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# Unprecedented Silver Resistance in Clinically Isolated *Enterobacteriaceae*: Major Implications for Burn and Wound Management

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**Increased utilization of inorganic silver as an adjunctive to many medical devices has raised concerns of emergent silver resistance in clinical bacteria. Although the molecular basis for silver resistance has been previously characterized, to date, significant phenotypic expression of these genes in clinical settings is yet to be observed. Here, we identified the first strains of clinical bacteria expressing silver resistance at a level that could significantly impact wound care and the use of silver-based dressings. Screening of 859 clinical isolates confirmed 31 harbored at least 1 silver resistance gene. Despite the presence of these genes, MIC testing revealed most of the bacteria displayed little or no increase in resistance to ionic silver (200 to 300 μM $Ag^+$). However, 2 isolates (*Klebsiella pneumonia* and *Enterobacter cloacae*) were capable of robust growth at exceedingly high silver concentrations, with MIC values reaching 5,500 μM $Ag^+$. DNA sequencing of these two strains revealed the presence of genes homologous to known genetic determinants of heavy metal resistance. Darkening of the bacteria's pigment was observed after exposure to high silver concentrations. Scanning electron microscopy images showed the presence of silver nanoparticles embedded in the extracellular polymeric substance of both isolates. This finding suggested that the isolates may neutralize ionic silver via reduction to elemental silver. Antimicrobial testing revealed both organisms to be completely resistant to many commercially available silver-impregnated burn and wound dressings. Taken together, these findings provide the first evidence of clinical bacteria capable of expressing silver resistance at levels that could significantly impact wound management.**

Clinicians have taken advantage of the antimicrobial and antifungal properties of inorganic silver compounds throughout history (1–3). The microbiocidal efficacy of silver can be accounted for by two primary mechanisms of action. First, silver ions can bind to the bacterial cell wall, disrupting polysaccharide integrity and membrane fluidity (4). Second, silver can bind directly to DNA, interfering with cell replication and transcription. Due to the unique characteristic of exhibiting broad-spectrum antimicrobial properties while lacking significant toxicity to mammalian cells, silver has become a popular additive for many medical applications, including surgical devices, implants, shunts, catheters, and wound dressings (1, 2). Consequently, the use of silver-based treatments has especially increased in the area of burn and wound care over the last 40 years (5, 6). As a result of increased silver utilization, questions concerning antimicrobial stewardship and fears of widespread silver resistance emerging in clinical bacteria have been raised (7).

Plasmid pMG101, isolated from a *Salmonella* species, provided the initial molecular basis for silver resistance (8, 9). As detailed by Gupta et al. (9, 10), pMG101 is a 180-kb plasmid that accounts for resistance to multiple antibiotics and metals, including silver. The gene cassette carrying silver resistance genes includes *silP*, *silA*, *silB*, *silC*, *silR*, *silS*, *silE*, ORF105, and *silABC* (ORF96) (11). Since their initial identification, *sil* genes have been identified in *Salmonella* (8, 9), *Enterobacter* (4, 12–14), *Escherichia coli* (11, 15–17), *Pseudomonas* (18), *Acinetobacter* (19), *Klebsiella* (20), and methicillin-resistant coagulase-negative *Staphylococcus aureus* (21). A number of other plasmids, typically those of incompatibility group H, have been reported to contain silver resistance determinants, including R478 (22), pAPEC-O1-R (23), R27 (10), and pUPI199 (19).

Endogenous silver resistance has also been reported to be due to the *cus* operon. Like the Sil system, the *cus* operon includes genes for a sensor kinase (Sil/CusS), a periplasmic efflux transporter (Sil/CusBCA), and a periplasmic $Ag^+$ binding protein (Sil/CusF). The two systems share >80% amino acid identity, and both are thought to function primarily via silver efflux (24, 25).

Despite clear evidence supporting the molecular basis for silver resistance, the level of phenotypic expression of these genes in clinical isolates remains unclear. Although the literature describing microorganisms displaying resistance to high silver concentrations is limited, evidence exists of adapted bacteria flourishing in microbicidal environments. *Pseudomonas stutzeri*, originally cultured from a silver mine (26), is able to grow in the presence of toxic levels of silver (0.05 M). This level of phenotypic silver-resistant expression is unique and remains unseen in active clinical bacteria isolated from patients. The few studies investigating silver resistance in clinical bacteria have shown a limited presence of *sil* genes and little to no evidence of clinically significant phenotypic

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expression. An investigation of silver resistance in methicillin-resistant *S. aureus* and methicillin-resistant coagulase-negative staphylococci isolated from humans and animals showed only 3 isolates that were positive for *sil* genes out of the 41 samples tested (21). Despite carrying silver-resistant determinants, the three positive strains remained sensitive to low silver concentrations. Additional clinical studies supported these findings, suggesting a limited presence of *sil* genes in patient settings. Bacteria collected from diabetic foot ulcers yielded only 2 out of 112 positive isolates that contained *sil* genes (27). In addition, a study investigating silver resistance in humans and horses showed only 6 of 172 isolates were *sil* gene positive. In both of these studies, all of the silver-resistant isolates were in the genus *Enterobacter* (27, 28).

Based on the increased use of silver in medicine and known genetic potentials, bacterial silver resistance should continue to be monitored for developing signs of phenotypic expression. The purpose of this study was to investigate and assess the prevalence of silver resistance in active clinical isolates collected at a level I trauma center.

## MATERIALS AND METHODS

**Bacterial isolates.** After IRB approval, 859 bacterial strains isolated from patients at a tertiary care facility in southwest Missouri were screened for resistance to ionic silver. These isolates were previously identified by using a Vitek 2 microbial identification system (bioMérieux, Durham, NC). Detailed information regarding these isolates is available on request. Isolates were received on blood agar plates (Remel, Lenexa, KS) and subcultured in lysogeny broth (LB) supplemented with 50 μM $Ag^+$. All isolates were initially screened for silver resistance by plating 10 μl of an overnight culture grown in LB supplemented with 50 μM $Ag^+$ onto freshly prepared LB agar plates supplemented with 250 μM $Ag^+$. Several control cultures were used for comparative purposes in MIC testing, including the following: *Klebsiella pneumoniae* ATCC 13883, *Enterobacter cloacae* ATCC 13047, *Escherichia coli* J53, and *E. coli* J53(pMG101). All control cultures were maintained on LB agar, with the exception of *E. coli*(pMG101), which was maintained on LB agar supplemented with 100 μg/ml ampicillin.

**Detection of *sil* genes.** Isolates capable of growth on LB agar supplemented with 250 μM $Ag^+$ were screened for the presence of *sil* genes (*silA*, *silB*, *silCBA*, *silF*, *silP*, and *silRS*) by using PCR, as described by Woods et al. (28). Individual colony PCRs for each primer pair were prepared using 2.0 μl cell lysate, 0.5 μM forward and reverse primer, 12.5 μl GoTaq 2× Green mastermix (Promega, Madison, WI), and nuclease-free water added for a total reaction volume of 25 μl. All PCR setups were performed in a PCR-dedicated Labconco workstation using barrier pipette tips to minimize the risk of contamination. Each group of reactions contained both positive and negative controls. The positive-control mixture contained cell lysate from *E. coli* J53(pMG101). The negative-control reaction mixture contained lysate from *E. coli* J53 cells, and there was also a no-template control. Amplification was performed in an Applied Biosystems Veriti thermal cycler (Life Technologies, Grand Island, NY). PCR products were separated by agarose gel electrophoresis and visualized via UV transillumination.

**MIC determinations.** MIC values for silver nitrate were determined for all *sil*-positive isolates and the control organisms. Growth experiments were conducted using an LB agar formulation with 0.0 g/liter NaCl supplemented with silver nitrate. Plates were prepared fresh before every trial, and 50 mM filter-sterilized silver nitrate was added once the medium had cooled to 50°C prior to pouring. Concentrations tested included 250 to 1,000 μM $Ag^+$ in 50 μM increments, 1,000 to 2,000 μM $Ag^+$ in 100 μM increments, and 2,000 to 6,000 μM $Ag^+$ in 500 μM increments. Prior to inoculation, all organisms were subcultured overnight in 5 ml of LB containing 50 μM $Ag^+$ and incubated at 37°C with shaking. $Ag^+$-supplemented plates were divided into quadrants and subsequently inoculated using a calibrated 10-μl inoculating loop in a zigzag manner. Plates were incubated for 24 h at 37°C. All MIC assays were performed in duplicate in two independent experiments ($n = 2$).

**TABLE 1** Silver-impregnated wound dressings used in the study

| Product | Manufacturer | Description |
|---|---|---|
| Acticoat | Smith-Nephew | Nanocrystalline silver |
| Acticoat-7 | Smith-Nephew | Nanocrystalline silver |
| Maxorb-Ag | Medline | Ionic silver alginate |
| Silverlon | Argentum Medical, LLC | Elemental silver |
| Aquacel-Ag | ConvaTec | Hydrofiber silver |
| Mepilex-Ag | Moinlycke | Ionic silver silicone |
| Silvercel | Systagenix | Hydro-alginate silver |
| Exsalt | Exciton | Silver oxysalts |
| Therabond | Choice Therapeutics | Elemental silver |

**DNA sequencing.** To confirm the presence of *sil* genes in two isolates which demonstrated a hyperresistance to ionic silver (isolate 141419, designated silver-resistant *Enterobacter cloacae* [SREC], and isolate 685129, designated silver-resistant *Klebsiella pneumoniae* [SRKP]), *sil* gene PCR products were subjected to DNA sequencing. PCR products from these isolates were purified using a PCR cleanup kit (Axygen, Union City, CA). The resultant DNA was diluted to 10 ng/μl and mixed with 2 μM forward primer. Sequencing was performed by MWG Operon (Louisville, KY) using an ABI 3737XL DNA sequencer. Returned sequences were analyzed using the NCBI BLASTN (Mega BLAST) algorithm.

**Sensitivity to silver-based dressings.** For an indication of clinical significance, resistance of SRKP and SREC isolates to nine commercially available silver-based burn and wound dressings was evaluated using the modified ASTM method E2149-01 (29). For comparative purposes, two *sil*-positive but low-MIC isolates, *E. cloacae* designation 16051951 (SSEC) and *K. pneumoniae* designation 28641251 (SSKP), were included in the assay. The dressings contained various forms of silver (Table 1). All dressings were die cut into circles 31 mm in diameter along with a control dressing containing no silver. Briefly, 50 ml of challenge bacterial inoculum at $10^5$ CFU/ml was transferred to a 250-ml jar with a control or silver-impregnated dressing. Jars were shaken horizontally for 24 h at 37°C. Reduction of bacteria was determined by serial dilutions in phosphate-buffered saline (PBS) and plated onto nutrient medium in triplicate. From this, the average number of CFU per milliliter from each dilution was used to calculate the percent reduction. Test series that resulted in the total killing of the test organism were reported as 1 CFU/ml. All data reported represent the averages of two independent experiments ($n = 2$).

To confirm the lack of phenotypic *sil* gene expression observed in MIC testing, we next tested the organisms against the same nine silver-based wound dressings in a corrected zone of inhibition (CZOI) assay. Similar to the standard zone of inhibition test, the CZOI test is a modification of the Kirby-Bauer test for antimicrobial sensitivity. In brief, 0.1 ml of a $10^8$ CFU/ml culture was streaked onto Mueller-Hinton II agar in three directions to form a confluent lawn. Silver dressing or control gauze was handcut into 20- by 20-mm squares and placed in the center of each lawn and allowed to incubate for 24 h at 37°C. The CZOI was then determined by measuring from the edge of the zone of inhibition to the edge of the dressing across both sides of the dressing. CZOI tests were conducted in duplicate. All reported CZOI measurements represent the average of two independent experiments ($n = 2$). To confirm that the silver dressing killed the microorganism under the dressing, a secondary culture was taken from underneath each silver dressing and incubated.

**SEM and EDS.** SRKP and SREC were grown on LB agar plates supplemented with 3.0 mM $Ag^+$ for 48 h. For comparative purposes, SRKP and SSKP were also grown on LB containing no added $Ag^+$ and at a sub-MIC level of 100 μM $Ag^+$. A small amount of growth from these plates was then suspended and washed in 0.1 M PBS before being fixed in 2.5% glutaraldehyde (prepared in 0.1 M PBS) overnight at 4°C. Cells were then washed

**TABLE 2** Summary of findings from *sil* gene PCR and MIC testing

| Identity | Designation | *sil* gene detected | | | | | | | MIC ($\mu$M $Ag^+$) |
|---|---|---|---|---|---|---|---|---|---|
| | | *silA* | *silB* | *silCBA* | *silE* | *silF* | *silP* | *silRS* | |
| *Candida tropicalis* | 994351 | + | + | + | | + | + | | 250 |
| *Citrobacter freundii* | 2253315 | + | + | + | | + | + | + | 250 |
| *Enterobacter aerogenes* | 761316 | + | + | + | + | + | + | + | 300 |
| *Enterobacter cloacae* | 9663501 | + | + | + | | + | + | | 300 |
| *Enterobacter cloacae* (SREC) | 141419 | + | + | + | | + | + | | 5,500 |
| *Enterobacter cloacae* | 473289 | + | + | + | | + | + | | 350 |
| *Enterobacter cloacae* | 20022641 | + | + | + | + | + | + | + | 1,000 |
| *Enterobacter cloacae* | 11162061 | + | + | + | | + | + | | 500 |
| *Enterobacter cloacae* (SSEC) | 16051951 | + | + | + | | + | + | | 300 |
| *Enterobacter cloacae* | 19362951 | + | + | + | + | + | + | | 600 |
| *Enterococcus* sp. | 221315 | | + | | | | | | 250 |
| *Enterococcus* sp. | 10022641 | + | + | + | + | + | + | | 300 |
| *Enterococcus* sp. | 14591051 | + | | | | | | | 250 |
| *Escherichia coli* | 1291301 | + | + | + | + | + | + | + | 300 |
| *Klebsiella oxytoca* | 2760274 | + | + | + | | + | + | | 300 |
| *Klebsiella oxytoca* | 9780161 | + | + | + | | + | + | | 300 |
| *Klebsiella pneumoniae* | 3471401 | + | + | + | + | + | + | + | 350 |
| *Klebsiella pneumoniae* | 833501 | + | + | + | | + | + | | 300 |
| *Klebsiella pneumoniae* | 2550359 | + | + | + | + | + | + | | 250 |
| *Klebsiella pneumoniae* (SRKP) | 685129 | + | + | + | | + | + | | 5,500 |
| *Klebsiella pneumoniae* | 196319 | | + | + | | + | + | | 300 |
| *Klebsiella pneumoniae* | 9352541 | + | + | + | + | + | + | | 300 |
| *Klebsiella pneumoniae* | 747184 | + | + | + | | + | + | | 300 |
| *Klebsiella pneumoniae* | 11041641 | + | + | + | + | + | + | | 300 |
| *Klebsiella pneumoniae* (SSKP) | 28641251 | + | + | + | | + | + | | 300 |
| *Klebsiella pneumoniae* | 7790451 | + | | | | | | | 300 |
| *Proteus mirabilis* | 6532841 | | + | | | | | | 250 |
| *Providencia rettgeri* | 1712441 | + | | | | | | | 250 |
| *Pseudomonas aeruginosa* | 598161 | + | + | + | | + | + | + | 250 |
| *Staphylococcus aureus* | 7183441 | + | + | + | | + | + | | 300 |
| *Stenotrophomonas maltophilia* | 160451 | + | + | + | | + | + | | 300 |
| *Escherichia coli* pMG101 | Control | + | + | + | + | + | + | + | 1,700 |
| *Escherichia coli* J53 | Control | | | | | | | | 200 |
| *K. pneumoniae* ATCC 13883 | Control | | | | | | | | 100 |
| *E. cloacae* ATCC 13047 | Control | + | + | + | + | + | + | | 200 |

twice with distilled water and dehydrated in a series of ethanol washes (10 to 100% in 10% increments). Cells were incubated at each concentration of ethanol for 20 min. The final suspension of cells in 100% ethanol was deposited onto a silicon wafer and dried in a vacuum desiccant chamber overnight at room temperature. The wafers were then deposited on a brass stub and examined by scanning electron microscopy (SEM; JEOL-7600F field emission SEM). Energy-dispersive X-ray spectroscopy (EDS; Oxford INCA X-ACT with a silicon drift detector) was carried out to detect any silver that had localized on the cell surface.

**Preparation of outer membrane proteins and analysis by SDS-PAGE.** Membranes were prepared from isolates SRKP, SREC, SSKP, SSEC, *E. cloacae* ATCC 13047, and *K. pneumoniae* ATCC 13883. Cultures were grown in LB containing 0.0 g/liter NaCl supplemented with 100 $\mu$M $Ag^+$ overnight. Cells were harvested from 50 ml of culture by centrifugation at 10,000 $\times$ *g* for 10 min at 4°C. The supernatant was removed and the pellet was resuspended in 7 ml of buffer (100 mM Tris-HCl, 500 mM sucrose, 1 mM EDTA; pH 8.0) and incubated on ice for 30 min. After incubation, spheroplasts were removed by centrifugation at 13,000 $\times$ *g* for 5 min at 4°C. The supernatant containing the soluble periplasmic components was loaded onto a Vivaspin 6 bioconcentrator (10-kDa molecular mass cutoff; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and centrifuged at 3,220 $\times$ *g* for 4 h at 4°C until the supernatant was reduced to approximately 100 $\mu$l. The concentrated membrane fractions were quantified using a Bradford assay, and equal amounts were analyzed using SDS-PAGE. Seven micrograms of protein was loaded onto a 4-to-15% Mini-Protean TGX gel (Bio-Rad, Hercules, CA) and electrophoresed for 45 min at 150 V. Gels were stained with Coomassie blue and visualized using white light transillumination.

## RESULTS

**Bacterial isolates.** The 859 clinical isolates consisted of 60 different species. The majority of isolates tested belonged to *Staphylococcus* ($n$ = 148), *Escherichia* ($n$ = 256), *Pseudomonas* ($n$ = 54), *Klebsiella* ($n$ = 69), *Enterococcus* ($n$ = 64), and *Enterobacter* ($n$ = 44) genera. In addition, 52 yeast isolates from the genus *Candida* were included in the survey. Most of the bacterial strains showed multiple-antibiotic drug resistance. Out of these isolates, 66 (7.7%) were able to grow on LB agar supplemented with 250 $\mu$M $Ag^+$ and were selected for further analysis.

**Detection of *sil* genes and DNA sequencing.** Findings from our PCR analysis revealed that one or more *sil* genes were present in 32/66 of the isolates capable of growth at 250 $\mu$M $Ag^+$ (Table 2). Four isolates tested positive for all seven primer pairs, indicating the presence of eight *sil* genes. Fourteen isolates yielded PCR products for all primer pairs excluding *silE* and *silRS*. Five isolates contained only one *sil* gene. It was noted that of the 32 *sil*-positive

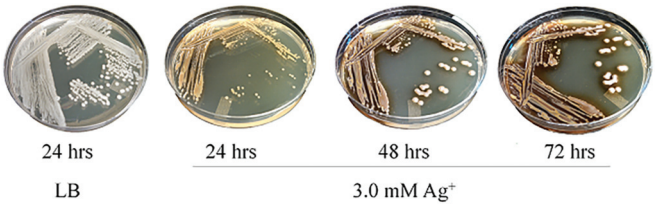


**FIG 1** SRKP grown on LB agar supplemented with 3.0 mM $Ag^+$ over a period of 3 days. Conditions were as follows (from left to right): 0.0 mM $Ag^+$ at 24 h, 3.0 mM $Ag^+$ at 24 h, 3.0 mM $Ag^+$ at 48 h, and 3.0 mM $Ag^+$ at 72 h. Colonies increased in size and developed a dark, metallic appearance over the period of incubation.

isolates, 8 (25%) were members of the genus *Enterobacter* and 12 (38%) were members of the genus *Klebsiella*. Of the 32 isolates containing *sil* genes, five were capable of growth in the presence of ≥500 μM $Ag^+$. Two of these isolates, *Klebsiella. pneumoniae* designation 685129 (SRKP) and *Enterobacter cloacae* designation 141419 (SREC), were capable of growth at a concentration of silver exceeding that of the control silver-resistant organism, *E. coli* pMG101. Both SRKP and SREC yielded a MIC value of 5,500 μM $Ag^+$. The positive control was capable of growth at 1,700 μM $Ag^+$. A complete listing of MIC values from the 32 isolates tested can be found in Table 2.

Due to the high level of resistance to ionic silver, the *sil* gene PCR products from SRKP and SREC were sequenced to confirm their identities. Sequence analysis revealed that PCR products from SRKP were ≥98% similar to plasmid pKPN3, a plasmid containing multiple genes for metal resistance (GenBank accession number CP000648). However, a high degree of similarity was also observed for several other megaplasmids in the *Klebsiella* taxid. Returned sequences from SRKP were ≥89% similar to *sil* genes on the control plasmid, pMG101 (GenBank accession number AF067954). PCR products from SREC were ≥95% identical to pMG101. Due to the genomic presence of the *cus* operon in many species of *Enterobacteriacae*, plasmid preparations from both SRKP and SREC were screened using the aforementioned *sil* gene PCR. Results from this PCR indicated that the *sil* genes present in these two isolates are located on extrachromosomal DNA.

**SEM and EDS findings.** Throughout the study, it was noted that SRKP and SREC became darkly colored when grown on LB containing a high concentration of silver (Fig. 1). In an effort to explore the interaction between the bacteria and ionic silver, SEM was used to image the isolates grown in a high-silver environment. SEM images revealed the presence of suspected silver nanoparticles embedded in the extracellular polymeric substance (EPS) of both isolates; EDS confirmed the presence of silver in the nanoparticle location (Fig. 2). Imaged cells grown in the absence of $Ag^+$ or at a sub-MIC of $Ag^+$ produced no nanoparticles (Fig. 2).

**Sensitivity to silver-based dressings.** Two different assays were used to explore the effects of commercially available silver-based wound dressings on SRKP and SREC. Both assays revealed that these isolates are resistant to various forms and concentrations of silver at a clinically significant level. Results from the dynamic exposure indicated that both SRKP and SREC are largely resistant to seven of the nine silver dressings used in the study after 24 h of contact. At most, SRKP was reduced 79.5% by the Therabond dressing. SREC was reduced >90% by the Exsalt-T7 and Therabond dressings. In contrast, both of the low-MIC isolates, SSEC and SSKP, were reduced 100% by all nine of the dressings used in the study (Fig. 3). Data from the CZOI assay supported these findings. Both SRKP and SREC produced no significant zones of inhibition after contact with the dressings, while the low-MIC organisms yielded a measurable zone of inhibition from eight of the nine dressings used in the study (Fig. 4). Swabs from underneath the dressings postincubation revealed that SRKP and SREC remained viable. In contrast, no secondary growth of the low-MIC organisms was observed in eight of the nine dressings (Table 3).

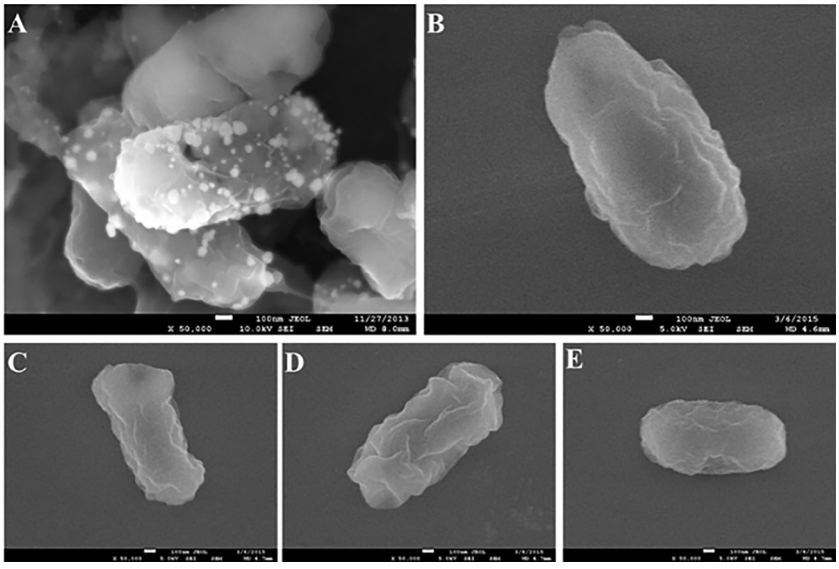


**FIG 2** SEM images of SRKP and SSKP grown in the presence or absence of $Ag^+$. Conditions were as follows: (A) SRKP grown on 3.0 mM $Ag^+$; (B) SRKP grown on 0 μM $Ag^+$; (C) SRKP grown on 100 μM $Ag^+$; (D) SSKP grown on 100 μM $Ag^+$; (E) SSKP grown on 0 μM $Ag^+$. Note the presence of silver nanoparticles when SRKP was grown in high concentrations of silver. Subsequent EDS analysis confirmed the presence of silver localized on the cell surface.

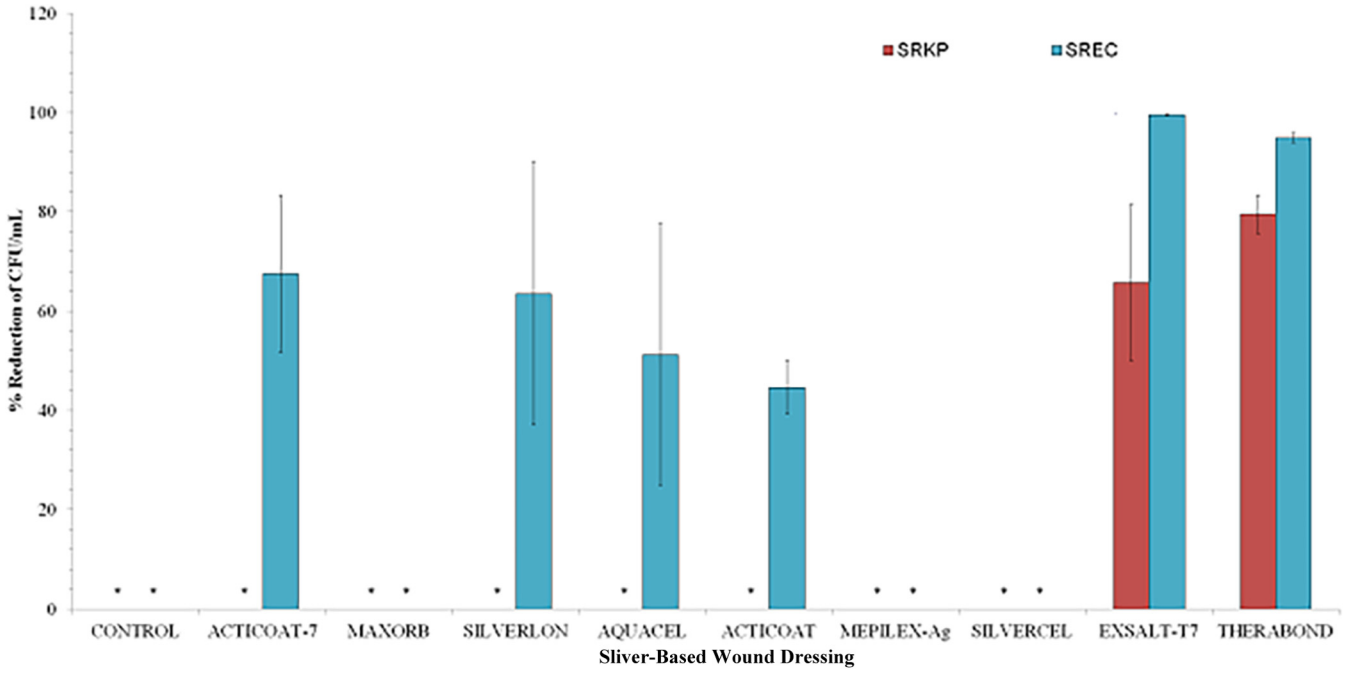


**FIG 3** Bar graph illustrating the percent reduction of challenge organisms after 24 h of dynamic contact with commercially available silver-impregnated wound dressings. Isolates SSKP and SSEC were reduced by 100% and were excluded from the figure for clarity. Error bars represent standard deviations. Asterisks (with absence of bars) indicate that there was no reduction in CFU per milliliter. Values are means of two independent experiments ($n = 2$).

**Analysis of outer membrane proteins by SDS-PAGE.** SDS-PAGE revealed the presence of a porin homologous to OmpC in *E. coli* that was also present in all of the *Klebisella* and *Enterobacter* strains analyzed. However, the OmpF homologue was either absent or underexpressed in all of the silver-resistant (SR) and silver-sensitive (SS) isolates (Fig. 5). Additional preparations were performed from cultures grown in LB without $Ag^+$ supplementation, and the OmpF homologue was still not expressed in either silver-resistant or silver-sensitive organisms at a level comparable to the controls (data not shown).

## DISCUSSION

Although the previous literature lacks evidence supporting widespread silver resistance, it remains a topic of interest warranted by the existence of silver resistance genetic determinants and increased antimicrobial stewardship practices. In this study, we identified the first two bacteria isolated from hospital patients displaying clinically relevant levels of silver resistance. Our findings suggest a low prevalence of these genes (3.6% minimum) occurring in hospital isolates and even fewer (0.2%) with clinically significant phenotypic expression of silver resistance.

However, the relatively low level of observed phenotypic silver resistance was not surprising, due to the mechanism by which *sil* genes on the plasmid pMG101 are transferred. Belonging to the incompatibility group H1 (IncH1), pMG101 and related plasmids are optimally transferred at or below 25°C. IncH1 plasmids have been shown to spread antibiotic resistance in environments with traditionally lower temperatures, such as soil and water (30). These environments provide the greatest probability of bacterial interactions with trace concentrations of heavy metals, warranting the need for minimal phenotypic expression for protection. From a clinical perspective, IncH1 plasmids are less worrisome, because genetic transfer becomes inhibited beyond 37°C, decreasing the risk of widespread resistance within and between species in patient populations. Additionally, the transfer of IncH1 plasmids requires the production of flexible H pili, whose production is inhibited at or above 37°C (30). Again, this supports the lack of concern for widespread transfer of IncH1 plasmids in patient populations.

Despite the unlikely transfer of IncHI in a clinical setting, there is evidence that *sil* gene homologues are carried on a multitude of different plasmids belonging to a variety of different incompatibility groups. Although this study did not fully investigate the plasmids retained in SRKP and SREC, analysis of plasmid extractions revealed the presence of several large plasmids in each isolate and *sil* genes present in extrachromosomal DNA (data not shown). Data obtained from sequencing of the *sil* gene PCR products from SRKP and SREC suggested that the *sil* gene homologues contained in these isolates differ from those of pMG101. Sequence analysis suggested that the *sil* genes from SRKP more likely originated from a plasmid such as pKPN3, one of many IncFII-type resistance plasmids commonly found in the *K. pneumoniae* taxid (31). From a clinical perspective, IncFII plasmids are persistent, highly mobile genetic elements that have the capability to spread *sil* gene homologs.

Both SRKP and SREC indicated the presence of multiple *sil* genes for a three-component efflux pump and a P-type cation ATPase.(10) However, compared to the pMG101 *sil* gene cassette, both isolates lacked *silRS* and *silE*, which carry genes for a two-component membrane-bound transcription regulator and a periplasmic silver cation binding protein, respectively. Additionally, the profile of silver resistance genes present in these two isolates cannot be fully explained by the endogenous Cus system, which lacks the P-type ATPase found in the Sil system (24, 25). Given the high degree of silver resistance observed in both isolates,

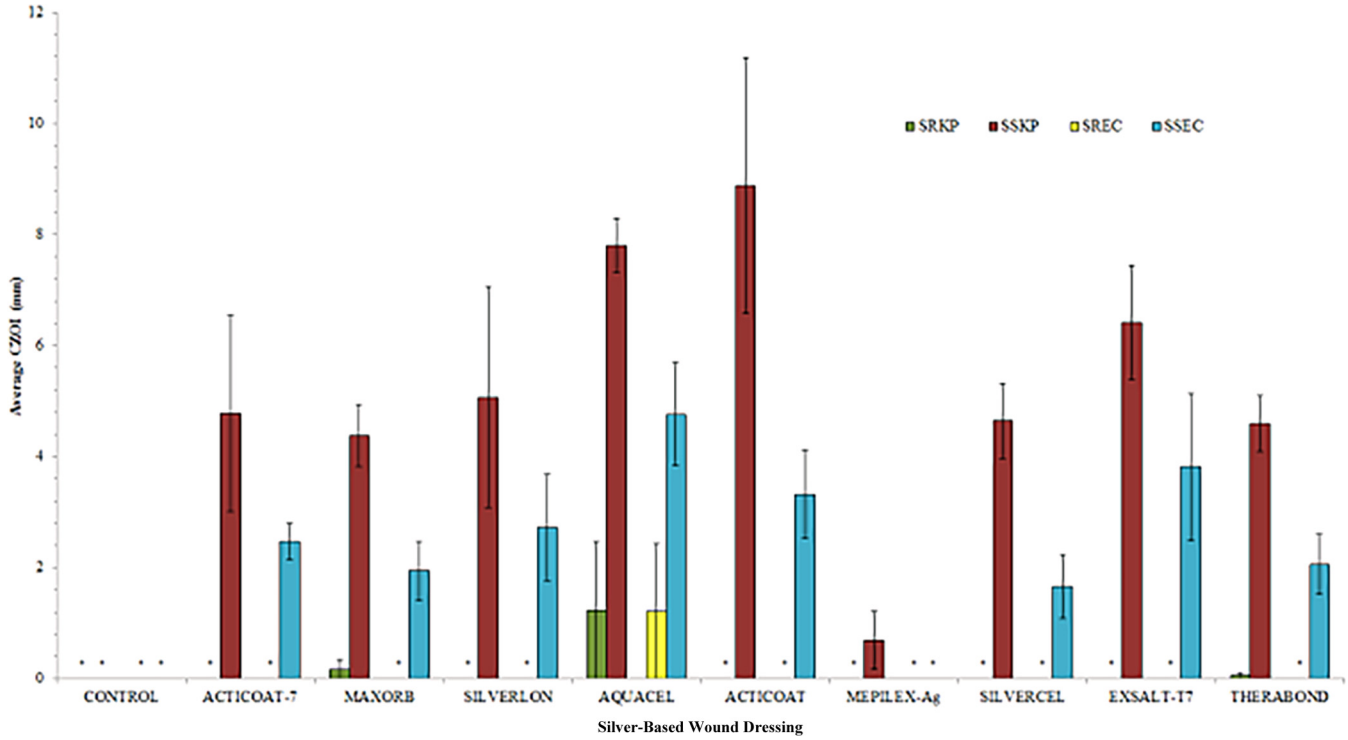


**FIG 4** Bar graph illustrating CZOI measurements of challenge organisms after 24 h of static contact with commercially available silver-impregnated wound dressings. Error bars represent standard deviations. Asterisks indicate no measurable CZOI. Values are means of two independent experiments ($n = 2$).

as well as the presence of nanocrystalline silver species embedded in the EPS, it is unlikely that these isolates are missing these two mechanisms of resistance. BLAST analysis of *sil* gene products from pMG101 and IncFII-type plasmids, such as pKNP3, revealed a total nucleotide difference of 8% and 6% for *silE* and *silRS*, respectively. These differences in nucleotide sequence likely account for the lack of detection by the pMG101 primer set and may also contribute to the elevated level of phenotypic silver resistance seen in both isolates. To our knowledge, no investigations have been conducted that have examined silver resistance as a result of IncFII-type *sil* gene cassettes.

As reported above, SRKP and SREC became darkly colored when grown on LB containing a high concentration of silver. This finding is similar to previous reports of blackening pigmentation of bacteria exposed to high ionic silver concentrations, including MBP-AgBP2p and *P. stutzeri* AG259 (9, 26, 32, 33). This color is proposed to be a result of silver particles building up on the surface of the cell after reduction of Ag(I) to the less toxic nanocrystalline silver species Ag(0). This finding was confirmed in SRKP and SREC through SEM and EDS. In an effort to further investigate the extracellular accumulation of silver nanoparticles, the expression levels of two porins (homologous to OmpC and OmpF in *E. coli*) were examined in SREC, SRKP, and control cultures of *K. pneumoniae* and *E. cloacae*. Analysis of the outer membrane porins in SRKP and SREC grown in the presence of both sub-MICs of silver (100 μM $Ag^+$) and low osmolarity (0.0 g/liter NaCl) revealed that only one porin, OmpF, was lost in these isolates. The losses of OmpC and OmpF in *E. coli* as a result of mutations in *ompR* and *cusS* have been shown to result in lower concentrations of intracellular silver and an overall greater resistance to silver (24). However, without the loss of both porins, the role of reduced membrane permeability in the development of hyper-silver resistance in SREC and SRKP remains unclear. Given that both of the resistant organisms identified in this work are capable of accumulating large amounts of extracellular silver, any change in outer membrane permeability is likely significant and warrants additional studies. The losses of OmpC and OmpF in *E. coli* as a result of mutations in *ompR* and *cusS* have been shown to result in lower concentrations of intracellular silver and an overall greater resistance to silver. (24) However, without the loss of both porins, it is unlikely that reduced membrane permeability is a major factor in the extracellular accumulation of silver or the hyper-silver resistance observed in SREC or SRKP.

Although the mechanism remains unclear, the redox reaction

**TABLE 3** Secondary growth observed after swabbing the area directly underneath each dressing following 24 h of incubation

| | Secondary growth observed with challenge microorganism? | | | |
|---|---|---|---|---|
| Bandage | SRKP | SSKP | SREC | SSEC |
| Control | Y | Y | Y | Y |
| Acticoat-7 | Y | | Y | |
| Maxorb | Y | | Y | |
| Silverlon | Y | | Y | |
| Aquacel | Y | | Y | |
| Acticoat | Y | | Y | |
| Mepiliex-Ag | Y | Y | Y | Y |
| Silvercel | Y | | Y | |
| Exsalt-T7 | Y | | Y | |
| Therabond | Y | | Y | |

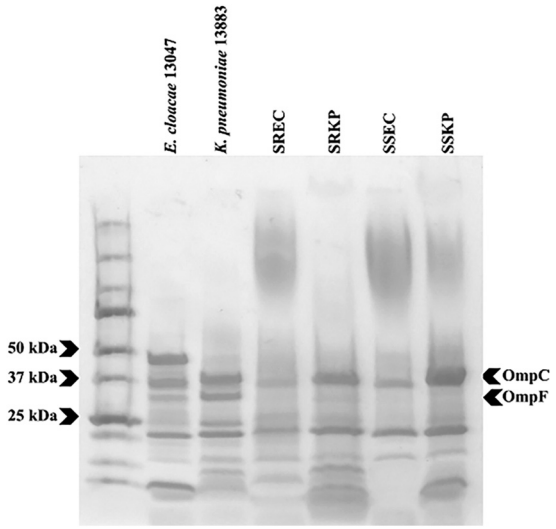


**FIG 5** SDS-PAGE results with outer membrane preparations from *K. pneumoniae* and *E. cloacae* strains used in this study. Isolates were grown in LB supplemented with 100 μM $Ag^+$. All silver-resistant and silver-sensitive isolates underexpressed or lacked the OmpF homologue, which was detected only from control cultures.

was suggested in early work to be (at least in part) a potential mechanism for the observed silver resistance (11). More recent work has provided details concerning metal binding mechanisms. Cytochromes, periplasmic polypeptides extending through the cell surface, have been shown to be the reducing agent driving electron transfer with heavy metals and other extracellular "oxidizers" (32, 34, 35). However, how the sequestering and reduction of silver relate to the observed increased resistance remains unclear. Simple sequestering of silver in EPS may be too parsimonious, and the silver resistance mechanism is more complex than initially proposed (32). It is important to note that while endogenous and exogenous mechanisms accounting for silver resistance have been put forth, detailed characterizations of this phenomenon remain unclear across Gram-positive and Gram-negative bacteria.

From a clinical perspective, SRKP and SREC demonstrate the potential to impact current silver-based therapies used in the areas of burn and wound treatment. Two different *in vitro* methods were used to assess the effectiveness of a cross-section of commercially available silver-based wound dressings against these silver-resistant strains. These methods were chosen to best utilize the different properties of each dressing, as the type of silver used by the manufacturer of the dressing varied greatly in terms of solubility, concentration, and antimicrobial effectiveness. Results from these assays indicated that both isolates demonstrated silver resistance at a level that is capable of greatly reducing, if not negating, the effectiveness of most commercially available silver dressings, compared to nonresistant counterparts of the same species.

Acute emergence of silver resistance would have extensive consequences on current burn and wound therapies. Silver has become a popular treatment option, in part because it may provide multiple benefits during soft tissue regeneration. Beyond its antimicrobial properties, silver may also improve wound healing by directly reducing local inflammation. Animal models have provided evidence of multiple wound healing benefits via treatment with silver, including reductions in matrix metalloproteinases and erythema (36), reductions in swelling and proinflammatory cytokines (37, 38), and active fibroblasts, thicker granulation beds, and limited inflammation (39). Regardless of the mechanism, the ability of silver to reduce inflammation and facilitate wound healing has resulted in a significant increase in its clinical utilization. In addition, the FDA has provided marketing clearance of many silver-impregnated wound dressings and topical agents which are now readily available.

Here, we identified the first strains of clinical bacteria expressing silver resistance at a level that could significantly impact the use of silver dressings in health care. Continued monitoring for silver resistance should be maintained and is warranted under increased antimicrobial stewardship practices.

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