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TRANSMISSIBLE SILVER RESISTANCE READILY EVOLVES IN HIGH-RISK CLONE ISOLATES OF *KLEBSIELLA PNEUMONIAE*

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Silver is used extensively in both hospitals and outpatient clinics as a disinfectant coating agent on various devices. Resistance to silver was recently reported as an emerging problem in *Enterobacteriaceae.* Multidrug-resistant high-risk clones of *Klebsiella pneumoniae* are common causes of serious healthcare-associated infections worldwide posing a serious threat to patients. In this study, we investigated the capacity of both high-risk (CG14/15 and CG258) and minor clone strains of *K. pneumoniae* to develop resistance to silver. Resistance was induced *in vitro* in silver-susceptible but otherwise multidrug-resistant clinical isolates. Genetic alterations in the silver-resistant derivative strains with regard to the silver-susceptible isolates were investigated by whole-genome sequencing. The transferability of high-level resistance to silver was also tested. We demonstrated that the high-level resistance to silver can quickly evolve as a consequence of a single-point mutation either in the *cus*S gene of the chromosomally encoded CusCFBARS efflux system and/or in the *sil*S gene of the plasmid-encoded Copper Homeostasis and Silver Resistance Island (CHASRI) coding also for a metallic efflux. The minimal inhibitory concentrations (MICs) of the strains increased from 4 mg/L (23.5 μM) $AgNO_3$ to >8,500 mg/L (>50,000 μM) $AgNO_3$ during induction. Harboring the CHASRI proved an important selective asset for *K. pneumoniae* when exposed to silver. Successful conjugation experiments using *Escherichia coli* K12 J5-3^{Rif} as recipient showed that high-level silver resistance can transmit between strains of high-risk clones of *K. pneumoniae* (ST15 and ST11) and isolates from additional species of *Enterobacteriaceae*. The lack of fitness cost associated with the carriage of the CHASRI in a silver-free environment and the presence of the RelEB toxin–antitoxin system on the conjugative plasmids could advance the dissemination of silver resistance. Our results show that multidrug-resistant high-risk clones of *K. pneumoniae* are capable of evolving and transmitting high-level resistance to

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silver. This observation should warrant a more judicious use of silver coated-devices to prevent the extensive dissemination of silver resistance.

Keywords: silver resistance, CHASRI, *Klebsiella pneumoniae*, high-risk clone, multidrug resistance

Introduction

Klebsiella pneumoniae is a primarily opportunistic pathogen capable of causing severe healthcare-associated infections. Neonates, elders, and immunocompromised patients are at greater risk. *K. pneumoniae* is one of the six "ESKAPE bugs" – the key pathogens (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) – which compromise the healthcare provision [1]. The treatment of *K. pneumoniae* infections has been complicated by the emergence of extensive antimicrobial resistance (AMR). In the past two decades, several important, horizontally spreading AMR genes were detected in this species: extended-spectrum β-lactamases (ESBLs), such as SHV- or CTX-M-type; carbapenemases, such as KPC, NDM, VIM, or OXA-48-like; and the plasmid-borne colistin resistance gene (*mcr-1*) [2].

While AMR can occur across a broad range of *K. pneumoniae* clones, only a few of them have become globally distributed. About 72% of all reported outbreaks were caused by one of the following five common clonal groups (CG258, CG14/15, CG17/20, CG43, and CG147) [2]. In 2016, CG258 and CG14/15 were the most prevalent CGs causing outbreaks all over the world. CG258 (main sequence types: ST11 and ST258) is undoubtedly the most widely recognized and globally distributed clonal group [2]. ST11 is a pandemic *K. pneumoniae* clone, an evolutionary precursor of ST258, and it is responsible for many outbreaks primarily in Europe and Asia. ST258 is a highly adaptive epidemic clone and is the major cause of carbapenem-resistant *K. pneumoniae* infections [2]. While the CG258 clonal group harbors a diverse arsenal of carbapenemases and ESBL genes (KPC, NDM-1, OXA-48, and CTX-M-15), the ST258 clone is predominantly associated with the KPC-2 and KPC-3 carbapenemases [3]. CG14/15 is the other globally distributed clonal group of *K. pneumoniae*, which has also been associated with a diverse selection of important β-lactamase genes (CTX-M-15, KPC, NDM-1, OXA-48, OXA-181, and VIM-1) [2].

The effectiveness of antibiotics has been severely compromised by the emergence of multidrug- or pandrug-resistant pathogens. With the increase of antibiotic resistance other alternative antimicrobial agents have gained increased attention in the past decades to prevent infections and silver compounds that

belong to the most popular ones. Silver has been used as an important antimicrobial agent throughout recorded history [4]. Nowadays, it is widely applied in hospitals (to treat burns, wounds, and ulcers, to coat indwelling medical devices to inhibit microbial colonization and biofilm development, in dental-amalgam and bone-cement, etc. [5, 6]) and in daily life (deodorants, underwear, sport and bed clothing, supermarket-available colloidal "silver-gelatinate" for washing salad, domestic water purification cartridges, etc. [7]), as well. Silver cations are usually microbicidal at low concentrations without adverse effects for humans. Ag^+ ions have multiple target sites within the bacterial cell; they attach to the cell membrane or envelope, destroy the membrane or get inside the pathogen through transmembrane proteins, bind to thiol groups (–SH) in key enzymes of ATP production, denature enzymes, inactivate them, or intercalate between the base pairs of DNA, disrupting the hydrogen bonding [8, 9].

Protection against the toxicity of heavy metals remains essential for all bacteria, thus sophisticated, intricate, and precisely regulated systems have evolved to maintain homeostasis. Due to the structure of their cell wall, Gram-negative bacteria need to deal with both cytoplasmic and periplasmic heavy metals. Several mechanisms such as intra- and extracellular sequestration, enzymatic detoxification, and active efflux can protect the bacteria from the toxicity of metals [10]. The main role of these native systems is to maintain the homeostasis of copper, which is an essential, but potentially toxic micronutrient. Copper and silver share very similar chemical- and ligand-binding properties; thus, systems that maintain copper homeostasis can also actively detoxify silver. The chromosomally encoded CusCFBARS system comprises of a tricomponent RND efflux system (CusCBA), a small periplasmic Cu and Ag ions binding protein (CusF), and a two-component regulatory system (CusRS) [11–14]. The operon of the efflux pump and that of the regulatory system is transcribed in a back-to-back fashion (Figure 1A). Under the conditions of elevated concentrations of Cu or Ag ions, CusS and CusR are essential for the induction of the copper efflux genes *cus*CFBA.

Silver/copper resistance genes can also be harbored on plasmids. The Copper Homeostasis and Silver Resistance Island (CHASRI) [11] contains two clusters (Figure 1B), the plasmid-borne copper-resistance system (Pco) and the silver-resistance system (Sil), which has sequence and functional homology with the copper sensing copper efflux system (Cus) [7, 15]. According to Staehlin et al. [11], the CHASRI was derived from a single evolving event by the linkage of the two copper resistance mechanisms, likely in a close relative of *Enterobacter cloacae*. The emergence of CHASRI could be a response to the previously unprecedented levels of copper stress caused by humans (tools, disinfection, plumbing, animal husbandry, crop protection, and preservation of perishable commodities) [11], but it can also serve for silver detoxification in bacteria.

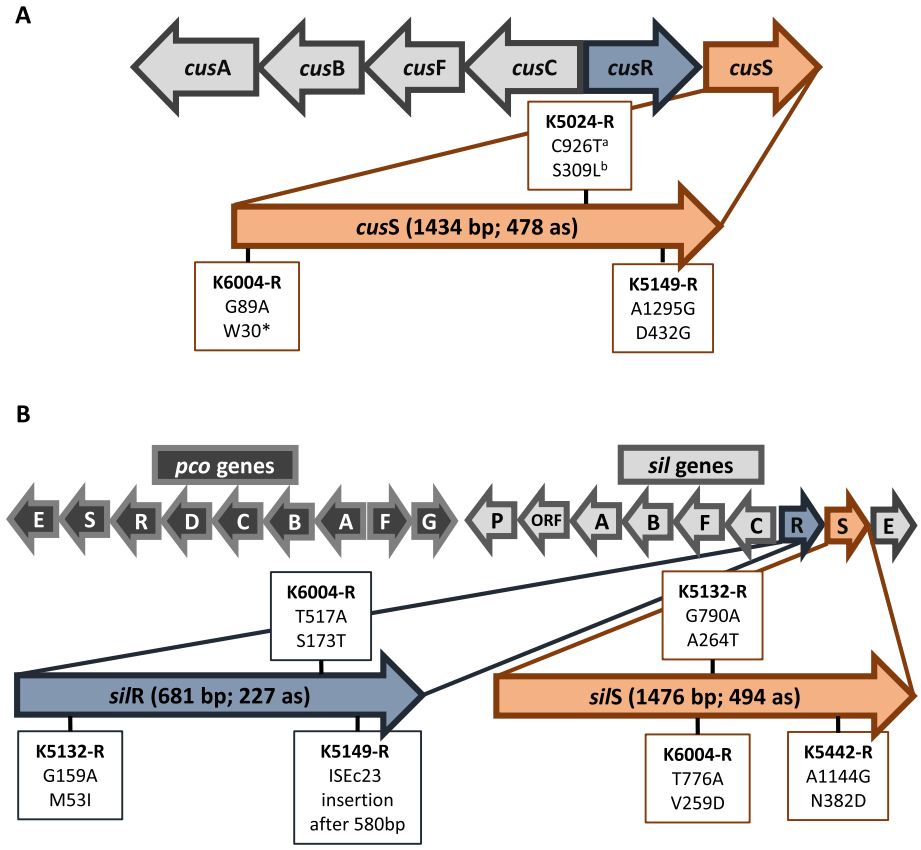

Figure 1. Schematic illustration of the silver efflux systems and the location of detected alterations in silver-resistant derivatives. A: chromosomally encoded CusCFBARS system and B: CHASRI island. Superscripts "a" and "b" mean single nucleotide polymorphism and consequent amino acid substitution, respectively. The symbol "*" means stop codon

Since its assembly, CHASRI has dispersed spottily by horizontal gene transfer among *Enterobacteriaceae* and other families of bacteria. The components of different chromosomally and plasmid-borne metal-resistance systems can cooperate, as they complement each other [10].

The carriage of these native heavy metal homeostasis systems does not basically affect the heavy metal sensitivity of bacteria, but even a single mutation event can lead to high-level resistance against both copper and silver [11, 16]. Silver-resistant *Escherichia coli* was already reported in 1969 [17]. Since then, silver-resistant bacteria have repeatedly been isolated from clinical environments, burn wounds, and teeth [13, 18]. Due to the increasing use of silver for medical

and non-medical applications, concerns have been raised about the development and spread of silver resistance.

The emergence and spread of heavy metal resistance could endanger the medical use of silver and copper; therefore, further investigations are necessary to better understand the phenomenon. It is especially important in the high-risk clones of *K. pneumoniae*, since treatment opportunities in infections caused by them have already been limited due to high AMR resistance.

The aim of this study was to determine the silver susceptibility of *K. pneumoniae* strains of both high-risk and minor clones and to investigate their inducibility of silver resistance using continuous exposure of subinhibitory concentrations of silver nitrate. The molecular mechanisms of silver resistance were established by whole-genome sequencing and the associated fitness cost was also investigated. The transferability of silver resistance was studied by mating out assays.

Materials and Methods

Bacterial strains

The investigated strain collection consisted of seven healthcare-associated, multidrug-resistant *K. pneumoniae* isolates originating from different areas of Hungary between 1998 and 2014 (Table I). We examined five strains from high-risk global clonal groups (CG) (CG14/15 and CG258) and two strains from minor clones (ST25 and ST274). The strains harbored a diverse array of β-lactamase genes with clinical and epidemiological importance representing the predominant multidrug-resistant *K. pneumoniae* clones (Table I).

Silver-susceptibility test

The minimum inhibitory concentration (MIC) values were determined for $AgNO_3$ solution by twofold serial dilution in Mueller–Hinton broth, according to the EUCAST broth microdilution method [19]. According to Sütterlin et al. [6], bacteria with a silver nitrate MIC >512 mg/L were classified as silver-resistant.

Inducing silver resistance

Passage experiments were performed by continuous exposure to increasing concentrations of $AgNO_3$, according to the method by Tóth et al. [20]. The obtained silver-resistant mutants were compared by pulsed-field gel

Table I. Detailed information about the investigated *K. pneumoniae* strains

Code of the isolate			K5149	K6004	K5825	K5442	K5132	K5024	K5312
Year of isolation			2005	2012	2012	2005	2009	2008	1998
Clonal lineage			CG14/15	CG14/15	CG14/15	CG258	CG258	minor	minor
Sequence type			ST15	ST15	ST15	ST11	ST258	ST274	ST25
Acquired antibiotic resistance genes and mutations	Aminoglycoside		*aac(3)-IIa, aph(6)-Id, aac(6′)Ib-cr, aph(3″)-Ib*	*aac(3)-IIa, aph(3″)-Ib, aph(6)-Id, aac(6′)-Ib, aac(6′)-Ib-cr*	*aac(3)-IIa, aph(3′)-Ia, aac(6′)Ib-cr*	*aph(3″)-Ib, aph(6)-Id, aac(6′)-Ib-cr, ant(3″)-Ia*	*aph(3′)-Ia, aac(6′)-Ib*	*aac(3)-IIa*	*aac(3)-IIa*
	β-lactam	ESBL	$bla_{\text{CTX-M-15}}$	$bla_{\text{CTX-M-15}}$	$bla_{\text{CTX-M-15}}$	$bla_{\text{CTX-M-15}}$	$bla_{\text{SHV-12}}$	$bla_{\text{SHV-2a}}$	$bla_{\text{SHV-2a}}$
		Carbapenemase		$bla_{\text{VIM-4}}$	$bla_{\text{OXA-162}}$		$bla_{\text{KPC-2}}$		
		Other	$bla_{\text{OXA-1}}$	$bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$	$bla_{\text{OXA-1}}$	$bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$	$bla_{\text{OXA-9}}$, $bla_{\text{TEM-1}}$	$bla_{\text{TEM-1}}$	$bla_{\text{TEM-1}}$
	Fluoroquinolone	Mutations in QRDR of *gyr*A	S83F and D87A	S83F and D87A	S83F and D87A	S83F and D87A	S83I	S83Y	
		QRDR of *par*C	S80I	S80I	S80I	S80I	S80I		
		PMQR	*aac(6′)-Ib-cr*	*aac(6′)-Ib-cr*	*aac(6′)-Ib-cr*	*aac(6′)-Ib-cr*			
	MLS (macrolid, lincosamide, and streptogramin B)			*mph(E), msr(E)*		*mph(A)*			
	Phenicol		*cat*B4 and *cat*A1	*cat*B4	*cat*B4 and *cat*A1	*cat*B4 and *cat*A1			
	Sulfonamide			*sul*1 and *sul*2		*sul*1			*sul*1
	Tetracycline		*tet*A		*tet*A			*tet*A	*tet*A
	Trimethoprim			*drf*A14	*drf*A30	*drf*A12			*drf*B3
Plasmid replicon type			IncFIB(K), IncFII(K), IncR, IncFII, and ColpVC	IncFIB(K), IncR, IncFII, and IncFIB	IncFII(K), IncL/M, IncFIB, and ColpVC	IncFII(K), IncFIB(K), IncR, and IncFIB	IncFII(K), IncX3, ColRNAI, and IncFIB(K)	IncL/M	IncL/M

Note: ESBL: extended-spectrum β-lactamase; QRDR: quinolone resistance determining region; PMQR: plasmid-mediated quinolone resistance.

electrophoresis (PFGE) with the original isolates. PFGE was performed in line with the standardized Centers for Disease Control and Prevention protocol [20]. The stability of the silver resistance was examined by subculture of the derivatives onto blood agar plates without silver. We measured the MIC values of the isolates after every 5th streaking.

Conjugation experiments

Conjugation experiments were performed by the filter mating procedure, according to the modified method of Werner et al. [21]. CHASRI harboring K5149-R, K6004-R, K5132-R, and K5442-R were used as donor strains and *E. coli* K12 J5-3^{Rif} was used as recipient strain. Selective agar plates contained 32 mg/L (188 μM) $AgNO_3$ and 300 mg/L rifampicin. All transconjugants were confirmed by species identification using MALDI-TOF and $AgNO_3$ susceptibility testing. Plasmid DNA from the donor strains and from transconjugants were obtained using the alkaline lysis method [22] and plasmid profile typing was performed in vertical agarose gels.

Determination of growth rates

Relative changes in the fitness of bacterial strains were determined in propagation assays. All strains were tested three times and the results were averaged. *In vitro* planktonic growth rates were measured for the parent strains and silver-resistant derivatives in monocultures with three different concentrations of silver nitrate (0, 5, and 315 μM Ag^+). Briefly, we adjusted the density of each bacterial suspension in brain–heart infusion broth (BHI; National Public Health Institute, Budapest, Hungary) supplemented by corresponding Ag^+ ion content to 0.5 McFarland (approximately 10^8 CFU/ml) using a McFarland turbidity meter. The working solutions were obtained after a 100-fold dilution. An amount of 200 μl of working solutions were pipetted into microtiter plates. Cultures were incubated at 35 °C with continuous shaking (260 rpm). Bacterial growth was measured after every 45 min by recording the absorbance at 620 nm using a SpectraMax 340 spectrophotometer (Molecular Devices, Sunnyvale, CA). Areas under curve (AUC) were computed to compare the relative fitness of the isolates.

Next-generation sequencing

We investigated the molecular mechanisms of silver resistance by next generation sequencing of the parent strains' and derivatives' bacterial genomes.

The genomic DNA extraction was performed using UltraClean Microbial DNA isolation kit (MO BIO Laboratories, USA) following the manufacturer's instructions. The DNA of plasmids from transconjugants was extracted by the alkaline lysis method and precipitated by ethanol [22]. Sequencing libraries were prepared with SureSelect QXT Library Prep Reagent Kit (Agilent Technologies, USA) according to the manufacturer's instructions. Sequencing was performed on a MiSeq system using the MiSeq reagent Kit v2 (Illumina, San Diego, CA, USA) generating 250-bp paired-end reads. Raw reads were processed using online tools of Illumina BaseSpace, which is a cloud-based genomics analysis and storage platform. We used FASTQC for quality control of sequencing, FASTQ toolkit for quality trimming, and SPAdes Genome Assembler 3.9.0. for *de novo* genome assembly. For *de novo* assembly of plasmids, plasmidSPAdes Genome Assembler 3.9.0. was applied. Assembled genomes were analyzed using online bioinformatics tools of Center for Genomic Epidemiology, such as ResFinder [23], MyDbFinder, PlasmidFinder [24], pMLST [24] (https://cge.cbs.dtu.dk/services/; Technical University of Denmark), and the plasmid MLST website (https://pubmlst.org/plasmid/; developed by Keith Jolley and sited at the University of Oxford [25]) and compared with GenBank data using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and to each other by SeqSphere+ (Ridom GmbH, Germany) using core and accessory genome gene sets. The analysis of CHASRIs was performed using SnapGene Viewer 3.3.3 software. Integron structure has been named according to INTEGRALL database (http://integrall.bio.ua.pt).

Results

Parent strains: Silver efflux systems and $AgNO_3$ MIC values

The antibiotic resistant determinants carried by our isolates were investigated previously [20, 26–30] or by whole-genome sequencing and are summarized in Table I.

Table II and Figure 1 show the silver efflux systems of the examined *K. pneumoniae* strains. Chromosomally encoded cusCFBASR system was found in all the seven strains, but only four of them harbored the CHASRI on plasmid (Figure 1 and Table II). Two minor clone strains (K5024: ST274 and K5312: ST25) and the K5825 (ST15) isolate did not carry CHASRI. The structure of CHASRI was identical in all of the *K. pneumoniae* strains regardless of their sequence types (Figure 1B).

The $AgNO_3$ MIC values of the seven parent strains proved similar (Table II) regardless of sequence types or CHASRI carriage. All of the strains showed a silver-sensitive phenotype.

Table II. Characteristics of *K. pneumoniae* parent and derivative strains

Code of isolate	MIC of $AgNO_3$	Chromosome: mutation in *cus*S of derivative	CHASRI island on plasmid			AUC		
			Yes/no	Alteration in *sil*S of derivative	Alteration in *sil*R of derivative	BHI without $AgNO_3$	5 µM 0.9 mg/L $AgNO_3$	315 µM 54 mg/L $AgNO_3$
K5149-S	23.5 µM 4 mg/L		Y			342	169	n.g.
K5149-R	>50,000 µM >8,500 mg/L	D432G		–	Insertion ISEc23	326	305	124
K6004-S	47 µM 8 mg/L		Y			380	227	n.g.
K6004-R	>50,000 µM >8,500 mg/L	W30*		V259D	S173T	391	260	247
K5132-S	23.5 µM 4 mg/L		Y			397	108	n.g.
K5132-R	>50,000 µM >8,500 mg/L	–		A264T	M53I	388	276	172
K5442-S	47 µM 8 mg/L		Y			400	177	n.g.
K5442-R	>50,000 µM >8,500 mg/L	–		N382D	–	360	224	228
K5024-S	23.5 µM 4 mg/L		N			382	15	n.g.
K5024-R	>50,000 µM >8,500 mg/L	S309L		–	–	338	204	204
K5825	47 µM 8 mg/L	–	N	–	–	353	40	n.g.
K5312	23.5 µM 4 mg/L	–	N	–	–	397	48	n.g.

Note: The columns show silver nitrate MIC, the mutations of silver efflux systems, and the growth data of the strains in three conditions. The symbol "*" means stop codon and "n.g." means the strain did not grow. AUC: area under the curve; CHASRI: Copper Homeostasis and Silver Resistance Island; BHI: brain–heart infusion broth; MIC: minimal inhibitory concentration.

Results of passage experiment, $AgNO_3$ MIC values of silver-resistant derivative strains

Resistance to silver could be induced in five of our seven strains (71%). Four of them harbored the CHASRI (Table II). Among the five silver-resistant derivatives, two belonged to ST15, two to CG258 (ST11 and ST258), and one to the ST25 sequence type (Table II). The silver-resistant derivatives of *K. pneumoniae* strains proved to be indistinguishable by PFGE from the original parent isolates (data not shown).

All silver-resistant derivatives were able to proliferate in broth with an $AgNO_3$ concentration >8,500 mg/L (>50,000 μM Ag^+) (Table II). The silver-resistant phenotype remained stable after 25 subcultures in the absence of silver.

Mutations in silver efflux systems

The sequencing data of the parent strains and the derivatives were compared. Table II and Figure 1 show different mutations of the strains in chromosomally encoded *cus*S and in plasmid encoded *sil*S, *sil*R genes of CHASRI. We found different missense mutations in three distinct genes, one nonsense mutation in *cus*S, and one insertion of an insertion sequence element in *sil*R gene. No differences in any of the *pco* genes were detected. The nucleotide sequences of CHASRIs from K5149-S/R, K6004-S/R, K5132-S/R, and K5442-S/R strains have been deposited in GenBank under accession numbers from MH130217 to MH130224.

Growth rate of parent and silver-resistant derivative strains

Figure 2 shows the growth curves of our isolates and Table II displays the calculated AUC values. Without silver, the parent strains showed similar growth curves and AUC values. In the presence of 0.9 mg/L $AgNO_3$ (5 μM, sub-MIC concentration), the AUC values of parent strains were reduced, the exponential phase of their growth delayed compared with those in silver-free BHI. Furthermore, in the presence of silver in sub-MIC concentration, the growth rates of the parent strains differed from each other (Figure 2). The three strains that did not carry CHASRI showed the lowest AUC values and the biggest delay in starting of exponential growth phase. In 54 mg/L (315 μM, supra-MIC) concentration of $AgNO_3$, the parent strains were not able to grow.

The derivative strains showed similar AUC values in BHI without silver nitrate (Table II). The presence of silver slightly reduced their AUC values due to the

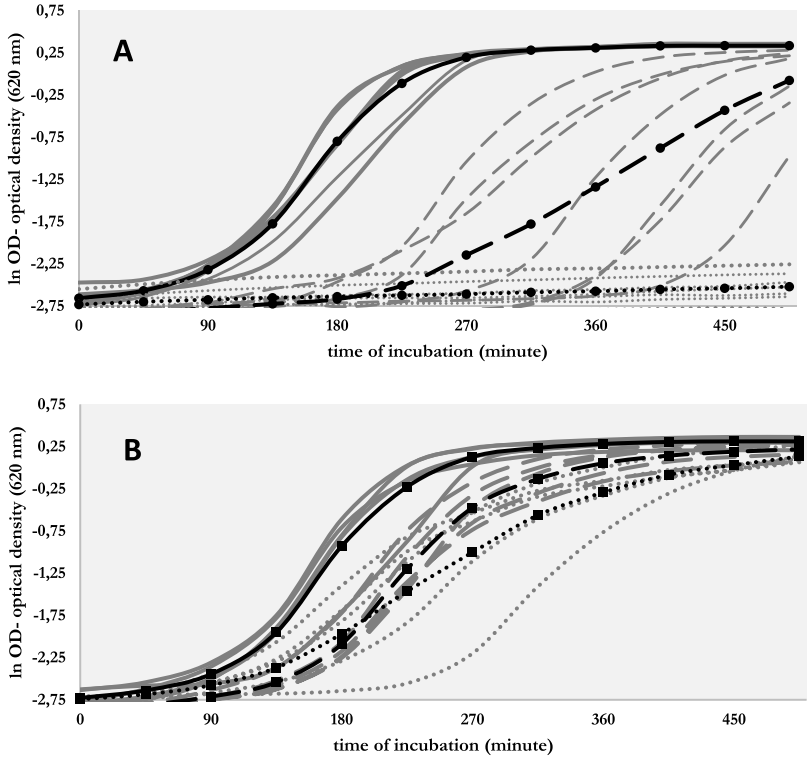

Figure 2. Growth curves of (A) parent strains and (B) silver-resistant derivatives in brain-hearth infusion with different concentration of silver nitrate [continuous line: without silver, dashed line: 5 μM Ag^+ (0.9 mg/L $AgNO_3$), and dotted line: 315 μM Ag^+ (54 mg/L $AgNO_3$)]. Gray curves show the individual results of the strains and black curves show the averaged values

delay in starting the exponential growth phase (Figure 2), but the growth rates were not always associated with the concentration of silver. The growth curves recorded in solutions with 5 and 315 μM of Ag^+ overlap each other, with the exception of K5149-R. The higher silver concentration substantially delayed the growth of K5149-R, but finally this strain reached the same optical density as those growing in silver-free BHI.

In a silver nitrate-free environment, the AUC values of the parent strains and of their silver-resistant derivatives remained similar to each other (Table II); thus, the derivatives did not suffer fitness cost due to the developing high-level silver resistance. In the presence of silver, the derivatives showed unequivocal growth advantage relative to the parent strains.

Results of conjugation

Conjugation was successful in case of K6004-R and K5442-R *K. pneumoniae* strains, which belonged to the widespread ST15 and ST11 sequence types, respectively. The $AgNO_3$ MIC values of the recipient *E. coli* K12 J-53^{Rif} and both the transconjugants (TR6004 and TR5442) were 4 (23.5 μM) and 8,500 mg/L (50,000 μM Ag^+), respectively. Plasmids obtained from TR6004 and TR5442 were different [p6004 – size: 197 kbp, replicon types: IncFII and IncFIB(K), pMLST: K9:A-:B-; p5442 – size: 160 kbp, replicon types: IncFII(K) and IncFIB(K), pMLST: K1:A-:B-]. A BLAST comparison of p6004 and p69-1 (GenBank: CP025457.1) revealed a 99% identity (82% query coverage) between the two plasmids. Plasmid p5442 showed 99% similarity to pKPN3 (GenBank: CP011577.1) with 92% query coverage. Compared to pKPN3 plasmid, p5442 harbored additional AMR genes (*aadA2, sul1, dfrA12*, and *catA1*), most of them are part of a Class-I integron (structure of In27 integron: 5′CS–dfrA12–gcuF–aadA2–qacEΔ1–sul1–orf5Δ–3′CS). Both plasmids contain RelBE type II toxin–antitoxin (TA) system.

Discussion

The silver MIC values of the parent *K. pneumoniae* strains were in the same to those reported by Randall et al. [16]. Similar to the study by Sütterlin et al. [6], we found no differences among the silver MIC values of parent strains with diverse genetic backgrounds. The MIC values of silver-resistant derivative strains increased dramatically upon induction (from 4–8 to >8,500 mg/L). Sütterlin et al. [6] detected similarly elevated MIC values (>512 mg/L) in other species of *Enterobacteriaceae*. According to Randall et al. [16], the silver resistance mediated by the Sil system had minimal impact on bacterial fitness when compared with silver-susceptible parental strains. Our results not only confirmed this observation but also complement it. In a silver-free environment, chromosomally *cus*S-mediated silver resistance did not show any fitness cost.

We showed that the development of silver-resistance is not clone-specific in *K. pneumoniae*. Resistance to silver could be quickly induced in both some high-risk clone strains (ST15, ST258, and ST11) and a minor clone isolate (ST274). In contrast, one of the ST15 strains and the ST25 *K. pneumoniae* isolate proved unable to evolve resistance to silver. The ability of globally distributed *K. pneumoniae* clones to develop resistance to silver is of concern as they could compromise the efficacy of silver in the healthcare setting.

The silver-resistant derivative of K5024 (ST274) proved that the presence of the CHASRI is not necessarily required for developing silver resistance.

Sütterlin et al. [6] were already able to induce silver resistance in one *sil*-negative *K. pneumoniae* strain, but the genetic mechanism was not investigated. According to Randall et al. [16], upregulation of chromosomally encoded CusCFBA efflux system itself is sufficient to remove silver ions from *E. coli*, provided it is associated with simultaneous loss of OmpC/F porins from the outer membrane. Comparing the genetic data of K5024-S and K5024-R, we found a missense mutation in *cus*S gene (Table II) and a nonsense mutation in *ompK*36 gene (Q261*, where * means stop codon), which is homolog to the *omp*C gene in *E. coli* [31]. These results confirm the conclusions of Randall et al. and prove that such combination of mutation events can also occur in *K. pneumoniae*.

Although the carriage of the CHASRI is not a prerequisite for the development of resistance to silver, it clearly promotes the evolvement of resistance. While all four strains carrying the CHASRI were able to attain high MIC values for silver, only one of the three isolates void of this genetic element proved capable of achieving similarly high level of resistance.

Our results are in agreement with the findings of Sütterlin et al. [6] obtained with *sil*S-positive *Enterobacteriaceae* isolates. In 12 isolates among the tested 17 strains, they found SNPs concentrated in two segments of the *sil*S gene (629–725 and 919–1054 bp). We suppose that due to the cooperation of chromosomally and plasmid-borne metal homeostasis systems, mutations of both *cus*S and *sil*S genes can cause constitutive expression of the CusCFBA and SilCFBA efflux transporters. In the K5149-R strain, we identified a missense mutation of *cus*S, but in K6004-R, K5132-R, and K5442-R strains alterations of *sil*S gene were responsible for silver-resistant phenotype (Figure 1 and Table II). Consequently, harboring *sil*S-encoded plasmid in one or more copies increases the probability of the emergence of suitable mutations.

Strains without CHASRI not only have less tools for developing silver resistance, but also have lower proliferation ability in the presence of silver. In the presence of sub-MIC concentration, $AgNO_3$ K5024-S, K5312, and K5825 strains showed lower and delayed growth compared with strains having CHASRI (Figure 2). We assume that the growth disadvantage is caused by the lower number of expressed membrane efflux pumps, which is the consequence of the missing CHASRI. Our theory is supported by the decreased growth of K5149-R strain in solution of 315 μM of $AgNO_3$ compared to BHI containing 5 μM of $AgNO_3$ (Table II). The *sil*R response regulator gene of K5149-R strain lost its function due to the insertion of ISEc23 element; thus, the expression of SilCFBA system was decreased, which made the proliferation of the bacterium slower relative to strains carrying a native *sil*R.

In the study of Deshpande and Chopade [32], silver resistance proved to be transferable from *A. baumannii* to *E. coli* K12 during conjugation, but according to

our knowledge, this is the first description of successful silver resistance transfer between *K. pneumoniae* and *E. coli* strains. In this investigation, strains belonging to both ST15 and ST11 *K. pneumoniae* were able to transmit silver resistance genes on two different plasmids. Analysis of p5442 plasmid showed that the CHASRI was cotransferred with AMR genes. This implies that spread of CHASRI among bacterial species could be helped by selection pressure of antibiotics. Both plasmids contained RelBE3 toxin–antitoxin system, which plays vital role in plasmid maintenance, due to the post-segregational killing effect. The RelBE3 system is composed of a stable toxin (protein RelE, which is capable of cleaving mRNA) and an unstable antitoxin (protein RelB, which neutralizes the toxin by forming a toxin–antitoxin complex) [33]. If the bacterium loses the TA system harboring plasmid, the stable toxin will kill it after the degradation of the unstable antitoxin. Presence of the RelBE system on CHASRI harboring plasmids ensures the maintenance of silver resistance in both donor strains and transconjugants.

In the presence of silver, the carriage of CHASRI could be an important selection asset for bacterial strains due to the higher growth rate and the multiple possibilities to develop a silver-resistant phenotype. According to Sütterlin et al. [6], the presence of *sil* genes was detected in 41% of the examined 129 healthcare-associated *K. pneumoniae* isolates. Originally, only 3% of the isolates showed phenotypic silver resistance, but based on the results of Sütterlin et al. and of this study a single mutation is sufficient to drastically elevate the silver MIC values. Our results proved that silver-resistant phenotype could be very stable and transferable by conjugation. All these observations emphasize the need for a prudent use of silver-containing products.

Conclusions

The mechanism of silver resistance is usually investigated using *E. coli* isolates. Although Sütterlin et al. [6] sequenced the whole genome of a ST20 *K. pneumoniae* strain before and after silver exposure, further research is required to understand the development of silver resistance in this species. We investigated the inducibility of silver resistance in seven *K. pneumoniae* strains, belonging to the widespread CG14/15 and CG258 clonal groups or to minor clones applying passage experiments. We also investigated the molecular background of silver resistance by whole-genome sequencing and tested the transferability of silver resistance by mating out assays.

Our findings contribute to the better understanding of the mechanism of silver resistance in high-risk *K. pneumoniae* strains. We proved that silver-resistance phenotype can arise after a single-point mutation in either *cus*S or

*sil*S genes, but carriage of the CHASRI could be an important selection advantage for bacterial strains in the presence of silver. The carriage of the CHASRI is not associated with any fitness cost in a silver-free environment. Successful mating out assays proved that plasmid-mediated silver resistance could spread among *K. pneumoniae* and other species. The presence of the RelBE toxin–antitoxin system on the plasmids harboring the CHASRI contributes to the maintenance of the silver-resistant phenotype.

In vivo developed/acquired resistance to silver was already detected among *Enterobacteriaceae* strains [6, 34]; moreover, commercially available silver-impregnated burn and wound dressings proved to be inefficient against two of these strains [34]. The prevalence of the *sil* genes in various *Enterobacteriaceae* species is common in the healthcare setting [6, 35]). These observations completed by our results draw attention to the prudent use of silver. When treating an infection caused by a *K. pneumoniae* high-risk clone isolate, not only antibiotic resistance but also resistance to silver should be considered. We have shown that a stable, transferable, high-level resistance to silver can readily develop in multi-drug-resistant *K. pneumoniae* high-risk clones.

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Conflict of Interest

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