	Silver nanoparticles immobilized on amorphous TiO_2 (TiO_2/Ag^0), silver size 20 nm	28
S3	Silver ions immobilized on crystalline TiO_2 (TiO_{2a}/Ag^+)	28
S4	Silver nanoparticles immobilized on crystalline TiO_2 (TiO_{2a}/Ag^0), silver size ≤10 nm	28
S5	Silver nanoparticles immobilized on amorphous TiO_2 (TiO_2:Ag^0), silver size ≤5 nm	5
S6	Silver nanoparticles immobilized on crystalline TiO_2 (TiO_{2a}:Ag^0), silver size ≤5 nm	5
S7	Aqueous dispersion of silver nanoparticles; stabilizers: trace amount of Tween and polyethyleneimine, silver size 20 nm	Commercially available

Notes: Forms had different physicochemical properties: lack or presence of carrier or stabilizers, carrier size, silver size, surface area, and chemical compounds.

Table 3 MIC Of Different Silver Nanoformulations Against Tested Gram-Negative Bacteria Strains

Silver nanoformulations / Bacteria strains	E. coli J53	E. coli ATCC 11229 wt	E. coli ATCC 11229 S2 V	E. coli ATCC 11229 S7 V	E. coli 475 wt	E. coli 475 S2 V	E. coli 475 S7 V	E. coli 555 wt	E. coli 555 S2 V	E. coli 555 S7 V	E. coli 408 wt	E. coli 408 S2 V	E. coli 408 S7V	E. coli 343 wt	E. coli 343 S7V	K. pneumoniae ATCC 4352 wt	K. pneumoniae ATCC 4352 S1 V	K. pneumoniae ATCC 4352 S2 V	K. pneumoniae ATCC 4352 S7 V	K. pneumoniae 626 wt	K. pneumoniae 626 S1 V	K. pneumoniae 626 S2 V	K. pneumoniae 626 S7 V	K. pneumoniae 268 wt	K. pneumoniae 268 S7 V	E. cloacae 233	E. cloacae 233 S7 V
MIC Ag^+ [µg/mL]	256	4	n/a	n/a	4	n/a	n/a	4	n/a	n/a	8	n/a	n/a	8	n/a	8	n/a	8	n/a	8	n/a	n/a	n/a	n/a	8	n/a	8	n/a
MIC S0[a] [µg/mL]	>2048	256	n/a	n/a	256	n/a	n/a	256	n/a	n/a	256	n/a	n/a	256	n/a	256	n/a	n/a	n/a	256	n/a	n/a	n/a	n/a	256	n/a	512	n/a
MIC S1 [µg/mL]	256	0.5	n/a	n/a	0.5	n/a	n/a	0.5	n/a	n/a	8	n/a	n/a	8	n/a	0.5	0.5	n/a	n/a	0.5	8	n/a	n/a	n/a	16	n/a	8	n/a
MIC S2 [µg/mL]	16	4*	32	n/a	4*	32	n/a	4*	8	n/a	4	16	n/a	16	n/a	4*	2*	64	n/a	64	64	n/a	n/a	n/a	16	n/a	16	n/a
MIC S7 µg/mL]	16	1	n/a	4	1	n/a	4	1	n/a	2	1	n/a	2	2	4	1	1	n/a	n/a	2	n/a	n/a	n/a	64	0.5	4	0.5	4

Notes: V, variants selected after long-term treatment with silver nanoformulations; S1, S2, S7, kind of silver form with physicochemical differences, physicochemical details described by Kędziora et al[5,28] and in Table 2; S0, silver nanoformulation commercially available (Table 2). [C]Positive control of pMG101 plasmid presence. *Converted to concentration of whole compounds in comparison to MIC values published previously[5,28]

Abbreviations: wt, wild type strain; n/a, not applicable.

Table 4 MIC Of Different Silver Nanoformulations Against Tested Gram-Positive Bacteria Strains

Silver nanoformulations / Bacteria strains	*S. aureus* ATCC 6538	*S. aureus* ATCC 6538 S7 V	*S. aureus* 173	*S. aureus* 173 S7 V	*S. aureus* 298	*S. aureus* 298 S7 V	*S. aureus* 187	*S. aureus* 340 MRSA	*S. aureus* 340 MRSA S0 V	*S. aureus* 340 MRSA S7 V	*S. aureus* 2	*S. aureus* 2 MRSA S7 V
MIC Ag^+ [μg/mL]	8	n/a	8	n/a	2	n/a	4	32	32	n/a	32	n/a
MIC S0 [μg/mL]	1600	n/a	1600	n/a	1600	n/a	1600	2048	4096	n/a	2048	n/a
MIC S1 [μg/mL]	0.5	n/a	0.5	n/a	0.5	n/a	0.05	16	n/a	n/a	8	n/a
MIC S2 [μg/mL]	4*	n/a	4*	n/a	4*	n/a	4*	16	16	n/a	16	n/a
MIC S7 [μg/mL]	1	2	1	2	1	2	1	1	n/a	2	1	2

Notes: V, variants selected after long-term treatment with silver nanoformulations; S1, S2, S7, kind of silver form with physicochemical differences, physicochemical details described by Kędziora et al[5,28] and in Table 2; S0, silver nanoformulation commercially available (Table 2). [C]Positive control of pMG101 plasmid presence. *Converted to concentration of whole compounds in comparison to MIC values published previously.[5,28]

Abbreviations: wt, wild type strain; n/a, not applicable.

population that adapted to the increasing concentration of silver nanoformulations grew on the agar plate (4 MIC). The MIC value was then determined again as described above and compared with that before long-term exposure.[30]

Analysis Of Whole Genome Sequences

Whole genome DNA was isolated with the Genomic Mini Kit according to the original instruction from 2 mL of overnight LB cultures. The product purity and concentration were verified with a Nano Photometer (Implen). Genomic libraries were prepared according to the NEBNext DNA Library Prep Master Mix Set for Illumina protocol, and sequencing (NGS) was performed at Genomed (Warsaw, Poland) using the Illumina MiSeq platform. NGS reads were trimmed with Cutadapt 1.9.1.[31] De-novo genome assembly was performed with SPAdes 3.13.0,[32] their quality was evaluated with QUASTt 5.0.2,[33] and resulting contigs were rearranged with progressiveMauve in Mauve 2.4.0[34,35] using the genome of *E. coli* K-12 substr. MG1655 (NC_000913.3) or *K. pneumoniae* subsp. *pneumoniae* HS11286 (NC_016845.1) as a reference. Newly assembled genomes were annotated with prokka 1.12.[36] Genomic mutations in adapted strains were identified and characterized with snippy 4.3.8.[37] The pre-processed reads and assembled genomes have been deposited in the NCBI SRA and Genome databases accordingly under the following accession numbers: SRR9733699, SRR9733700, SRR 9733697, SRR9733698, SRR9733703, SRR9733704, SRR 9733701, SRR9733702, SRR9733705, SRR9733706, SRR9 733707 and VLTC00000000, VLTB00000000, VLTA000 00000, VLSZ00000000, VLSY00000000, VLSX00000 000, VLSW00000000, VLSV00000000, VLSU00000000, VLST00000000, VLSS00000000.

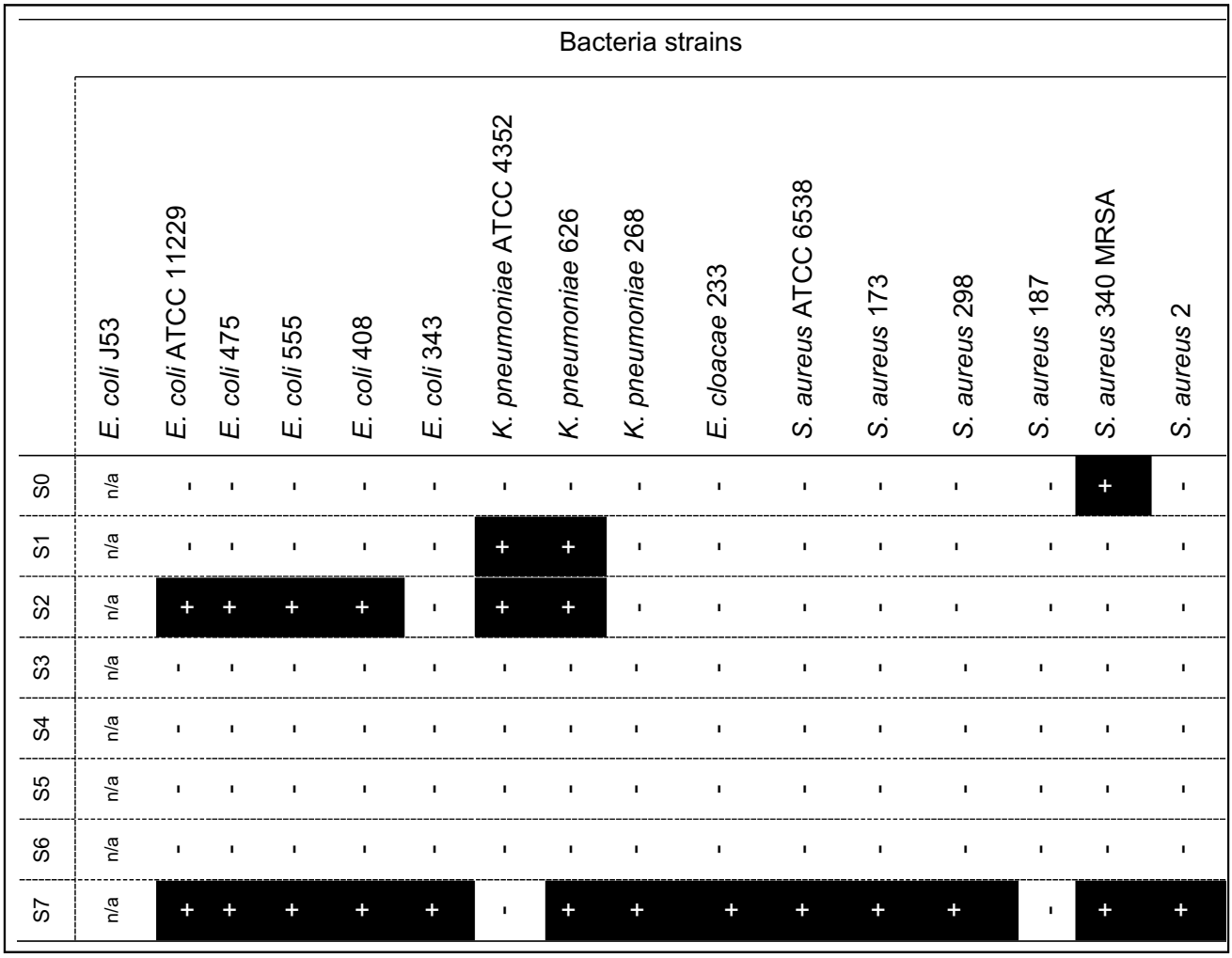
Detection Of *Sil* Genes

To verify the exogenous silver resistance in all tested bacteria strains, the prevalence of *sil* genes located on pMG101 plasmid was determined in the following steps.

Plasmid Isolation

Plasmid pMG101 was isolated with the Plasmid Mini Kit according to the original instruction. The product quantity of this extraction was confirmed with the Nano Photometer (Implen). Plasmid DNA was isolated from all of the tested strains (described above), and the one from the *E. coli* J53 strain was used as a positive

Table 5 The Changes Of Bacterial Sensitivity To Silver Nanoformulations After Their Repeated Exposure



Notes: "+", increases of MIC after 6 days of exposition to appropriate silver nanoformulations; "–", no changes of MIC noticed after 6 days of exposition to appropriate silver nanoformulations, no variants obtained. Variants with changed sensitivity to silver formulations (S0–S7).
Abbreviation: n/a, not applicable.

control. All bacteria strains grew on Luria Broth before DNA extraction.

Primer Preparation

In the first step, lyophilized primers were centrifuged (12,500 rpm, 2 min); next, the required volume of water (MQ) was poured to the final concentration of 100 µM. The primers were vortexed and left for 10 min at room temperature. Then, they were diluted 10 times and stored at −20 °C.

PCR Mixture

The following reagents were mixed for every reaction: 5 µL Phusion Hot Start II High Fidelity PCR Master Mix, 0.5 µL forward primer, 0.5 µL reverse primer, 1 µL plasmid, and the required volume of MQ. The final volume of all reactions was 10 µL. The positive (with plasmid from *E. coli* J53) and negative (sample without any of plasmid) controls were performed.

PCR Conditions

The following steps were established for the best final products of PCR: 30 sec at 98 °C (initial denaturation) followed by 30 cycles of 98 °C for 5 sec (denaturation), 60 °C for 10 sec (annealing), and 72 °C for 12 sec (elongation); and 72 °C for 10 min (final elongation). PCR was carried out on a T100™ Thermal Cycler (BioRad).

DNA Electrophoresis

5 µL of each sample and 1 µL of heavy buffer (30% glycerol stained bromophenol blue) were loaded to a 2% agarose Tris–acetate–EDTA (TAE) gel containing 0.002%

Table 6 Number And Distribution Of Genomic Mutations Acquired By Selected Test Variants (V) Due To Long-Term Exposure For Different Silver Nanoformulations

Silver Nanoformulations	Variants (V)	Genomic Region									Genes With Mutations		
		CDS				RNA			Intergenic			Nonconservative	
		SNP	Del	Ins	Comp	SNP	Del	Ins	Comp	SNP	Del	Ins	Comp	In Total	Number	Examples
S1	*K. pneumoniae* ATCC 4352	20,375	29	26	1663	26	2	2	3	2680	87	88	476	19,008	829	Inc. cusS tamB
S2	*E. coli* ATCC 11229	15,639	5	6	2429	–	–	2	3	1331	34	29	298	16,477	388	Inc. cusS
S1	*K. pneumoniae* 626	2	–	–	–	–	–	–	–	–	–	–	–	2	1	Only cusS
S2	*K. pneumoniae* 626	2	–	–	–	–	–	–	–	2	–	–	3	2	1	Only cusS
S2	*K. pneumoniae* ATCC 4352	1	–	–	–	–	–	–	–	–	–	–	–	1	1	Only tamB
S7	*K. pneumoniae* ATCC 4352	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S7	*E. coli* ATCC 11229	3	–	–	–	–	–	–	–	–	–	–	–	2	–	–
S7	*K. pneumoniae* 626	–	–	–	–	–	–	–	–	2	–	–	3	–	–	–

Notes: 2–53 nt in length, average 4 nt.
Abbreviations: CDS, protein coding sequences; SNP, single nucleotide polymorphism; Del, deletion; Ins, insertion; Comp, complex.

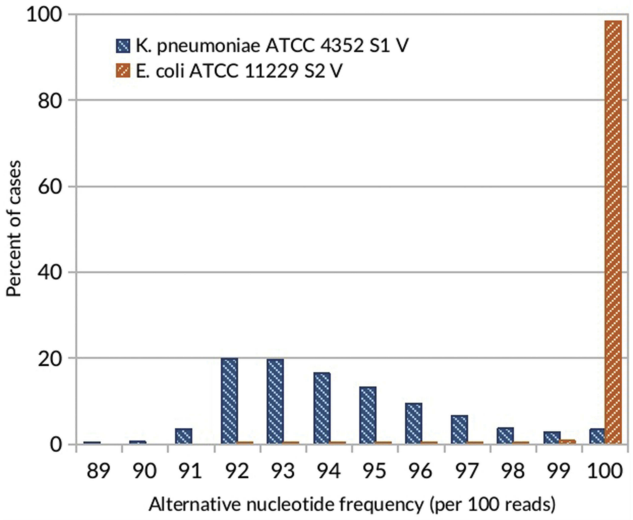


Figure 2 A histogram showing mutation frequency counts in genomic sequencing of *Escherichia coli* ATCC 11229 S2 V and *Klebsiella pneumoniae* ATCC 4352 S1 V strains.

Midori Green. Electrophoresis was carried out during 0.5 h at 7.5 V/cm. A 5 µL DNA marker was applied on the gel. Images of the gels were carried out on a Gel Doc XR$^+$ (BioRad).

Antibiotic Susceptibility Testing

Sensitivity of the tested bacterial strains to antibiotics was determined with disc diffusion methods. The criteria for selection of antimicrobials were based on the EUCAST recommendation.[38] Bacteria (0.5 McFarland) were inoculated and antibiotic discs were placed on the MHA plate. Plates were incubated at 37 °C during 18 h, followed by zone diameter measurement and breakpoint estimation.

Results And Discussion

The changes of bacterial sensitivity to silver nanoformulations were determined after bacteria strains' exposition to increasing concentrations of silver nanoformulations. After comparison of the primary (in wild type strains, wt) and secondary (among variants, V) MIC values, the changes of bacterial sensitivity were determined and the selection of variants was possible. The obtained MIC values are described in Tables 3 and 4. The susceptibility of bacteria depended on the kind of silver nanoformulations (their size, shape, surface area, compounds, etc.) and bacterial strains. Among some of the tested bacterial strains, the changes of sensitivity to certain silver nanoformulations were noticed. It is interesting that almost all strains

Table 7 Substitution Counts From Whole Genome Sequencing As Divided For Transitions And Transversions, Followed By Distribution Of Substitutions In Coding Regions And Possible Impact On Coded Protein Structure

	E. coli K-12 PFM2 wt [43]	*E. coli* K-12 PFM2 mutL [43]	*E.coli* ATCC 11229 S2 V	*K. pneumoniae* ATCC 4352 S1 V	*K. pneumoniae* 626 S1 V	*K. pneumoniae* 26 S2 V	*K. pneumoniae* ATCC 4352 S2 V	*E. coli* ATCC 11229 S7 V	*K. pneumoniae* 626 S7 V
Substitutions in total	233	1 625	17 001	23 081	2	4	1	3	2
Type of substitution									
Transitions									
A:T>G:C	49	1 141	5 988	8 676	1	2	–	–	1
G:C>A:T	82	447	6 681	8 153	–	2	–	1	1
Transversions									
A:T>T:A	17	14	1 143	1 128	–	–	1	1	–
A:T>C:G	38	10	1 192	1 682	1	–	–	–	–
G:C>T:A	30	10	1 220	1 594	–	–	–	1	–
G:C>C:G	17	3	777	1 848	–	–	–	–	–
Consequences of substitutions									
Position									
Noncoding	54	213	1 362	2 706	–	2	–	–	2
Coding	179	1 412	15 639	20 375	2	2	1	3	–
Within coding sequences									
Synonymous	55	482	13 732	16 727	1	1	1	3	–
Nonsynonymous	124	930	1 907	3 648	1	1	–	–	–
Amino acid changes									
Conservative	57	644	15 154	19 299	1	1	–	3	–
Nonconservative	67	286	477	1 077	–	1	10	–	–

Notes: Green and blue marks illustrate relative abundance of particular substitution type.

changed their susceptibility level to S7, 4 of 5 *E. coli* changed sensitivity to S2, 2 of 3 *K. pneumoniae* changed sensitivity to S1 and S2, and only 1 of 6 *S. aureus* changed the sensitivity to S0. All of the selected variants are summarized in Table 5. To verify the reasons for changing the variants' sensitivity, whole genome sequences were analyzed. DNA from 11 of the tested wt strains and their phenotype variants (V) was isolated: *K. pneumoniae* ATCC 4352 wt, *K. pneumoniae* ATCC 4352 S1 V, *K. pneumoniae* ATCC 4352 S2 V, *K. pneumoniae* ATCC 4352 S7 V, *K. pneumoniae* 626 wt, *K. pneumoniae* 626 S1 V, *K. pneumoniae* 626 S2 V, *K. pneumoniae* 626 S7 V, *E. coli* ATCC 11229 wt, *E. coli* ATCC 11229 S2 V, *and E. coli* ATCC 11229 S7 V. The above strains were selected on the basis of the obtained results described in Table 5. For the analysis, mutations were identified in protein coding sequences (CDS), RNA coding sequences, and intergenic regions. All mutations recorded in the above genomic regions were divided into nucleotide substitutions, insertions, deletions, and other complex mutations (Table 6). The most mutations (mainly SNP) were noticed among *K. pneumoniae* ATCC 4352 and *E. coli* ATCC 11229 strains treated with S1 and S2, respectively. Definitely fewer genomic changes were indicated among *K. pneumoniae* 626 treated with S1 and S2, and *K. pneumoniae* ATCC 4352 treated with S2. In general, all mutations can be divided into conservative and nonconservative. Mutation is considered as conservative when it does not introduce any change in primary protein structure or the introduced amino acid change has score ≥0 in BLOSUM62.[39] Otherwise, mutation is considered as nonconservative.[40] Counts of genes harboring at least one mutation (including conservative and nonconservative) are listed in Table 6. In the case of the strains with the highest number of mutations (*K. pneumoniae* ATCC 4352 S1 V and *E. coli* ATCC 11229 S2 V), more than 1200 genes were identified with nonconservative mutations (only a few examples are shown in Table 6), while among the strains *K. pneumoniae* 626 S1 and S2 V and *K. pneumoniae* ATCC

Table 8 Presence Of *sil* Genes In MDR Bacteria Strains wt

Bacteria Strains	*sil* Genes						
	silE	***silRS***	***silCBA***	***silF***	***silB***	***silA***	***silP***
E. coli J53	+	+	+	+	+	+	+
E. coli ATCC 11229	+	+	+	+	+	–	–
E. coli 475	–	–	–	–	–	–	–
E. coli 555	–	–	–	–	–	–	–
E. coli 408	–	–	–	–	–	–	–
E. coli 343	–	–	–	–	–	–	–
K. pneumoniae ATCC 4352	–	–	–	–	–	–	–
K. pneumoniae 626	–	–	–	–	+	–	–
K. pneumoniae 268	–	–	–	–	–	–	–
E. cloacae 233	–	–	–	–	–	–	–

Notes: +, presence of gene; –, lack of gene.

4352 S2 V only one gene was identified, cusS and tamB appropriate. It is worth underlining that the same genes were noticed in cases of strains with a huge number of mutations. The obtained results can be divided into three clusters, where each one has a distinctive feature: a) high number of mutations (*E. coli* ATCC 11229 S2 V, *K. pneumoniae* ATCC 4352 S1 V), b) single gene mutations (*K. pneumoniae* 626 S1 and S2 V, and *K. pneumoniae* ATCC 4352 S2 V), c) no gene mutations (*E. coli* ATCC 11229 S7 V and, *K. pneumoniae* ATCC 4352 S7 V and 626 S7 V) (Table 6). The mutation frequency counts in genomic sequencing of *E. coli* ATCC 11229 S2 V and *K. pneumoniae* ATCC 4352 S1 V strains are shown in Figure 2. As indicated by these data, all recorded mutations occurred with a frequency between 82 and 100 per 100 reads, suggesting some level of heterogeneity gained in silver-treated

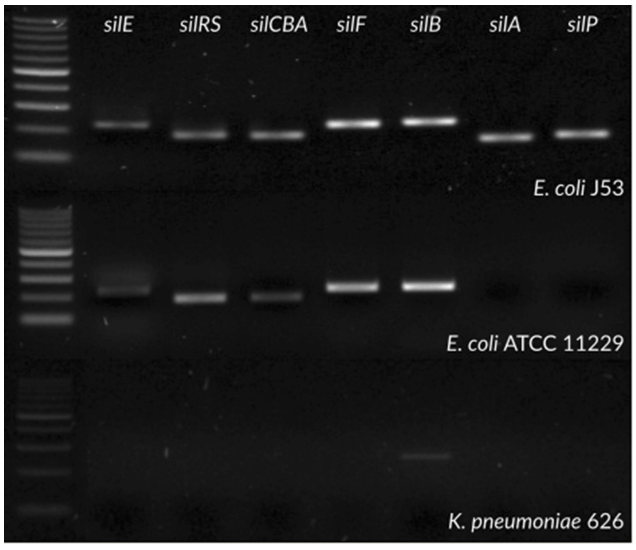


Figure 3 Electropherograms of *Escherichia coli* ATCC 11229 wt and *Klebsiella pneumoniae* 626 wt *sil* genes.

populations, more noticeable in the case of *K. pneumoniae* ATCC 4352 S1 V. Therefore, this post-treatment population may be considered not as a uniform strain, but as a mix of differently adapted organisms, harboring varying sets of genomic mutations. In summary, S1 and S2 samples may be considered as silver nanoformulations that exert the highest selective pressure depending on the individual features of bacterial strains, in contrast to S7. To explain the mutation rates among obtained variants after S0–S7 exposition, the substitution counts from whole genome sequencing followed by the distribution of substitutions in coding regions and possible impact on coded protein structure are described in Table 7. Since the two variants (*E. coli* ATCC 11229 S2 V and *K. pneumoniae* ATCC 4352 S1 V) gained an extraordinarily high number of mutations, they may be premised as mutator strains.[41] This observation can be explained by a possible decrease of DNA repair efficiency in obtained variants, resulting from nonconservative mutations in genes coding proteins involved in mismatchrepair (MMR): *mutL* (Pro389Ser, Leu418Pro) in *E. coli* ATCC 11229 S2 V, and *mutL* (Pro371Thr) and *mutM* (Ala148Glu) in *K. pneumoniae* ATCC 4352 S1 V. As MMR deficiency promotes not only quantitative, but also qualitative changes in the mutational profile, substitution rates from this experiment are compared with corresponding data obtained by Lee et al.[40] In brief, the wt strain gains transitions and transversions with comparable ratios (56% vs 44%), with a slight domination of G:C>A:T transitions. In contrast, the MMR defective strain (*mutL*) gains mostly transitions (over 90%) with clear dominance of A:T>G:C transitions (70%). The two mutator-like variants from this experiment, carrying spontaneous mutation in *mutL*, do not fully fall into any of these categories, although the transition ratio (73–75%) is notably higher than the transversion ratio

Table 9 Antibiotics Susceptibility Of Gram-Negative Bacteria Strains

Bacteria Strains		Am	AMC	CXM	CTX	CAZ	ATM	AN	CIP	SXT	Imp	Mem	TZP
E. coli J53[C]		–	R	R	R	S	R	–	S	S	S	S	R
E. coli ATCC 11229 wt		S	S	S	S	S	S	S	S	S	S	S	S
E. coli ATCC 11229 S2 V		S	S	S	S	S	S	S	S	S	S	S	S
E. coli ATCC 11229 S7 V		S	S	S	S	S	S	S	S	S	S	S	S
E. coli 475 wt	MR	R	S	S	S	S	S	S	S	S	S	S	S
E. coli 475 S2 V		R	S	S	S	S	S	S	R	S	S	S	S
E. coli 475 S7 V		S	S	S	S	S	S	S	S	S	S	S	S
E. coli 555 wt		R	R	R	S	S	S	S	S	S	S	S	S
E. coli 555 S2 V		R	R	S	S	S	S	S	S	S	S	S	S
E. coli 555 S7 V		R	R	S	S	S	S	S	S	S	S	S	S
E. coli 574 wt	MDR	R	R	R	S	S	S	S	S	S	S	S	S
E. coli 574 S7 V		S	S	S	S	S	S	S	S	S	S	S	S
E. coli 408 wt		R	R	R	R	S	S	S	S	R	S	S	S
E. coli 408 S2 V		R	R	R	R	S	S	S	S	R	S	S	S
E. coli 408 S7 V		S	S	S	S	S	S	S	S	S	S	S	S
E. coli 343 ESBL wt		R	R	R	R	R	R	R	R	R	S	S	S
E. coli 343 ESBL S7 V		S	R	R	R	S	S	S	R	R	S	S	S
K. pneumoniae ATCC 4352 wt		R	R	R	R	R	R	S	S	S	S	S	S
K. pneumoniae ATCC 4352 S2 V		R	R	R	R	R	R	S	S	S	S	S	S
K. pneumoniae ATCC 4352 S7 V		S	S	S	S	S	S	S	S	S	S	S	S
K. pneumoniae 626 wt		R	R	R	R	R	R	S	S	S	S	S	S
K. pneumoniae 626 S2 V		R	R	R	R	R	R	S	S	S	S	S	S
K. pneumoniae 626 S7 V		R	R	R	R	R	S	S	S	S	S	S	S
K. pneumoniae 268ESBL wt		R	R	R	R	R	R	S	R	S	S	S	S
K. pneumoniae 268ESBL S7 V		S	R	R	R	S	S	S	R	S	S	S	S
E. cloacae 233 wt		R	R	R	R	R	R	S	S	R	S	S	S
E. cloacae 233 S7 V		S	R	R	R	S	S	S	R	R	S	S	S

Notes: V, variants selected after long-term treatment with silver nanoformulations; S2, S7, etc., kind of silver form with physicochemical differences. [C]Positive control of pMG101 plasmid presence; green marks, increasing the susceptibility of variants to antibiotic; red marks, decreasing the susceptibility of variants to antibiotic; gray marks, no changes, no differences between wild type and variants.[I] No variants obtained.

Abbreviations: wt, wild type strain; S, susceptible; R, resistant; Am, ampicillin; AMC, amoxicillin-clavulanic acid; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; AN, amikacin; ATM, aztreonamum; CIP, ciprofloxacin; Imp, imipenem; Mem, meropenem, TZP, piperacillin-tazobactam; SXT, trimethoprim-sulfamethoxazole.

(25–27%), what is a resemblance to the *mutL* strain, where A:T>G:C and G:C>A:T transition ratios are close to equal. Considering the effect of substitution, in both WT and *mutL* strains, the nonsynonymous mutation ratio outweighed the synonymous mutation ratio. Interestingly, in silver-resistant variants the trend is opposite and most substitutions do not affect coded proteins. Therefore, the mutator phenotype in *E. coli* ATCC 11229 S2 and *K. pneumoniae* ATCC 4352 cannot be explained with a single *mutL* dysfunction, and may be the result of more complex changes and specific selective pressure

Table 10 Antibiotics Susceptibility Of Gram-Positive Bacteria Strains

Bacteria Strains	P	AMC	CXM	CTX	CAZ	Ge	AN	CIP	SXT	Imp	Mem	E	CC	Va	Fox	Link	Tet	C
S. aureus ATCC 6538	R	S	S	S	S	S	S	R	R	S	S	R	R	S	S	R	R	R
S. aureus ATCC 6538 S7 V	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R
S. aureus 173 wt	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	S
S. aureus 173 S7 V	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
S. aureus 298 wt	R	S	S	S	S	S	S	R	S	S	S	R	R	S	S	R	S	R
S. aureus 298 S7 V	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
S. aureus 187 MLSB MRSA wt	R	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	S
S. aureus 340 MRSA wt	R	R	R	R	R	R	S	S	R	R	R	S	S	S	R	S	S	R
S. aureus 340 MRSA S0 V	R	R	R	R	R	R	R	S	S	R	R	R	R	S	R	R	R	S
S. aureus 340 MRSA S7 V	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
S. aureus 2 MRSA wt	R	R	R	R	S	R	R	R	R	R	R	R	R	S	R	R	S	R
S. aureus 2 MRSA S7 V	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Notes: V, variants selected after long-term treatment with silver nanoformulations; S2, S7, etc., kind of silver form with physicochemical differences. [C]Positive control of pMG101 plasmid presence; green marks, increasing the susceptibility of variants to antibiotic; red marks, decreasing the susceptibility of variants to antibiotic; gray marks, no changes, no differences between wild type and variants.

Abbreviations: wt, wild type strain; S, susceptible; R, resistant; AMC, amoxicillin-clavulanic acid; CXM, cefuroxime; CTX, cefotaxime; CAZ, ceftazidime; AN, amikacin; Ge, gentamicin; CIP, ciprofloxacin; Imp, imipenem; Mem, meropenem; SXT, trimethoprim-sulfamethoxazole; P, penicillin; E, erythromycin; CC, clindamycin; VA, vancomycin; FOX, cefoxitin; Link, lincomycin; Tet, tetracycline; C, chloramphenicol.

of silver nanoformulations. Other examined variants do not harbor mutations in any known gene related to DNA repair, consistently the mutator phenotype is missing in them. Table 7 illustrates the relative abundance of particular substitution types within the sample. Genotoxicity of silver nanoparticles toward Gram-positive and Gram-negative bacteria was confirmed.[42] Panacek et al[43] showed that Gram-negative bacteria (including *E. coli* strains) can develop resistance to silver nanoparticles after repeated exposure but without any genetic changes. It resulted from phenotypic changes involving inhibition of the flagellum protein production. Panacek et al[43] confirmed that strains which changed sensitivity to silver nanoparticles did not change sensitivity to silver ions. Those results suggest different response of bacteria cells to various types of silver but is the opposite of studies performed by Randall et al[13] that proved the genomic changes in *E. coli* strains after 6 days of exposure to a subinhibitory concentration of silver ions.

Among all of the bacteria strains tested by us, both Gram-negative and Gram-positive, the presence of *sil* genes, located on the plasmid pMG101, was checked. We indicated that 2 strains possess some *sil* genes: *silE*, *silRS*, *silCBA*, *silF*, and *silB*were present in *E. coli* ATCC 11229 and *silB*in *K. pneumoniae* 626 (Table 8). It is worth underlining that those strains showed high sensitivity to silver ions and tested silver nanoformulations (excluding S0) (Table 3). Probably, this results from incompleteness of the pMG101 plasmid. In most of these strains the lack of all checked *sil* genes was confirmed. All results were visualized with electrophoresis (Figure 3).

Finally, the antibiotic susceptibility of variants was checked. Results are summarized in Tables 9 and 10. Results indicated that 4 variants (*E. coli* 475 S2 V and *E. cloacae* 233 S7 V among Gram-negative bacteria and *S. aureus* ATCC 6538 S7 V and *S. aureus* 340 S7 V among Gram-positive bacteria) became more resistant to antibiotic (ciprofloxacin in the case of Gram-negative bacteria and few classes in Gram-positive strains) (Tables 9 and 10). All of them were obtained after exposition to different kinds of silver nanoformulations: S2 and S7. Remaining Gram-negative and Gram-positive variants maintained the level of antibiotic susceptibility or became more sensitive to some of the antibiotic classes. Almost all of the S7 variants became more sensitive to certain classes of antibiotics. One of the possible mechanisms of bacterial resistance to antibiotics is the inhibition of antibiotic penetration into the bacteria cell.[44–48] In this case it may be related to higher envelope permeability after silver nanoformulation treatment. On the other hand, Barras et al[49] reviewed that incorporation of silver into antibiotics can increase their antibacterial activity. Anuj et al[50] confirmed that cationic particles of nanosilver disturb the membrane integrity in bacteria strains, increasing their permeability in *E. coli* for linezolid, its higher intracellular concentration, and better antibacterial efficacy. A similar study was performed by Kaur et al[51] who conjugated vancomycin with silver nanoparticles and enhanced antibacterial activity against both classes of bacteria: Gram-positive *S. aureus* and Gram-negative *E. coli*. However, Mühling et al[52] showed that environmental exposure of resistant bacteria to silver nanoparticles did not increase antibiotic resistance in naturally occurring strains. They proved that there is no interaction between the antibiotics and Ag. As we reviewed,[9] the interaction of bacteria with silver nanoformulations and consequences depends on the physicochemical properties of silver. The size, shape, biodiversity, and active surface determine their mode of action and the cell answer.

Conclusion

Silver nanoformulations with different physicochemical properties exert selective pressure on the bacterial strain population. Application of silver in the case of MR or MDR bacterial infection may cause different bacterial responses to antibiotics. Based on our results and the analyzed literature we may conclude that the response of bacteria cells to silver nanoparticles depends on the physicochemical properties of the nanoformulations (such as size, shape, charge, surface area, compounds) and individual features of bacteria strains (such as structural compounds and metabolism). The incorporation of silver into industrial products should be under strong control. Moreover, each silver nanomaterial should be considered as a separate agent with a potential different mode of antibacterial action.

Highlights

1. Silver nanoformulations, in dependence on physicochemical properties, can exert selective pressure on the bacterial cells, decreasing the sensitivity to silver.
2. Phenotypical changes, as a consequence of long-term exposure to silver nanoformulations, can change their sensitivity to antibiotics, including increasing bacterial susceptibility to some classes of antibiotics.
3. Silver nanoformulations may cause mutational or phenotypical changes in dependence on physicochemical properties.
4. Development of bacterial resistance to silver nanoformulations depends on their physicochemical

properties and individual features of the bacteria cell (e.g. cell structure, cell compounds, and metabolism)

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