Supplementary Information for

Silver Enhances Antibiotic Activity Against Gram-negative Bacteria

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Table S5. Raw data showing potentiation of vancomycin using Ag^{+} as an adjuvant compound.

Supplementary Materials and Methods

Antibiotics and Chemicals

All experiments were performed in Luria Bertani (LB) medium (Fisher Scientific). Unless otherwise specified, the following concentrations were used for the antibacterial experiments with *E. coli:* 10, 20, 30, 60 and 120 μM silver nitrate (Fisher Scientific), 1 μg/mL and 10 μg/mL ampicillin (Fisher Scientific), 0.25 μg/mL and 5 μg/mL gentamicin (Fisher), 0.03 μg/mL and 3 μg/mL ofloxacin (Sigma), and 30 μg/mL vancomycin (MP Pharmaceuticals). Additional chemicals used in this work include: phosphate buffer saline (PBS) (Sigma), Tris/HCl buffer pH 7.5 (Sigma), Ferene-S (Fisher Scientific), 3'-p-hydroxyphenyl fluorescein dye (Invitrogen), propidium iodide (Invitrogen), and thiourea (Fluka).

Media and Growth Conditions

Exponential Phase Experiments

The growth and survival of untreated log-phase *E. coli* strains were compared with the antibiotic-treated strains. Briefly, cultures from frozen stock were grown overnight. The overnight culture was diluted 1:250 with 25 mL of LB medium in 250 mL flasks and incubated at 37 ºC, 300 rpm and 80% humidity. Cells were grown until an OD_{600} of 0.3. 500 μL samples were taken from the flasks and transferred to a 24-well plate for antibiotic treatment. For the hydroxyl radical (OH•) quenching experiments, thiourea (150 mM) was added simultaneously with the antibiotics. Measurements of OD_{600} were taken using a SpectraFluor Plus (Tecan). For the cfu/mL measurement, 100 μL of culture was collected and then serially diluted in PBS (pH 7.2). A 5 μL portion of each dilution was plated in LB-agar plates and incubated overnight at 37 ºC. The colonies were counted and cfu/mL was calculated using the following formula: ((#colonies)*(dilution factor))/(amount plated).

Bacterial Persister Cell Experiments

Survival of untreated *E. coli* persister cells was compared with that of antibiotic-treated persister cells. Briefly, cultures from frozen stock were grown to stationary phase for 16 h in 25 mL of LB medium in 250 mL flasks and incubated at 37 ºC, 300 rpm and 80% humidity. Cells were then treated with 5 μg/mL ofloxacin for 4 h to kill non-persister cells(*1*). The samples were then washed with PBS and suspended in M9 minimal media, and treated with the different antibiotics. To determine bacterial persister counts, 100 μL of culture was collected and then serially diluted in PBS (pH 7.2). A 5 μL portion of each dilution was plated in LB-agar plates and incubated overnight at 37 ºC. The colonies were counted and cfu/mL was calculated using the following formula: ((#colonies)*(dilution factor))/(amount plated).

Biofilm Experiments

Survival of *E. coli* in untreated biofilms was compared with that of *E. coli* in antibiotic-treated biofilms. *E. coli* were grown for 24 h at 30 ºC, 0 rpm and 80% humidity in MBEC Physiology and Genetic Assay wells (MBEC BioProducts, Edmonton, Canada) previously inoculated with a 1:200 dilution of overnight grown *E. coli* cells. Wells containing biofilms were then treated with the different antibiotics. After treatment, the wells were washed with PBS 3X. To determine viable cell counts following treatments, the plates were sonicated for 45 min to disrupt the biofilms. 100 μL of the disrupted biofilm in the PBS was collected and serially diluted in PBS (pH 7.2). A 5 μL portion of each dilution was plated in LB-agar plates, which were then incubated overnight at 37 ºC. The colonies were counted and cfu/mL was calculated using the following formula: ((#colonies)*(dilution factor))/(amount plated).

Quantifying Synergy Using the Bliss Model

Drug synergism was calculated using the Bliss Independence Model, which calculates a degree of synergy using the following formula: $S = (f_{X0}/f_{00})(f_{0Y}/f_{00})-(f_{XY}/f_{00})$, where f_{XY} refers to the wildtype growth rate in the presence of the combined drugs at a concentration X, for one of the drugs, and Y for the other; f_{X0} and f_{0Y} refer to the wildtype growth rates in the presence of the individual drugs at a concentration of X and Y, respectively; f_{00} refers to the wildtype growth rate in the absence of drugs; and S corresponds to the degree of synergy, a parameter that determines a synergistic interaction for positive values and an antagonistic interaction for negative ones(*2*). Growth rates at different time points are determined by calculating the slope of the growth or kill curve being analyzed.

Bacterial Strains

All *E. coli* experiments were performed with MG1655 (ATCC 700926)-derived strains. The knockouts were constructed using P1 phage transduction and derived from the Keio single-gene knockout collection(*3*). Removal of the kanamycin-resistance cassette was accomplished using the pcp20 plasmid and confirmed by PCR prior to experimentation.

Construction of Measurement of the sodA Overexpression Vector

The native *sodA* gene was amplified from the wildtype MG1655 genome using PCR amplification and the following primers: 5′-CGG CGG ATC CTT ATT TTT TCG CCG CAA AAC GTG CC-3′ and 5′- GCG CGC AAG CTT ATG AGCTAT ACC CTG CCATC -3′. The resulting PCR product was then cloned into the BamHI and HindIII sites of the plasmid vector pZE21(*4*), which placed the *sodA* gene under control of a pLTetO promoter. Restriction digests and ligations were performed with enzymes from New England Biolabs. For treatments, MG1655Pro cells (F^-, λ^-, Sp^R, *lacR*, *tetR*) from an overnight culture were diluted 1:500 into LB

plus 100 μg/mL ampicillin and grown to an OD_{600} of 0.3. Cells were then induced with 50 ng/mL anhydrotetracycline (Sigma Aldrich) for 1 h. Samples were collected for time 0 h measurements and then cultures were treated with 30 μM $AgNO_3$. Samples were then collected every hour for 3 h for serial dilution and plated for cfu determination.

Determination of Minimum Inhibitory Concentrations

Overnight cultures were diluted 1:10,000 into 200 μL of Luria-Bertani media in 96-well plates. Serial dilutions of antibiotics were added to the individual wells. For the re-sensitization experiments with the *AG100* and *AG112* strains, $AgNO_3$ and tetracycline alone as well as in combination were added at various concentrations. Plates were then grown overnight in a plate shaker shaking at 900 rpm at 37 °C. The minimum inhibitory concentration was determined as the lowest concentration of drug that inhibited 90% of growth compared to the untreated strain (MIC90) after 16 h.

Transmission Electron Microscopy

Samples analyzed with transmission electron microscopy were prepared using the following procedure: 100 μL samples were taken, centrifuged and resuspended in 1X PBS twice. To fix the bacterial cells, the samples were centrifuged and resuspended in 1 mL of 2.5% glutaraldehyde solution in PBS for 30 min. The cells were then dehydrated by exposing them for 10 min to a series of ethanol concentration solutions (50, 60, 80, 90 and 100% ethanol/PBS solutions). The cells were finally embedded into a Spurr resin and left to polymerize in an oven at 60 ºC for 24 h. The polymerized samples were sectioned into slices of thickness of ~60 nm using a microtome. The sliced samples were analyzed in a Jeol 1200EX – 80kV.

Iron Detection Ferene-S Colorimetric Assay

The release of protein-bound iron in an *E. coli* cell lysate was measured using a Ferene-S assay. The lysate was prepared by first growing 150 mL of cells to an OD_{600} of 0.7. The cells were then lysed by sonication in 20 mM Tris/HCl pH 7.2 buffer. The samples were centrifuged and the supernatants containing the cell lysates were collected. Lysates were treated either with heat (90 ºC for 20 min) or 30 μM $AgNO_3$. 10 mM Ferene-S was added to each sample, and samples were then incubated at room temperature for 1 h. Absorbance at 593 nm was then measured.

Iron Misregulation Superoxide Production and Disulfide Bond Formation Measurements

Iron regulation and superoxide production were assessed using previously constructed genetic reporter strains(*5*). A disulfide bond formation sensor was constructed based on activation of the OxyR protein, which forms a disulfide bond in the presence of H_2O_2. The disulfide bond formation sensor was constructed by PCR amplifying the native *dps* promoter and cloning it into the BamHI and XhoI restriction sites of the pZE21 vector(*4*). The forward primer for PCR was GCGCCTCGAGCCGCTTCAATGGGGTCTACGCT, and the reverse primer was GGCCGGATCCTCGGAGACATCGTTGCGGGTAT. The data were collected using a Becton Dickinson FACSCalibur flow cytometer with a 488-nm argon laser and a 515-545 nm emission filter (FL1) at high flow rate. Calibrite beads (Becton Dickinson) were used for instrument calibration. FlowJo was used to process flow cytometric data.

OH• Production Measurements

In Vitro Measurements of OH•

The fluorescent reporter dye 3'-(p-hydroxyphenyl fluorescein (HPF), which is oxidized by hydroxyl radicals with high specificity, was used for radical detection. Overnight cultures were diluted 1:250 with 25 mL of LB medium in 250 mL flasks and incubated at 37 ºC, 300 rpm, and 80% humidity. Cells were grown until reaching an OD_{600} ~0.3. 500 µL samples were taken from the flasks and transferred to a 24-well plate for treatments. After 1 h of treatment, 100 µL samples were collected, centrifuged at 10,000 rpm, and the media was removed and replaced with PBS + 5 mM HPF. Samples were incubated in the dark at room temperature for 15 min and then centrifuged at 10,000 rpm. The supernatant was removed and replaced with 1X PBS for flow cytometry measurements. The data were collected using the FL1 filter of the Becton Dickinson FACSCalibur flow cytometer described previously. The following equation was used to determine percentage change due to increase in hydroxyl radical production: $((Fluorescence_{HPF} - Fluorescence_{no\ HPF})/(Fluorescence_{no\ HPF}))*(100)$. These values were compared with the same formula between treated and untreated samples to obtain a percent change. FlowJo was used to process flow cytometric data. The same procedure was followed for the fluorescence measurements in persister cells, with the difference that it was measured 3 h after treatment with antibiotics.

In Vivo Measurements from a Peritoneal Infection in Mice

Inbred, wildtype male C57BL/6 mice (6 weeks; ~20 g) were used. After one week of quarantine, inoculation was performed by intraperitoneal (i.p.) injection of 500 µL of $5x10^6$ *E. coli* cells using a 26-gauge syringe. The inoculum was delivered in suspension with 8% (w/v) mucin in sterile saline. One hour after introduction of the inoculum, 50 µL of either PBS or Ag^+

(35μM) was administered intraperitoneally using water as the vehicle. Three mice per group received the treatments. 1 h after treatment, the mice were euthanized and peritoneal washes were performed by injecting 1.0 mL of sterile saline in the peritoneum followed by a massage of the abdomen. Subsequently, the abdomen was opened and 200 μL of peritoneal fluid (PF) was recovered from the peritoneum for OH• production determination in the intraperitoneal *E. coli* cells. OH• production was determined using the previously described HPF dye. Each of the intraperitoneal washes were collected and centrifuged at 10,000 rpm. The media was removed and replaced with PBS + 5 mM HPF. The samples were incubated in the dark at room temperature for 15 min and then centrifuged at 10,000 rpm. The supernatant was removed and replaced with 1X PBS for flow cytometry measurements. In order to eliminate fluorescence from mammalian cells found in the intraperitoneal wash, an *E. coli* cell culture was grown in parallel in flasks and treated in the same way as the intraperitoneal washes. Data were collected using the FL1 filter of the Becton Dickinson FACSCalibur flow cytometer described previously. The data were gated by size by using the *E. coli* grown in cultures. The following equation was used to determine percentage change due to increase in ROS production: $((\text{Fluorescence}_{\text{HPF}} - \text{Fluorescence}_{\text{no HPF}})/(\text{Fluorescence}_{\text{no HPF}}))*(100)$. These values were compared with the same formula between treated and untreated samples to obtain a percent change. FlowJo was used to process flow cytometric data.

Membrane Permeability Measurements

In Vitro Measurements of Membrane Permeability

The fluorescent reporter dye propidium iodide (PI) was used to monitor membrane permeability. Increased PI fluorescence is correlated with increased membrane permeability(*6, 7*). Overnight cultures were diluted 1:250 with 25 mL of LB medium in 250 mL flasks and

incubated at 37 ºC, 300 rpm and 80% humidity. Cells were grown until reaching an OD_{600nm} ~0.3. 500 µL samples were taken from the flasks and transferred to a 24-well plate for treatments. After 1 hour of treatment, 100µL samples were collected, centrifuged at 10,000 rpm, and the media was removed and replaced with PBS + 1mM PI. For the persister cell experiments, the samples were collected after 3 h of treatment instead of 1 h. Samples were incubated in the dark at room temperature for 15 min and then centrifuged at 10,000 rpm. The supernatant was removed and replaced with 1X PBS for flow cytometry measurements. The data were collected using the FL2 filter of the Becton Dickinson FACSCalibur flow cytometer described previously. The following equation was used to determine percentage change due to increase in cell permeability: $((Fluorescence_{PI} - Fluorescence_{no\ PI})/(Fluorescence_{no\ PI}))*(100)$. These values were compared with the same formula between treated and untreated samples to obtain a percent change. FlowJo was used to process flow cytometric data.

In Vivo Measurements from a Peritoneal Infection in Mice

The same procedure was used for determining *in vivo* production of ROS in bacteria, except that staining was performed with PBS + 1mM PI. The following equation was used to determine percentage change due to increase in cell permeability: $((Fluorescence_{PI} - Fluorescence_{no\ PI})/(Fluorescence_{no\ PI}))*(100)$. These values were compared with the same formula between treated and untreated samples to obtain a percent change. FlowJo was used to process flow cytometric data.

Tissue Culture

Primary human keratinocytes were obtained from neonatal foreskins as described previously(*8*). Briefly, neonatal foreskins were trypsinized overnight at 4 ºC. The keratinocytes were maintained in a serum-free basal medium made of MCDB-153 plus the following amino

acids: L-tryptophan (60 μM), L-tyrosine (90 μM), L-phenylalanine (120 μM), L-methionine (120 μM), L-isoleucine (770 μM), and L-histidine (320 μM). The basal medium was further supplemented with 0.15 mM calcium, 3 μg/mL bovine pituitary extract, 25 ng/mL EGF and 5 μg/mL prostaglandin E1, to make complete medium. For initial plating of cells, complete medium was supplemented with 0.1 μg/mL cholera toxin and 200 ng/mL of hydrocortisone. Cells were fed with complete medium three times per week and passaged using trypsin/EDTA. The SH-SY5Y human neuroblastoma cell line (Lonza) was cultured in DMEM/F12 plus 10% FBS. Cells were fed three times per week and passaged using trypsin/EDTA.

Human primary hepatocytes (CryoHepatocytes, BD Biosciences) were prepared using the Cryopreserved Hepatocyte Purification Kit: Two Steps (BD Bioscience) per the manufacturer's instruction. The cells were plated at a concentration of $4x10^5$ cells per well in 24-well plates coated with rat tail collagen I (BD Biosciences). Cells were fed with HepatoSTIM media (BD Biosciences) three times per week.

MTT Assay

Mitochondrial dehydrogenase enzyme activity is used as a proxy for metabolic activity. The assay tests the ability of cells to enzymatically convert 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium (MTT) to formazan. Formazan is an insoluble colorimetric indicator that can be detected by measuring absorbance at 570 nm.

All three cell types were seeded in 24-well plates at a concentration of $4x10^4$ cells per well. SH-SY5Y cells were differentiated using 10 μM retinoic acid for 72 hours prior to $AgNO_3$ treatment. Hepatocytes were plated on collagen I and incubated with HepatoSTIM (BD Biosciences) 24 hours prior to $AgNO_3$ treatment to induce differentiation.

Primary human keratinocytes, differentiated SH-SY5Y human neuronal cells and differentiated primary human hepatocytes were treated with vehicle alone or 30 μM, 40 μM, 60 μM $AgNO_3$. 24 hours after treatment, the MTT assay was performed. MTT was prepared at a concentration of 1 mg/mL in PBS. Media was aspirated out of each well and replaced with 400 μL of the MTT solution plus 1600 μL of DMEM without phenol red. The MTT-treated cells were incubated for 4 hours at 37 ºC, 5% CO_2. MTT was removed from each well and replaced with 800 μL DMSO plus 200 μL of Sorenson's glycine buffer (0.1 M glycine, 0.1 M NaCl, pH 10.5). The plates were incubated at room temperature for 5 min. Absorbance was measured at 570 nm, with a reference wavelength of 630 nm to quantify the amount of formazan produced by the metabolically active cells

In Vivo Toxicity Studies

Median Lethal Dose (LD_{50}) of Parenterally Delivered Silver in Mice

6-week-old C57BL6 male mice (body weight, ~20 g) were used. 10 mice per group were treated with 50 μL of intraperitoneally administered Ag^+ at 30, 60, 120, and 240 μM concentrations using water as the vehicle. The animals were observed for seven days. The LD_{50} was determined by measuring the concentrations at which only 50% of the recipient mice survived.

Analysis of Diverse Blood Metabolites to Determine Mouse Health

6-week-old C57BL6 male mice (body weight, ~20 g) were used. Three mice per group were treated with parenterally administered PBS, 30 mg vancomycin/kg body weight, 6 mg $AgNO_3$/kg body weight (35 μM), and 30 mg vancomycin/kg body weight in combination with 6 mg $AgNO_3$/kg body weight. The animals were observed for two days and retro-orbital blood

sample collection was performed 6, 24 and 48 h after treatment. Blood was collected in heparinized whole blood test tubes and further analyzed using the Piccolo Comprehensive Metabolic Reagent Disks in a Piccolo Blood Chemistry Analyzer (Abaxis).

Peritoneal Mouse Infection Model

Minimum Lethal Dose (MLD) of E. coli for Peritonitis Mouse Model

For all animal experiments, 6-week-old C57BL6 male mice (body weight, ~20 g) were used. Serial dilutions of *E. coli* ranging from $1x10^6$ – $1x10^9$ cfu/mouse were introduced into the peritoneal cavity of the mice in 500 µL aliquots of sterile saline supplemented with 8% mucin. The animals were observed for seven days. The MLD was determined to be $5x10^6$ cfu/mouse by measuring the lowest concentration of *E. coli* that killed 100% of the recipient mice.

Mouse Peritonitis Model

Inbred, wildtype male C57BL/6 mice (6 weeks; ~20 g) were used. After one week of quarantine, inoculation was performed by intraperitoneal (i.p.) injection of 500 µL of either the MLD *E. coli* inoculums (acute peritonitis model) or $1x10^4$ *E. coli* cells (mild peritonitis model) using a 26-gauge syringe. The inoculum was delivered in suspension with 8% (w/v) mucin in sterile saline. Either 1 h (acute infection model) or 24 h (mild infection model) after introduction of the inoculum, the untreated control group (t=0) was euthanized and the antibacterial therapy was initiated by intraperitoneal injection of 50 µL aliquots for the rest of the groups. Ten mice per group received antibacterial treatments. At time 0 (control only), 8, 16 and 24 h, mice were euthanized. Peritoneal washes were performed by injecting 1.0 mL of sterile saline in the intraperitoneal followed by a massage of the abdomen. Subsequently, the abdomen was opened and 200 µL of peritoneal fluid (PF) was recovered from the peritoneum for analysis of *E. coli* cfu/mL. For the cfu/mL measurement, the peritoneal fluid was serially diluted in PBS (pH 7.2).

A 5 μL portion of each dilution was plated in LB-agar plates and incubated overnight at 37 ºC. Colonies were counted and cfu/mL was calculated using the following formula: ((#colonies)*(dilution factor))/(amount plated).

Survival Assays

Male C57BL6 6-week old mice (weighing ~20 g) received intraperitoneal injections of the MLD of *E. coli* in a volume of 500 μL with 8% mucin. After 1 h, ten mice per group received 50 μL intraperitoneal (i.p.) injections of either vehicle (PBS) only or the different antibacterial treatments. The mice were observed for 5 days to evaluate survival.

UTI Mouse Model

6-week old C57BL/6 female mice were inoculated with 50 μL of 8% (w/v) mucin solution in sterile saline containing $2x10^9$ *E. coli* (MG1655) cells, via transurethral catheterization into their bladders, as previously described(*9*). Briefly, mice were anesthetized using 2-4% isoflurane. Urinary catheters (30G x ½ inch hypodermic needle aseptically covered with polyethylene tubing) were coated in medical grade sterile lubricating jelly. The bladder of the mouse was gently massaged to expel urine. The lubricated catheter was inserted into the urethral opening. It was then pushed into the urethra until the base of the needle reached the urethral opening. Once fully inserted, 50 μL of the inoculum (containing $2x10^9$ *E. coli* cells) was injected directly into the bladder.

Infected animals received designated drug treatments or vehicle (PBS) only via intraperitoneal delivery 24 h post-inoculation. Following treatment, animals were observed for an additional 24 h. At the end of the experiment, animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. Bladders were collected in 1 mL of PBS and homogenized for

30 sec for subsequent quantification of bacterial load. For the cfu/bladder measurements, the homogenized bladder was serially diluted in PBS (pH 7.2). A 200 μL portion of each dilution was plated in LB-agar plates and incubated overnight at 37 ºC. The colonies were counted and cfu/bladder was calculated using the following formula: (((#colonies)*(dilution factor))/(amount plated))*5.

Mouse Biofilm Infection Model

Briefly, intramedic polyethylene tubing (PE10, BD Biosciences) measuring 1 cm in length was incubated in *E. coli* cultures for 24 h to form biofilms. The biofilm-coated tubing was surgically implanted subcutaneously on the back of 8-week old C57BL/6 female mice under 2-4% isoflurane. 48 h after surgery, infected animals received designated drug treatments or vehicle (PBS) only via intraperitoneal (i.p.) delivery. Following treatment, animals were observed for an additional 24 h. At the end of the experiment, animals were euthanized by CO_2 asphyxiation followed by cervical dislocation. The catheter tubing was surgically removed and collected in 1 mL of PBS. The biofilm on the tubing was disrupted through sonication for 45 min for subsequent quantification of bacterial load. For the cfu/catheter measurements, the PBS was serially diluted in PBS (pH 7.2). A 200mL portion of each dilution was plated in LB-agar plates and incubated overnight at 37 ºC. The colonies were counted and cfu/catheter was calculated using the following formula: (((#colonies)*(dilution factor))/(amount plated))*5.

Supplementary Results

Ag^+ Toxicity and Previous Clinical Applications

The EPA has reported tolerances for Ag^+ administered through distinct routes. Orally administered Ag^+ has an LD_{50} of 1206 μM in rats(*10*). In humans, at least 1335 μM of orally administered Ag^+ was required to induce minor side effects, such as skin pigmentation(*11*). These concentrations are much higher (close to 100 times) than those found in our work to be effective for enhancing the efficacy of existing antibiotics. Furthermore, Ag^+ at concentrations of approximately 6000 μM (200–400 times higher than our effective adjuvant concentration) is currently approved by the FDA as an antibacterial for treating topical infections(*12*).

Peritonitis Infection and Urinary Tract Infection Models

We used an acute peritonitis mouse infection model to validate the ability of Ag^+ to potentiate commonly used antibiotics *in vivo*. The use of this peritonitis model to test antibiotic efficacy is widely documented in the literature. This model has contributed significantly to our understanding of host response to infection and the activity of various antibiotics in the treatment of infections(*13*)(*14*). The model depends significantly on the initial load of bacteria administered. High doses of bacteria, in acute models like the one we used in this work, allow for rapid migration of the bacteria into the bloodstream leading to sepsis and death within 24 to 48 hours. In the literature, treatment of these infections is usually administered immediately or up to 8 hours after initial bacterial inoculation, depending on the bacterial strain and load(*9, 15-19*). We chose to treat after one hour because of the high bacterial load required to achieve sepsis.

In order to decrease mice mortality, while extending the time between initial inoculation and antimicrobial treatments, we lowered the initial bacterial load. As expected, the lower load

prevented the infection from going systemic and the mice did not die from the infection. However, this mild infection model was an excellent way to monitor bacterial eradication. Using this model, we observed that when treatment was administered 24 h after the initial inoculation, Ag^+ had a robust potentiation effect in combination with vancomycin (Fig. 7A and B).

Urinary tract infections (UTI) are one of the most common infections diagnosed in clinical settings(*9*). Although these infections are rarely lethal, they represent a great socioeconomic burden since they tend to recur at a high frequency – 25% of the patients that acquire a UTI experience multiple infections within the following six months(*9*). We therefore tested whether Ag^+ could potentiate the antibiotic gentamicin *in vivo* using a urinary tract infection model. We found that while low doses of Ag^+ and gentamicin individually had no effect on *E. coli* cell counts, these were significantly reduced when used in combination (Fig. 6A to C). These results further demonstrate in an additional infection model that Ag^+ is capable of potentiating antibiotics *in vivo*.

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Supplementary Figures

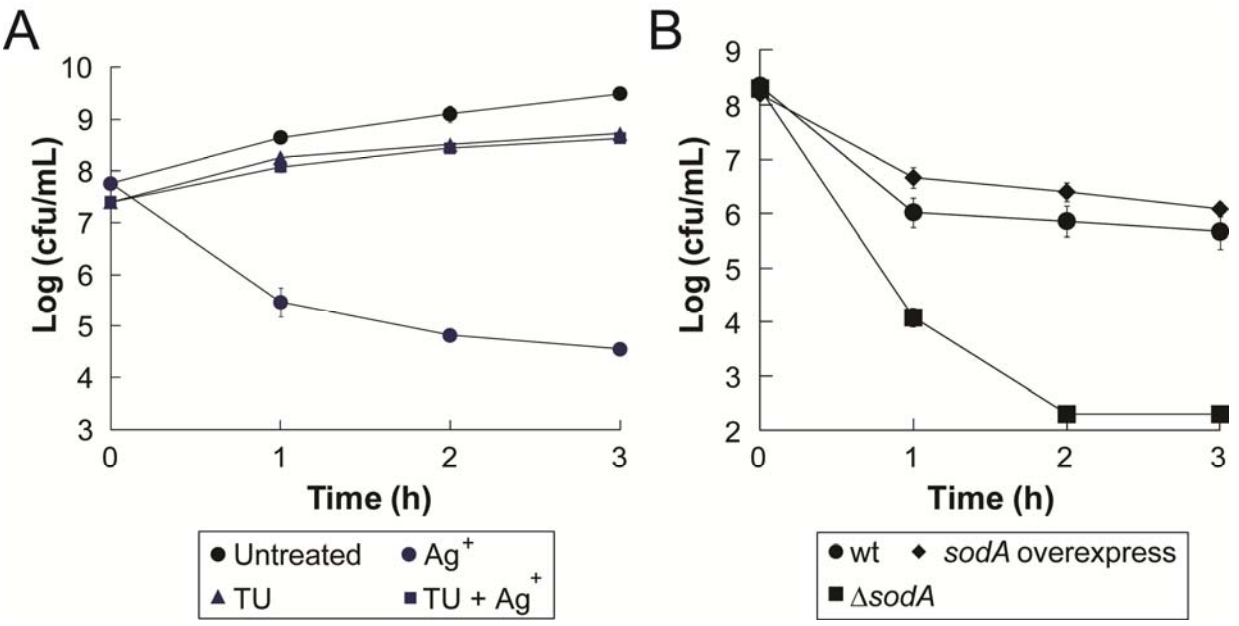


Fig S1. Scavenging OH• within the cell during antibiotic treatment inhibits cell death. **(A)** Kill curves for wildtype *E. coli* treated with 30 μM $AgNO_3$, 150 mM thiourea (TU), or a combination of both. **(B)** Survival of the *ΔsodA* and *sodA* overexpression strain relative to wildtype when treated with 30 μM $AgNO_3$. Error bars represent mean ± SEM for at least 3 biological replicates.

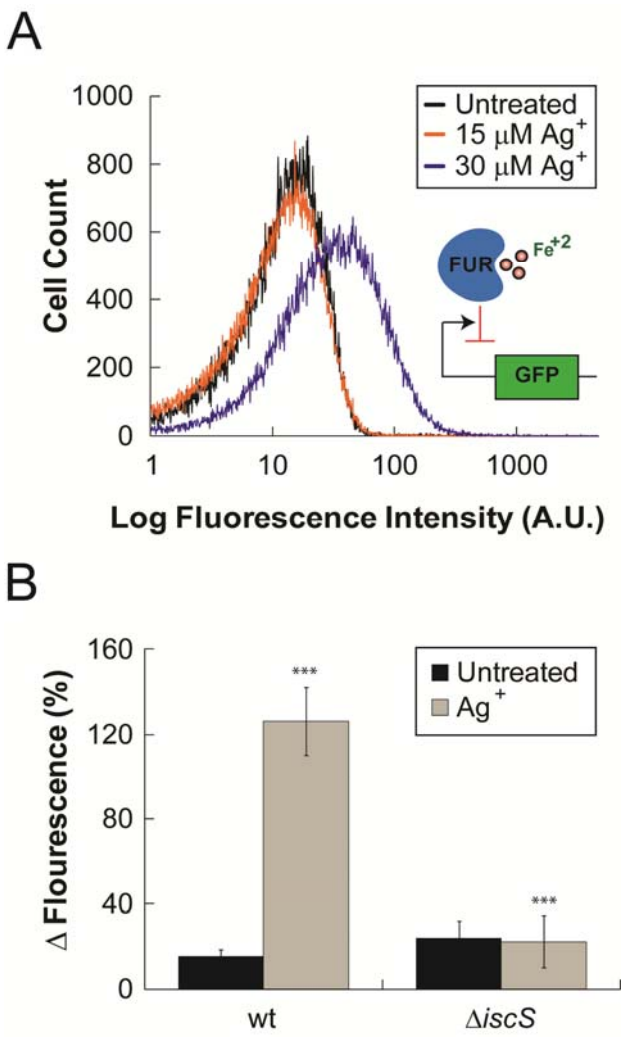


Fig S2. Direct interaction between Ag^{+} and Fe-S clusters leads to iron misregulation, leaking of Fe^{+2} and the overproduction of OH• radicals. **(A)** GFP fluorescence histogram from the Fur reporter strain after 1 h treatment with 15 and 30 μM $AgNO_3$. (*Inset*) Schematic of the reporter strain, which is based on activation of the *fur* gene. **(B)** Change in fluorescence of the HPF-stained wildtype and *ΔiscS* strain after 1 h treatment with 30 μM $AgNO_3$. *** indicates a p-value of <0.001 as determined by a t-test, indicating a significant difference from the treated wildtype strain. Error bars represent mean ± SEM for at least 3 biological replicates.

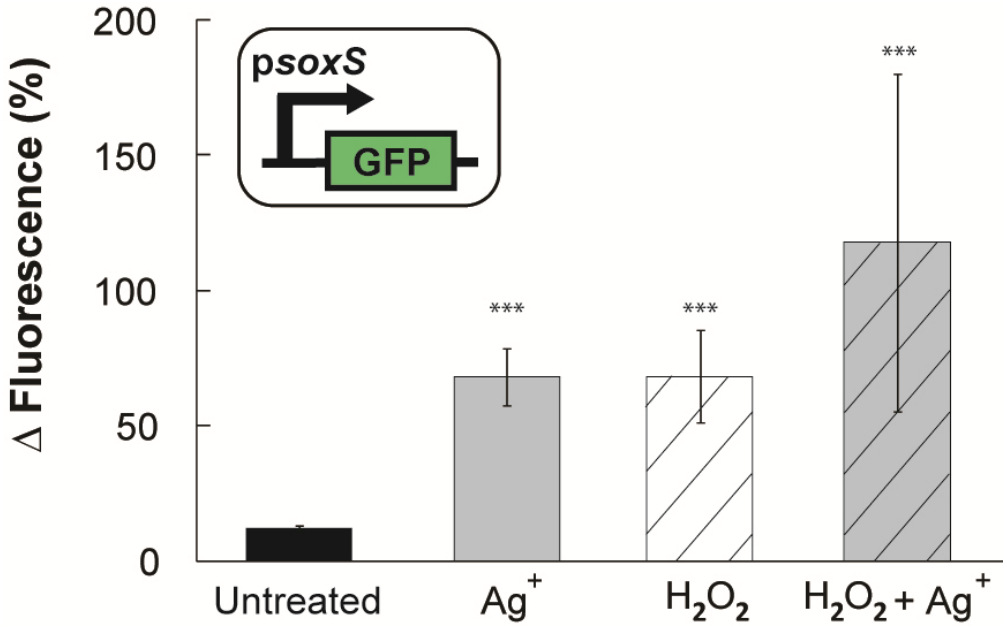


Fig S3. Ag^+ induces activation of *soxS*, indicating production of ROS. Change in GFP fluorescence from the *soxS* reporter strain after 1 h of the indicated treatments. Cells were treated with 30 μM $AgNO_3$, 0.5 mM H_2O_2, or a combination of both. Values shown are relative to fluorescence at time zero before treatment. (*Inset*) Schematic of the reporter strain, which is based on activation of the *soxS* promoter. *** indicates a p-value of <0.001 as determined by a t-test, indicating a significant difference from the untreated strain. Error bars represent mean ± SEM for at least 3 biological replicates.

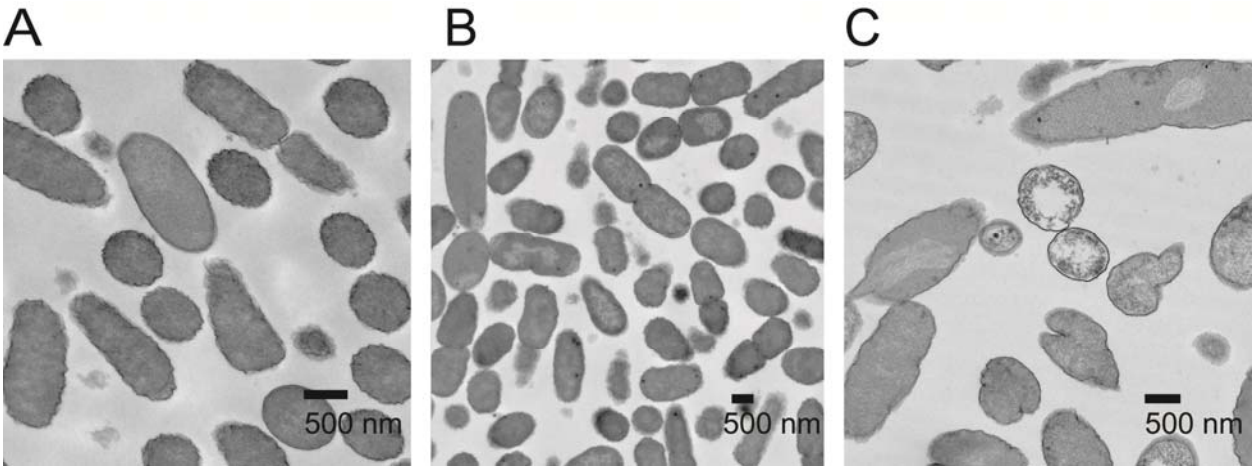


Fig S4. Ag^{+} induces moderate morphological changes and protein aggregate formation when delivered at sublethal concentrations. Transmission electron microscopy micrographs showing: **(A)** 0 μM, **(B)** 10 μM, and **(C)** 20 μM $AgNO_3$-treated *E. coli*.

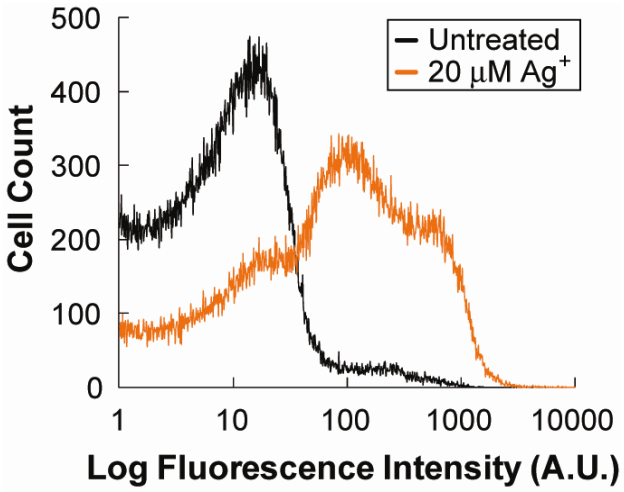


Fig S5. Propidium iodide fluorescence emitted by cells treated with a sublethal concentration of Ag^+. Propidium iodide (PI) fluorescence histogram for wildtype *E. coli* after 1 h treatment with sublethal Ag^+ concentrations (20 μM).

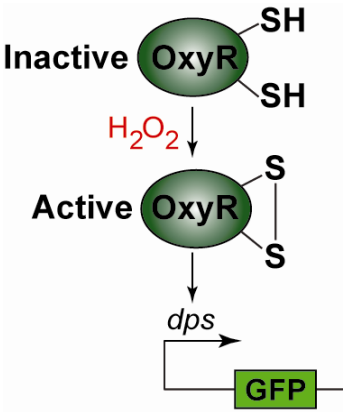


Fig S6. Schematic of the disulfide bond reporter construct. In the presence of intracellular H_2O_2, OxyR is activated via the formation of a disulfide bond. The activated OxyR then binds and activates the *dps* promoter, which is ligated to GFP, to create a reporter construct that is sensitive to the formation of disulfide bonds.

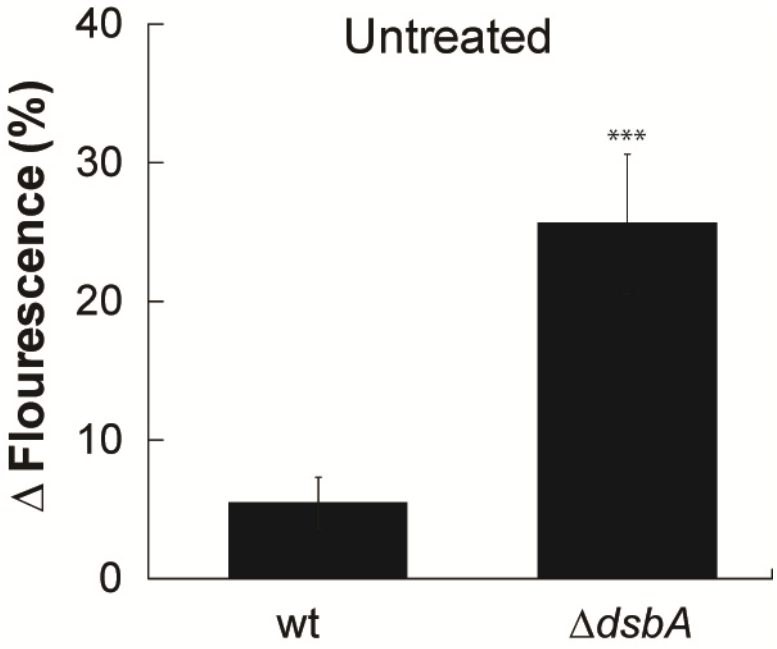


Fig S7. Impairment of disulfide bond formation leads to increased permeability of the cellular outer membrane. Change in fluorescence of PI-stained wildtype and *ΔdsbA* strains after 1 h of untreated growth. *** indicates a p-value of <0.001 as determined by a t-test, indicating a significant difference from the wildtype strain. Error bars represent mean ± SEM for at least 3 biological replicates.

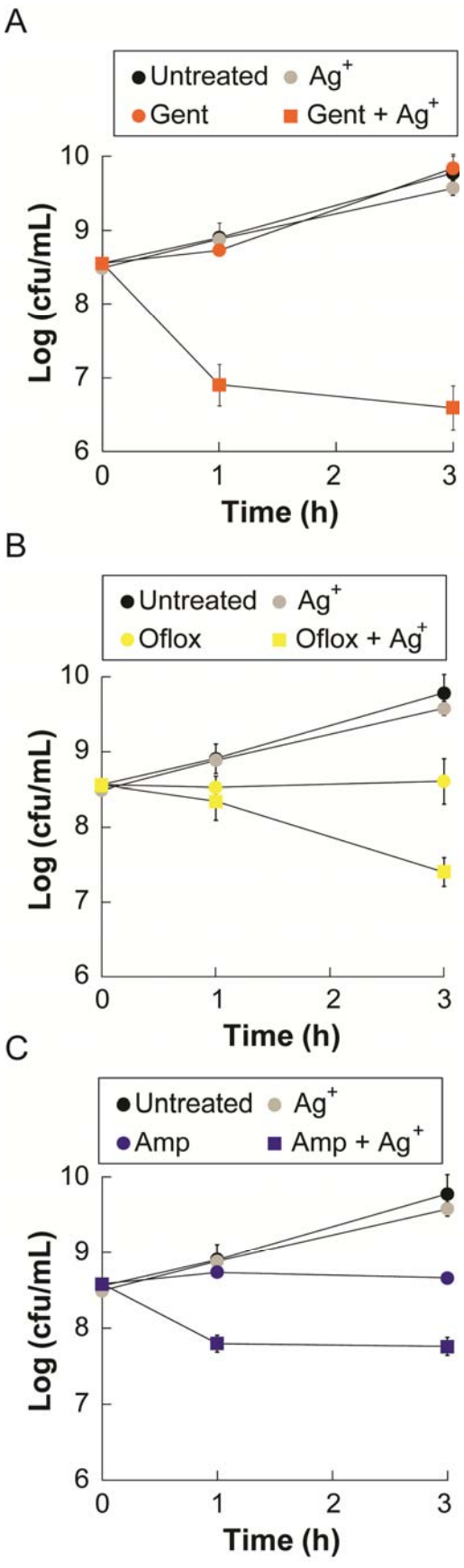

A
Untreated
Ag+
Gent
Gent + Ag+
Log (cfu/mL)
Time (h)
B
Untreated
Ag+
Oflox
Oflox + Ag+
Log (cfu/mL)
Time (h)
C
Untreated
Ag+
Amp
Amp + Ag+
Log (cfu/mL)
Time (h)

Fig S8. Addition of sublethal concentrations of Ag^{+} potentiates bactericidal antibiotics. Kill curves of wildtype *E. coli* after treatment with the following: **(A)** 15 μM $AgNO_3$, 0.25 μg/mL gentamicin, and the combination of both; **(B)** 15 μM $AgNO_3$, 0.03 μg/mL ofloxacin, and the combination of both; and **(C)** 15 μM $AgNO_3$, 1 μg/mL ampicillin, and the combination of both. Error bars represent mean ± SEM for at least 3 biological replicates.

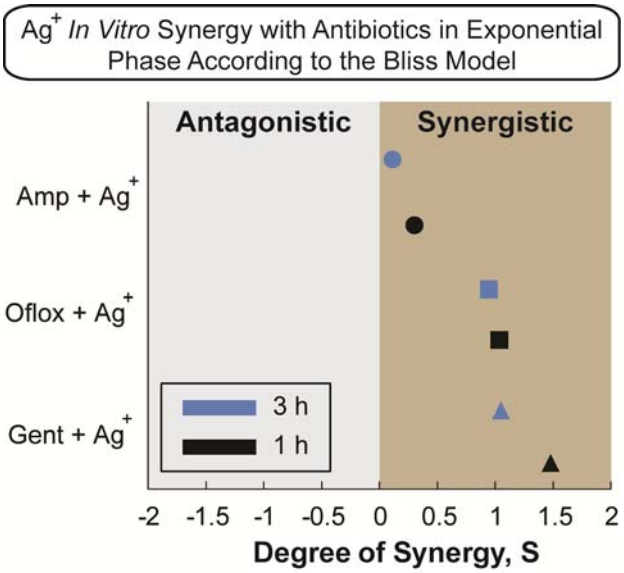


Fig S9. The Bliss Model for Synergy confirms a synergistic effect, between Ag^+ and three different families of bactericidal antibiotics, against Gram-negative bacteria. Degree of synergy quantified, using the Bliss Model for Synergy, after 1 and 3 h of treatment with 15 μM $AgNO_3$ in combination with the following individual antibiotics: 0.25 μg/mL gentamicin, 0.03 μg/mL ofloxacin, and 1 μg/mL ampicillin. (Circle) Ampicillin + Ag^+, (Square) Ofloxacin + Ag^+, (Triangle) Gentamicin + Ag^+.

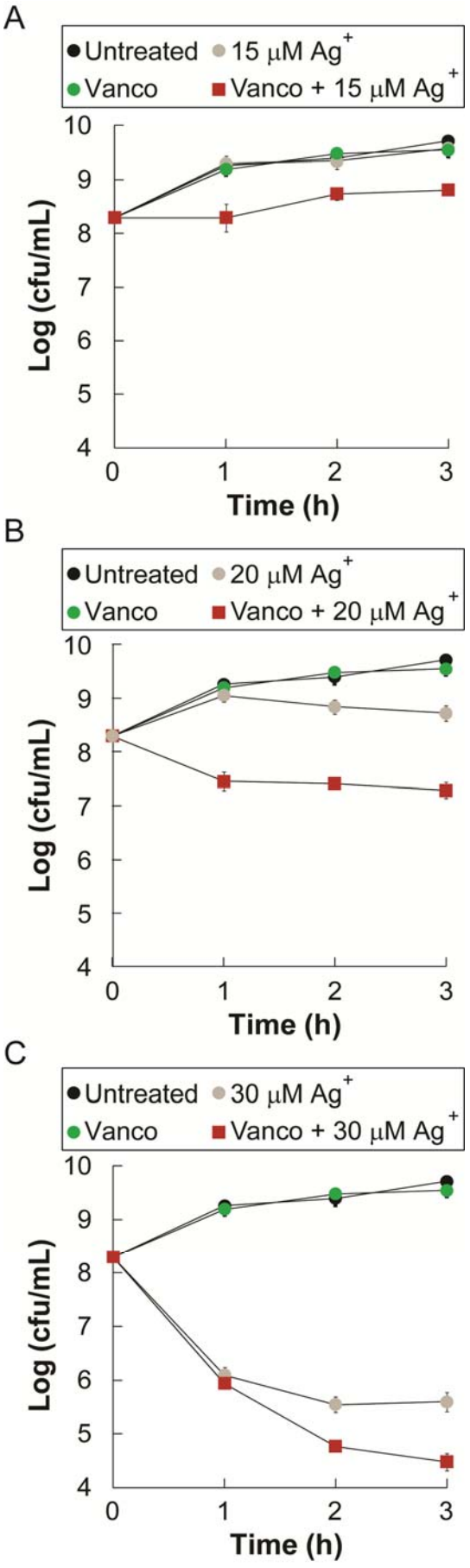

A
Untreated
15 μM Ag+
Vanco
Vanco + 15 μM Ag+
Log (cfu/mL)
Time (h)
B
Untreated
20 μM Ag+
Vanco
Vanco + 20 μM Ag+
Log (cfu/mL)
Time (h)
C
Untreated
30 μM Ag+
Vanco
Vanco + 30 μM Ag+
Log (cfu/mL)
Time (h)

Fig S10. Addition of Ag^+ broadens the spectrum of vancomycin. Kill curves of wildtype *E. coli* after treatment with Ag^+, vancomycin and the combination of both, at the following concentrations: **(A)** 30 μg/mL vancomycin and 15 μM $AgNO_3$; **(B)** 30 μg/mL vancomycin and 20 μM $AgNO_3$; and (**C)** 30 μg/mL vancomycin and 30 μM $AgNO_3$. Error bars represent mean ± SEM for at least 3 biological replicates.

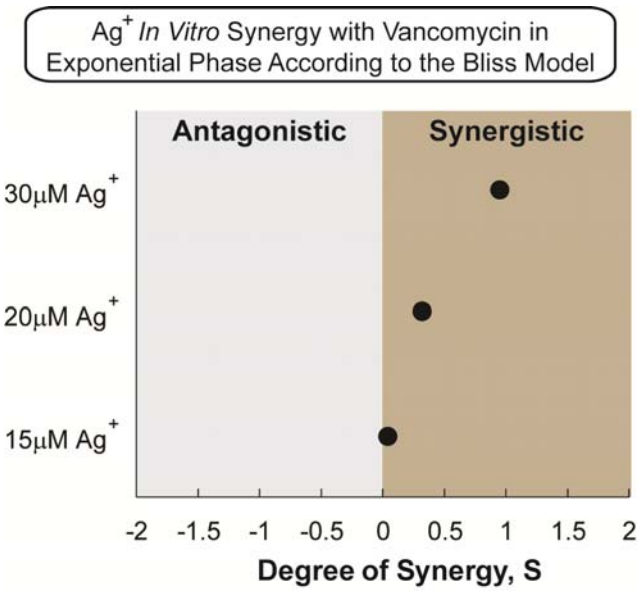
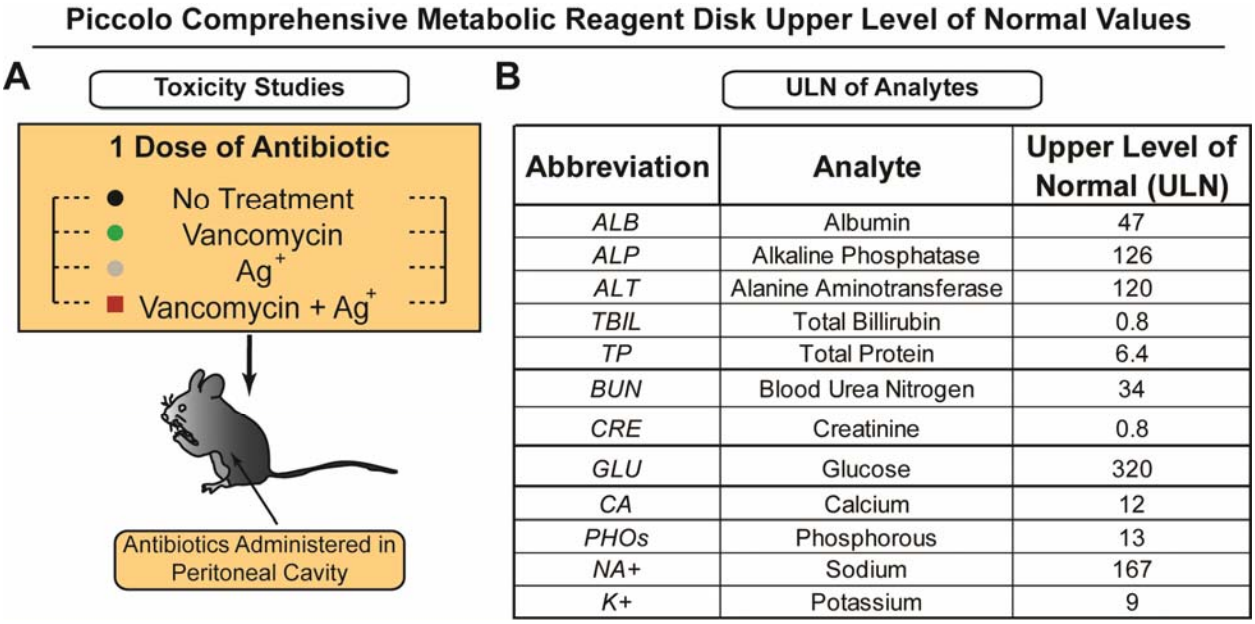


Fig S11. The Bliss Model for Synergy confirms a synergistic effect, between Ag^+ and vancomycin, against Gram-negative bacteria. Degree of synergy quantified, using the Bliss Model for Synergy, after 3 h of treatment with 30 μg/mL vancomycin in combination with $AgNO_3$ at 15, 20 and 30 μM.



Abbreviation	Analyte	Upper Level of Normal (ULN)
ALB	Albumin	47
ALP	Alkaline Phosphatase	126
ALT	Alanine Aminotransferase	120
TBIL	Total Billirubin	0.8
TP	Total Protein	6.4
BUN	Blood Urea Nitrogen	34
CRE	Creatinine	0.8
GLU	Glucose	320
CA	Calcium	12
PHOs	Phosphorous	13
NA+	Sodium	167
K+	Potassium	9

Fig S12. Reference upper level of normal (ULN) levels for mice when using the Piccolo® Metabolic Disk from Abaxis. **(A)** Schematic of the *in vivo* mouse experimental design. Piccolo® Metabolic Disks from Abaxis were used to analyze blood metabolites and enzyme activities. **(B)** ULN values determined for mice using the Abaxis Metabolic Disks.

Comprehensive Metabolic Analysis to Determine Theapeutic Toxicity

A Ion and Trace Metals

	Analyte	6 h	24 h	48 h
Untreated	*CA*	9.3	9.6	9.3
	PHOs	8.2	6.5	8.2
	NA+	148.0	148.3	148.0
	K+	7.1	6.3	7.1
[Ag^+]	*CA*	9.1	9.5	9.8
	PHOs	8.2	5.6	6.2
	NA+	146.3	149.7	149.3
	K+	7.9	7.9	4.7
Vanco	*CA*	9.3	9.3	9.3
	PHOs	6.7	6.1	6.1
	NA+	150.3	148.3	148.3
	K+	7.1	6.3	6.3
Vanco + [Ag^+]	*CA*	8.9	9.3	9.5
	PHOs	6.9	6.7	6.3
	NA+	146.7	149.0	148.0
	K+	8.0	7.9	4.7

B Liver Function

	Analyte	6 h	24 h	48 h
Untreated	*ALB*	3.8	3.9	3.8
	ALP	119.7	125.7	112.3
	ALT	26.7	25.7	26.7
	TBIL	0.3	0.3	0.3
	TP	5.1	5.2	5.1
[Ag^+]	*ALB*	3.5	3.3	3.6
	ALP	137.0	91.0	66.0
	ALT	496.7	167.3	29.0
	TBIL	0.5	0.3	0.3
	TP	4.6	4.6	5.3
Vanco	*ALB*	4.0	3.6	3.6
	ALP	124.7	109.0	109.0
	ALT	21.7	29.3	29.3
	TBIL	0.3	0.3	0.3
	TP	5.2	4.8	4.8
Vanco + [Ag^+]	*ALB*	3.7	3.5	3.6
	ALP	150.7	126.0	101.0
	ALT	590.0	357.0	19.3
	TBIL	0.5	0.3	0.3
	TP	4.5	4.4	5.1

C Kidney Function

	Analyte	6 h	24 h	48 h
Untreated	*BUN*	22.0	29.7	27.0
	CRE	0.2	0.2	0.2
[Ag^+]	*BUN*	29.0	20.0	25.7
	CRE	0.2	0.2	0.2
Vanco	*BUN*	19.0	26.0	26.0
	CRE	0.2	0.2	0.2
Vanco + [Ag^+]	*BUN*	28.7	20.7	23.7
	CRE	0.2	0.2	0.2

D Pancreatic Function

	Analyte	6 h	24 h	48 h
Untreated	*GLU*	313.0	303.0	263.7
[Ag^+]	*GLU*	254.7	263.3	238.3
Vanco	*GLU*	264.3	294.7	294.7
Vanco + [Ag^+]	*GLU*	203.0	237.0	224.7

Fig S13. Blood chemistry analysis of murine peripheral blood following Ag^+ and antibiotic treatments. **(A)-(D)** Average levels (observed in 3 mice per group at 0, 6, 24 and 48 h) in metabolites and enzymes involved in **(A)** ion and trace metals, **(B)** liver function, **(C)** kidney function, and **(D)** pancreatic function after treatment with 30 mg vancomycin/kg body weight, 6

mg $AgNO_3$/kg body weight (35 μM), and 30 mg vancomycin/kg body weight in combination with 6 mg $AgNO_3$/kg body weight.

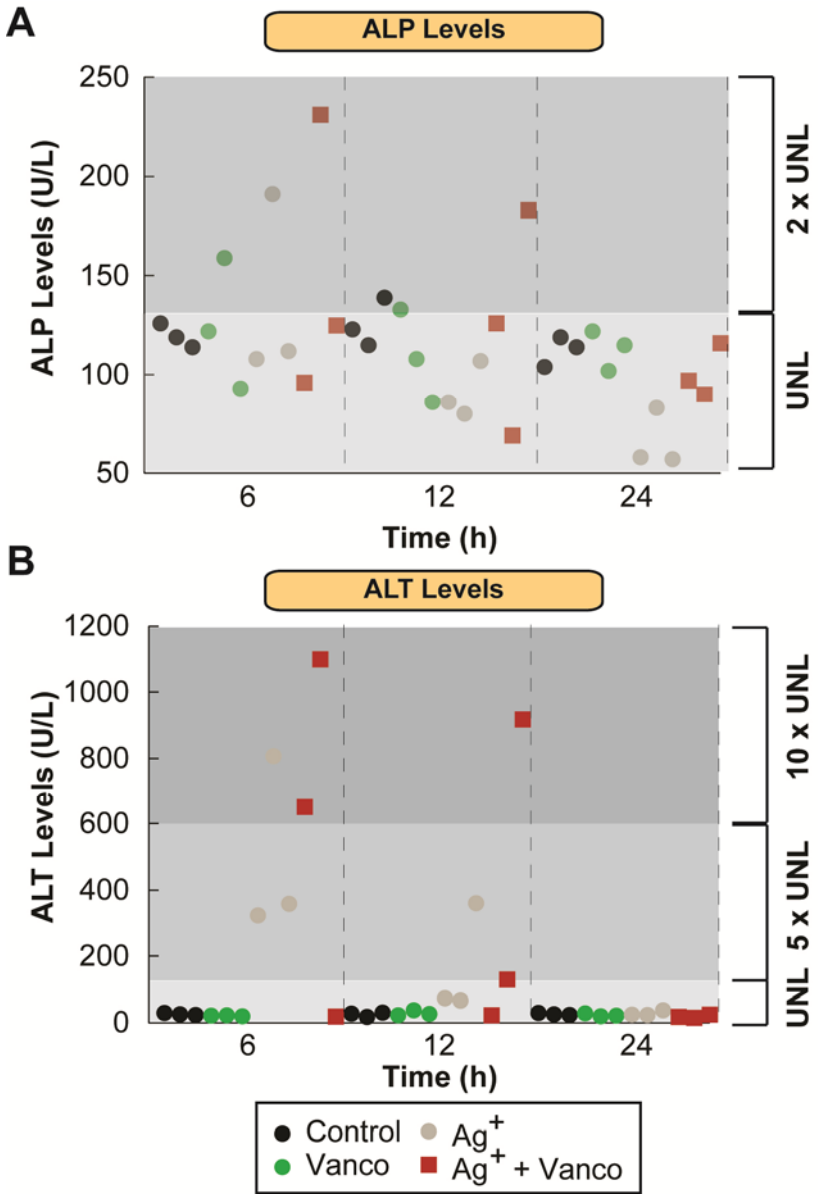


Fig S14. Blood chemistry analysis showing that liver function enzymes detected in peripheral blood soon recover to normal levels after treatment with Ag^{+}, vancomycin and Ag^{+} plus vancomycin. **(A)** Alkaline phosphatase (ALP) levels and **(B)** Alanine aminotransferase (ALT) levels of individual mice.

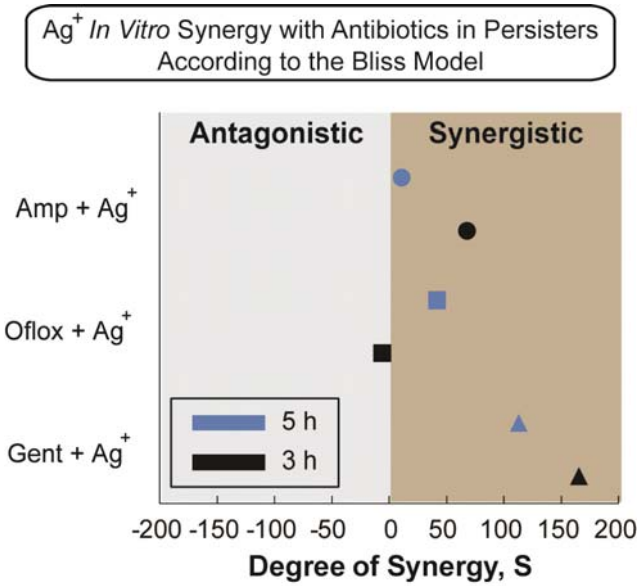


Fig S15. The Bliss Model for Synergy confirms a synergistic effect, between Ag^+ and three different families of bactericidal antibiotics, against persister Gram-negative bacteria. Degree of synergy quantified, using the Bliss Model for Synergy, after 3 and 5 h of treatment with 30 μM $AgNO_3$ in combination with the following individual antibiotics: 5 μg/mL gentamicin, 5 μg/mL ofloxacin, and 10 μg/mL ampicillin. (Circle) Ampicillin + Ag^+, (Square) Ofloxacin + Ag^+, (Triangle) Gentamicin + Ag^+.

Supplementary Tables

	Drug	MG1655
MIC90	$AgNO_3$ (μM)	15
	AMP (μg/mL)	1
	GENT (μg/mL)	0.75
	OFLOX (ng/mL)	110

Table S1. MICs for different antibiotics for the MG1655 *E. coli* strain used in this study. MICs were determined as described in the Methods. The MIC is the lowest concentration of drug that after 16 h inhibits 90% of bacterial growth relative to an untreated strain. AMP refers to ampicillin, GENT to gentamicin, and OFLOX to ofloxacin.

Strain	Concentration	ΔLog (T0-T3)	SEM
MG1655	Untreated	1.63	0.07
	Treated	-2.67	0.31
iscS	Untreated	1.06	0.03
	Treated	0.78	0.11
tonB	Untreated	1.62	0.02
	Treated	-3.50	0.18
cydB	Untreated	0.71	0.03
	Treated	-2.00	0.52
acnB	Untreated	0.62	0.02
	Treated	-2.73	0.24
icdA	Untreated	1.47	0.03
	Treated	-1.08	0.21
sucB	Untreated	0.77	0.11
	Treated	0.02	0.18
mdh	Untreated	0.94	0.04
	Treated	0.65	0.09
dsbA	Untreated	1.49	0.05
	Treated	-4.63	0.16
dsbC	Untreated	1.56	0.04
	Treated	-3.77	0.61
secG	Untreated	1.46	0.03
	Treated	1.64	0.06

Table S2. Raw data showing antimicrobial effects of Ag^+ observed in different mutants. Log change in cfu/mL, from time zero, of different *E. coli* mutant strains after treatment for 3 h with 30 μM $AgNO_3$ or untreated. The SEM indicates the standard error mean of at least 3 biological replicates.

Treatment	Δ Log (T0-T3)	SEM
Untreated	1.22	0.03
Ag^+	0.87	0.09
Gent	1.29	0.16
Oflox	0.05	0.03
Amp	0.08	0.05
Gent + Ag^+	-1.97	0.3
Oflox + Ag^+	-1.16	0.19
Amp + Ag^+	-0.83	0.12

Table S3. Raw data showing potentiation of antibiotics using Ag^+ as an adjuvant compound. Log change in cfu/mL, between time zero and 3 h post-treatment with 15 μM $AgNO_3$, 0.25 μg/mL gentamicin, 0.03 μg/mL ofloxacin, 1 μg/mL ampicillin and the combinations of the same concentrations of antibiotics plus $AgNO_3$. The SEM indicates the standard error mean of at least 3 biological replicates.

	Drug	AG100	AG112
MIC90	$AgNO_3$ (μM)	25	25
	TET (μg/mL)	0.92	3
	$AgNO_3$ (15 μM) + TET	0.3	1.3

Table S4. MICs for different antibiotics and different *E. coli* strains used in this study. MICs were determined as described in the Methods. The MIC is the lowest concentration of drug that after 16 h inhibits 90% of bacterial growth relative to an untreated strain. TET refers to tetracycline.

Treatment	Δ Log (T0-T3)	SEM
Control	1.42	0.08
Vanco	1.25	0.07
Ag^+ 15 μM	1.28	0.04
Ag^+ 20 μM	0.42	0.14
Ag^+ 30 μM	-2.70	0.18
Ag^+ 15 μM + Vanco	0.52	0.03
Ag^+ 20 μM + Vanco	-1.01	0.15
Ag^+ 30 μM + Vanco	-3.82	0.16

Table S5. Raw data showing potentiation of vancomycin using Ag^+ as an adjuvant compound. Log change in cfu/mL, between time zero and 3 h post-treatment with 30 μg/mL vancomycin, 15 μM, 20 μM, 30 μM $AgNO_3$ and the combinations of each $AgNO_3$ concentration with 30 μg/mL vancomycin. The SEM indicates the standard error mean of at least 3 biological replicates.