Combatting Bacterial Infections by Killing Persister Cells with Mitomycin C

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ABSTRACT

Persister cells are a multi-drug tolerant subpopulation of bacteria that contribute to chronic and recalcitrant clinical infections such as cystic fibrosis and tuberculosis. Persisters are metabolically dormant, so they are highly tolerant to all traditional antibiotics which are mainly effective against actively-growing cells. Here, we show that the FDA-approved anti-cancer drug mitomycin C (MMC) eradicates persister cells through a growth-independent mechanism. MMC is passively transported and bioreductively activated, leading to spontaneous crosslinking of DNA, which we verify in both active and dormant cells. We find MMC effectively eradicates cells grown in numerous different growth states (e.g., planktonic cultures and highly robust biofilm cultures) in both rich and minimal media. Additionally, MMC is a potent bactericide for a broad range of bacterial persisters, including commensal *Escherichia coli* K-12 as well as pathogenic species of *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. We also demonstrate the efficacy of MMC in an animal model and a wound model, substantiating the clinical applicability of MMC against bacterial infections. Therefore, MMC is the first broad-spectrum compound capable of eliminating persister cells, meriting investigation as a new approach for the treatment of recalcitrant infections.

INTRODUCTION

There are 17 million new biofilm infections every year in the U.S.A., which lead to 550,000 fatalities (Wolcott and Dowd, 2011), and biofilms are difficult to treat due to the presence of persister cells (Lewis, 2008). Persisters arise due to metabolic inactivity (Lewis, 2007; Kwan *et al.*, 2013; Wood *et al.*, 2013) and are highly tolerant against all traditional antibiotic classes, which are primarily effective against actively growing cells. Bacterial persistence is a non-hereditary phenotype (Bigger, 1944) which occurs both stochastically (Balaban *et al.*, 2004) or through environmental influence (Dörr *et al.*, 2010; Möker *et al.*, 2010; Vega *et al.*, 2012; Kwan *et al.*, 2013; Hu *et al.*, 2014; Kwan *et al.*, 2014) in a small sub-population of all tested bacterial species (Lewis, 2008) (~1% during stationary phase and in biofilm cultures (Lewis, 2007, 2008)).

Few distinctly new antibiotics have been discovered recently (Mills and Dougherty, 2012), and current antibiotics are ineffective against persister cells. Thus, we searched for a compound which could eradicate persister cells. Mitomycin C (MMC) is used as an FDA-approved (Doll *et al.*, 1985) chemotherapeutic agent for a wide range of cancer treatments (e.g., bladder, gastric, and pancreatic) (Bradner, 2001). As an amphipathic molecule, MMC passively diffuses into cells (Byfield and Calabro-Jones, 1981). Bacterial cytoplasm is a reducing environment (Szybalski and Iyer, 1964), so after entering into cells, the quinone functional group of MMC is reduced spontaneously, initiating crosslinking of adjacent guanine residues in 5′-CG sequences to join two opposing strands of DNA (Tomasz, 1995). Because transport is passive and the reaction is spontaneous, we reasoned that MMC would be effective against cells in the persister state, a state of metabolic dormancy, since MMC activity would not require active metabolism.

We found that MMC is effective against persister cells in a broad range of bacteria including commensal *Escherichia coli* K-12 as well as pathogenic strains of *E. coli*, *Staphylococcus aureus* (frequently found in wounds), and *Pseudomonas aeruginosa*. We also demonstrated that MMC eradicates

bacteria in biofilms, communities of notoriously difficult to treat cells present in a majority of infections. Furthermore, we verified that MMC kills persister cells by crosslinking DNA, and we demonstrated the efficacy of MMC in an animal model and in a wound model. Therefore, MMC has broad-spectrum activity against growing, non-growing, and persister cells, and should be used for the treatment of recalcitrant infections.

RESULTS AND DISCUSSION

MMC kills active and persister cells in rich medium. MMC activity is decreased at high pH (Kennedy *et al.*, 1985); hence, we buffered the medium to avoid high pH fluctuations and to match the physiological resting pH of ~7.4 and exercising pH of ~7.1 and ~6.4 (Hermansen and Osnes, 1972). For this work, we compared MMC with ciprofloxacin, a fluoroquinolone that inhibits DNA replication and kills both growing and non-growing cells but not persister cells (Sanders, 1988) and which is commonly used in persister studies (Conlon *et al.*, 2013). Therefore, throughout this work, ciprofloxacin tolerance represents the baseline level of persistence. Additionally, antibiotic treatments were generally at least 5x the minimum inhibitory concentration (MIC) (**Table 1**) to ensure eradication of non-persisters and to minimize the survival of potential spontaneous resistant mutants.

As evidence that ciprofloxacin tolerance is due to persistence rather than spontaneous genetic resistance, we measured the tolerance of *E. coli* K-12 cultures after three rounds of ciprofloxacin treatment (5 μg/mL for 3 h) and subsequent regrowth of persisters in fresh media. There was no observable increase in ciprofloxacin survival after each round of regrowth (survival at 0.026 ± 0.007%, 0.048 ± 0.002%, and 0.044 ± 0.001% for rounds 1, 2 and 3, respectively). In addition, no colonies were detectable with 5 μg/mL ciprofloxacin, showing absolutely that there were no resistant strains. These results confirm the reliability of using ciprofloxacin tolerance as an indicator of persistence.

We initially evaluated MMC with *E. coli* K-12 and found, compared to ciprofloxacin, that MMC was 2,300-fold more effective against exponentially-growing cells (**Fig. 1A**) and 150,000-fold more effective against mid-stationary-phase cells in buffered lysogeny broth (LB) medium (**Fig. 1B**). As evidence of the

ability of MMC to kill persister cells, we found that treatment of a late-stationary phase culture with MMC does not show the bi-phasic death curve that is characteristic of a persister population (**Fig. 1C**). We then utilized a rifampicin pretreatment which we previously demonstrated to induce high levels of persistence (~10-100%) (Kwan *et al.*, 2013) and found that MMC was highly effective against rifampicin-induced persister cells, in stark contrast to ciprofloxacin (**Fig. 1D**). Therefore, MMC kills non-persister cells and dormant persister cells.

MMC kills active and persister cells in minimal medium, in anaerobic cultures, and in biofilms. The previous assays were performed in planktonic cultures grown in rich medium; however, these growth conditions are a poor representation of ecological bacterial growth. Thus, we investigated MMC activity against cultures grown in minimal medium, in biofilms, and in anaerobic cultures. Exponential-phase cultures in M9-glucose were similarly susceptible to MMC and ciprofloxacin (**Fig. 1E**); however, during late-stationary phase in M9-glucose, we found that while the population was highly persistent against ciprofloxacin (10 ± 1 %) and the aminoglycoside gentamicin (44 ± 5 %), MMC eradicated cells (**Fig. 1F**).

Biofilms more accurately model clinical bacteria growth with a high population of persisters (Lewis, 2008), and we found that MMC was effective against biofilms in M9-glucose, killing 100,000-fold more cells than ciprofloxacin and nearly eliminating cells after 24 h of treatment (**Fig. 1G**). Additionally, MMC did not cause biofilm dispersal, confirming that efficacy against biofilms was in fact due to eradication of cells (**Fig. S1**). Bacterial infections have a propensity to exist under anaerobic conditions, and we found that MMC eradicated anaerobic, late-stationary phase cells in rich medium beyond the limit of detection, in comparison to 0.44 ± 0.08 % survival against ciprofloxacin treatment (**Fig. 1H**). Furthermore, anaerobic biofilm cultures in M9-glucose were 2,500-fold more susceptible to MMC than ciprofloxacin (**Fig. 1I**).

MMC kills viable but non-culturable cells. Numerous species of bacteria enter the viable but non-culturable (VBNC) state, another state of metabolic dormancy closely related to persistence (Ayrapetyan *et al.*, 2014), as a survival response to environmental stresses, and these cells do not resuscitate and become culturable unless exposed to suitable stimuli (Li *et al.*, 2014). VBNC cells exhibit high antibiotic

tolerance, similarly to persisters, and pose a risk to human health because they can avoid detection in goods, leading to infection (Li *et al.*, 2014). Hence, we generated VBNC cultures by starving cells in saline solution for 36 days until there were ~1,000-fold more VBNC cells than culturable cells. Respiratory activity is a commonly used criterion for viability (Ramamurthy *et al.*, 2014), so RedoxSensor Green, a fluorescent dye for detection of actively respiring cells, was used to enumerate the VBNC population. Upon antibiotic treatment of these cultures, we found that MMC was 7-fold more effective at killing VBNC cells than ciprofloxacin (**Fig. 1J**), while also eradicating the culturable population (**Fig. S2**), unlike ciprofloxacin (0.40 ± 0.05 % survival). Therefore, we have demonstrated that MMC is highly effective against metabolically dormant cells in both the persister and VBNC states.

MMC kills persister cells by crosslinking DNA. To verify that MMC kills bacteria via DNA crosslinks, we investigated MMC activity against single-gene deletion mutants lacking *uvrA*, *uvrB*, and *uvrC*. The UvrABC complex is part of the bacterial SOS response in *E. coli* (Michel, 2005) to repair DNA crosslinks (Weng *et al.*, 2010), and contributes to MMC tolerance (Salem *et al.*, 2009). We found that the Δ*uvrA*, Δ*uvrB*, and Δ*uvrC* mutants were much more sensitive to MMC than the wild-type strain, and were rapidly eradicated (beyond the limit of detection) within less than 30 min of treatment (**Fig. 2A**). Additionally, we were able to complement the high sensitivity to MMC of a Δ*uvrA* mutant with production of UvrA via plasmid (**Fig. 2B**). These results confirm that DNA crosslinking is the basis for MMC bactericidal activity in actively-growing cells.

However, persister cells are dormant, thus having a different metabolic state than non-persisters. Therefore, we sought to verify that MMC was in fact forming DNA crosslinks within persisters, rather than killing persisters through an unknown mechanism. Genomic DNA (gDNA) was isolated from both exponential phase cells (i.e., non-persisters) and rifampicin-induced persisters (Kwan *et al.*, 2013) before and after MMC treatment. We hypothesized that crosslinks within DNA should inhibit amplification via quantitative PCR (qPCR). qPCR was performed with primers designed to amplify a 234 nt region of *rrsG* and a 302 nt region of *murB*, containing 18 and 10 potential MMC crosslinking sites, respectively. As a positive control, qPCR was performed on gDNA crosslinked by MMC *in vitro*, verifying that DNA

crosslinking inhibited amplification dramatically (*rrsG*: -1,456 ± 1 fold and *murB*: -1,621 ± 28 fold). Our *in vivo* results revealed the presence of gDNA crosslinks based on reduced quantities of PCR-amplifiable DNA after MMC treatment for non-persisters (*rrsG*: -5.95 ± 0.20 fold and *murB*: -5.13 ± 0.14 fold) and persisters (*rrsG*: -5.01 ± 0.51 fold and *murB*: -5.99 ± 0.50 fold) (**Table S3**).

Crosslinked DNA runs differently from non-crosslinked DNA after denaturation because the crosslinks covalently bind the two strands, preventing separation (Matsumoto *et al.*, 1989). As confirmation of DNA crosslinking within persister cells found with qPCR, we isolated plasmid DNA (pDNA) from both exponential-phase cells (i.e., non-persisters) and rifampicin-induced persisters (Kwan *et al.*, 2013) before and after MMC treatment and performed agarose gel electrophoresis under denaturing conditions to allow uncrosslinked DNA to migrate as single strands. Under denaturing conditions, pDNA samples from cells without MMC treatment migrated as single-stranded DNA, while samples for both non-persisters and persisters treated with MMC showed a high percentage of crosslinking, based on migration as double-stranded DNA (**Fig. 2C**). DNA treated *in vitro* with MMC migrated in the expected manner as double-stranded DNA. Therefore, we have demonstrated by two independent means that MMC does in fact crosslink the DNA of persister cells.

MMC kills persister cells of pathogens. Clinical application of MMC is dependent on efficacy against pathogenic bacteria. Therefore, we tested the ability of MMC to kill *E. coli* O157:H7 (EHEC) a common pathogenic strain of Gram-negative *E. coli*. MMC was substantially more effective than ciprofloxacin against EHEC, eradicating both exponential (**Fig. 3A**) and mid-stationary phase cells (**Fig. 3B**) in rich medium beyond the limit of detection. Additionally, MMC eradicated late-stationary phase EHEC cells in M9-glucose beyond the limit of detection, while 1.2 ± 0.1 % of cells survived against ciprofloxacin (**Fig. 3C**). Biofilm cultures of EHEC were also eradicated (beyond the limit of detection) after 24 h of MMC treatment, in comparison to 3 ± 1 % of cells surviving ciprofloxacin (**Fig. 3D**).

MMC was also tested against Gram-positive *S. aureus* (methicillin sensitive) and Gram-negative *P. aeruginosa*, two other common species of human pathogens. Against planktonic cultures of *S. aureus* grown in rich medium, MMC was highly effective, eradicating both exponential (**Fig. 3E**) and mid-

stationary phase cultures (**Fig. 3F**), in comparison to ciprofloxacin (0.55 ± 0.04 % survival and 2.2 ± 0.1 % survival, respectively). MMC was also highly effective against biofilm cultures of *S. aureus* grown in minimal medium, eradicating cells beyond the limit of detection after 24 of treatment, compared with 18 ± 2 % survival against ciprofloxacin (**Fig. 3G**). MMC also killed cultures of *P. aeruginosa* PA14 grown planktonically in rich medium to exponential (**Fig. 3H**) and mid-stationary phase (0.0038 ± 0.0005 % survival) and in minimal medium to late-stationary phase (**Fig. 3I**), although the extent of killing activity was similar to that of ciprofloxacin. Therefore, MMC is significantly more effective in eradicating EHEC and *S. aureus* and is similar to other potent antibiotics against *P. aeruginosa*, demonstrating the efficacy of MMC against several species of human pathogens.

MMC kills persister cells in a wound model. In clinical infections, bacteria are exposed to drastically different growth conditions from those generally used within laboratory cultures. The *in vitro* Lubbock chronic wound pathogenic biofilm model was previously developed to closely represent growth conditions of polymicrobial infections (Sun *et al.*, 2008). We used this *in vitro* wound model to test MMC activity against cultures of EHEC, *S. aureus*, and *P. aeruginosa* PAO1 as well as a co-culture of *S. aureus* and *P. aeruginosa* PAO1. Our strain of *S. aureus* is coagulase-positive (Hsueh *et al.*, 1999), causing the medium to form a jelly-like mass consisting of insoluble fibrin (Zajdel *et al.*, 1975). Cultures were grown statically so that coagulated plasma served as a scaffold for bacterial growth (DeLeon *et al.*, 2014) in cultures containing *S. aureus*, while biofilms formed at surface interfaces served as scaffolds for bacterial growth in cultures without *S. aureus*. We found that MMC was more effective than ciprofloxacin and ampicillin against all three species under wound-like conditions in mono- and co-cultures (**Fig. 4A-D**). These results show that MMC is a significantly more effective treatment than other antibiotics against pathogenic strains of several species (e.g., EHEC, *S. aureus*, and *P. aeruginosa*) grown using an *in vitro* wound model. This substantiates the efficacy of MMC as a clinical treatment for clearing infections.

MMC is effective in an animal model. In order to test the efficacy of MMC treatment *in vivo*, we used an EHEC infection within the nematode *Caenorhabditis elegans*. *C. elegans* was fed on lawns of EHEC on nematode growth media (NGM) agar plates for 2 days in order to establish an infection. Nematodes

were then exposed to MMC, ciprofloxacin, ampicillin, or no treatment, transferred to lawns of avirulent *E. coli* OP50, and monitored for viability. All three antibiotic treatments enhanced the survival of the EHEC-infected worms; however, survival with MMC was higher than with either ciprofloxacin or ampicillin based on four experimental replicates (10 worms per replicate) (**Fig. 4E**), likely because MMC eradicates persisters that can reestablish infection. Of note, we obtained similar results from four additional replicates performed with different antibiotic treatments and EHEC infection conditions. Therefore, MMC is consistently more effective than other antibiotics at clearing EHEC infection within an animal model.

CONCLUSION

Traditional antibiotics (e.g., fluoroquinolones, aminoglycosides, and β-lactams) are ineffective against persister cells due to their mechanisms which rely on cellular activity. Here, we found that MMC is highly effective because of its unique mechanism of action, which is independent of the metabolic state, by demonstrating its activity against slow-growing, non-growing, and dormant (e.g., persister and VBNC) cells, as well as its activity on cells grown planktonically, in biofilms, in an *in vitro* wound model, and in an *in vivo* animal model. In comparison, several methods have been proposed for eradicating persister cells, including increasing aminoglycoside uptake via glycolysis intermediates (Allison *et al.*, 2011), altering membranes via Trp/Arg-containing antimicrobial peptides (Chen *et al.*, 2011), activating ClpP-mediated self-digestion via rifampicin and ADEP4 (Conlon *et al.*, 2013), and converting persisters to non-persisters via *cis*-2-decenoic acid (Marques *et al.*, 2014). However, the potential application of these treatments against clinical infections is distant due to limited levels of *in vivo* testing. These treatments are also likely limited to a small range of species that are susceptible to the compounds. In contrast, MMC has been an FDA-approved chemotherapeutic cancer drug for over forty years (Doll *et al.*, 1985) with a well-characterized biochemical mechanism (Tomasz, 1995). Additionally, MMC passively diffuses into cells, and the DNA crosslinking activity of MMC is spontaneous, so MMC treatment should be effective against many bacterial species which cannot be fully cleared with traditional antibiotics such as recalcitrant internal and external (wound) infections.

Throughout the majority of this study, MMC was effectively applied at a concentration of 10 μg/mL, although the effective concentration should be much lower against strains with low MICs (e.g., EHEC and *S. aureus*; **Table 1**). For various cancer treatments, intravenously infused MMC dosages are often administered at concentrations between 0.5-2.0 μg/mL (20-80 mg/m^2) (Bradner, 2001) and topical dosages have been safely applied at concentrations up to 400 μg/mL (Shields *et al.*, 2002). Therefore, the bactericidal concentrations of MMC are similar to the therapeutic concentrations that have been established for cancer treatments, which validates MMC as a readily applicable treatment for clinical infections.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

REFERENCES

Allison, K.R., Brynildsen, M.P., and Collins, J.J. (2011) Metabolite-enabled eradication of bacterial persisters by aminoglycosides. *Nature* **473**: 216-220.

Ayrapetyan, M., Williams, T.C., and Oliver, J.D. (2014) Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends Microbiol*.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M. et al. (2006) Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol Syst Biol* **2**: 2006.0008.

Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004) Bacterial persistence as a phenotypic switch. *Science* **305**: 1622-1625.

Bernier, S.P., Labeaux, D., DeFrancesco, A.S., Valomon, A., Soubigou, G., Coppée, J.-Y. et al. (2013) Starvation, together with the SOS response, mediates high biofilm-specific tolerance to the fluoroquinolone ofloxacin. *PLoS Genet* **9**: e1003144.

Bigger, J.W. (1944) Treatment of staphylococcal infections with penicillin by intermittent sterilisation. *Lancet* **244**: 497-500.

Bradner, W.T. (2001) Mitomycin C: a clinical update. *Cancer Treat Rev* **27**: 35-50.

Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* **77**: 71-94.

Byfield, J.E., and Calabro-Jones, P.M. (1981) Carrier-dependent and carrier-independent transport of anti-cancer alkylating agents. *Nature* **294**: 281-283.

Chen, X., Zhang, M., Zhou, C., Kallenbach, N.R., and Ren, D. (2011) Control of bacterial persister cells by Trp/Arg-containing antimicrobial peptides. *Appl Environ Microbiol* **77**: 4878-4885.

Conlon, B.P., Nakayasu, E.S., Fleck, L.E., LaFleur, M.D., Isabella, V.M., Coleman, K. et al. (2013) Activated ClpP kills persisters and eradicates a chronic biofilm infection. *Nature* **503**: 365-370.

DeLeon, S., Clinton, A., Fowler, H., Everett, J., Horswill, A.R., and Rumbaugh, K.P. (2014) Synergistic interactions of *Pseudomonas aeruginosa* and *Staphylococcus aureus* in an *in vitro* wound model. *Infect Immun* **82**: 4718-4728.

Doll, D.C., Weiss, R.B., and Issell, B.F. (1985) Mitomycin: ten years after approval for marketing. *J Clin Oncol* **3**: 276-286.

Donegan, K., Matyac, C., Seidler, R., and Porteous, A. (1991) Evaluation of methods for sampling, recovery, and enumeration of bacteria applied to the phylloplane. *Appl Environ Microbiol* **57**: 51-56.

Dörr, T., Vulić, M., and Lewis, K. (2010) Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLoS Biol* **8**: e1000317.

Hermansen, L., and Osnes, J.-B. (1972) Blood and muscle pH after maximal exercise in man. *J Appl Physiol* **32**: 304-308.

Hsueh, P.-R., Teng, L.-J., Yang, P.-C., Pan, H.-J., Chen, Y.-C., Wang, L.-H. et al. (1999) Dissemination of two methicillin-resistant *Staphylococcus aureus* clones exhibiting negative staphylase reactions in intensive care units. *J Clin Microbiol* **37**: 504-509.

Hu, Y., Kwan, B.W., Osbourne, D.O., Benedik, M.J., and Wood, T.K. (2014) Toxin YafQ increases persister cell formation by reducing indole signaling. *Environ Microbiol*.

Jacobs, M.A., Alwood, A., Thaipisuttikul, I., Spencer, D., Haugen, E., Ernst, S. et al. (2003) Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100**: 14339-14344.

Kennedy, K.A., McGurl, J.D., Leondaridis, L., and Alabaster, O. (1985) pH dependence of mitomycin C-induced cross-linking activity in EMT6 tumor cells. *Cancer Res* **45**: 3541-3547.

Kitagawa, M., Ara, T., Arifuzzaman, M., Ioka-Nakamichi, T., Inamoto, E., Toyonaga, H., and Mori, H. (2005) Complete set of ORF clones of *Escherichia coli* ASKA library (a complete set of *E. coli* K-12 ORF archive): unique resources for biological research. *DNA Res* **12**: 291-299.

Kwan, B.W., Valenta, J.A., Benedik, M.J., and Wood, T.K. (2013) Arrested protein synthesis increases persister-like cell formation. *Antimicrob Agents Chemother* **57**: 1468-1473.

Kwan, B.W., Osbourne, D.O., Hu, Y., Benedik, M.J., and Wood, T.K. (2014) Phosphodiesterase DosP increases persistence by reducing cAMP which reduces the signal indole. *Biotechnol Bioeng* **112**: 588-600.

Lee, J., Maeda, T., Hong, S.H., and Wood, T.K. (2009) Reconfiguring the quorum-sensing regulator SdiA of *Escherichia coli* to control biofilm formation via indole and *N*-acylhomoserine lactones. *Appl Environ Microbiol* **75**: 1703-1716.

Lewis, K. (2007) Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**: 48-56.

Lewis, K. (2008) Multidrug tolerance of biofilms and persister cells. *Curr Top Microbiol Immunol* **322**: 107-131.

Li, L., Mendis, N., Trigui, H., Oliver, J.D., and Faucher, S.P. (2014) The importance of the viable but non-culturable state in human bacterial pathogens. *Front Microbiol* **5**: 258.

Liberati, N.T., Urbach, J.M., Miyata, S., Lee, D.G., Drenkard, E., Wu, G. et al. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci USA* **103**: 2833-2838.

Marques, C.N.H., Morozov, A., Planzos, P., and Zelaya, H.M. (2014) The fatty acid signaling molecule *cis*-2-decenoic acid increases metabolic activity and reverts persister cells to an antimicrobial susceptible state. *Appl Environ Microbiol* **80**: 6976-6991.

Matsumoto, A., Vos, J.-M.H., and Hanawalt, P.C. (1989) Repair analysis of mitomycin C-induced DNA crosslinking in ribosomal RNA genes in lymphoblastoid cells from Fanconi's anemia patients. *Mutat Res* **217**: 185-192.

Michel, B. (2005) After 30 years of study, the bacterial SOS response still surprises us. *PLoS Biol* **3**: e255.

Mills, S.D., and Dougherty, T.J. (2012) Cell-based screening in antibacterial discovery. In *Antibiotic discovery and development*. Dougherty, T.J., and Pucci, M.J. (eds). New York, NY: Springer, pp. 901-930.

Möker, N., Dean, C.R., and Tao, J. (2010) *Pseudomonas aeruginosa* increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. *J Bacteriol* **192**: 1946-1955.

Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.

Ramamurthy, T., Ghosh, A., Pazhani, G.P., and Shinoda, S. (2014) Currect perspectives on viable but non-culturable (VBNC) pathogenic bacteria. *Front Public Health* **2**: 103.

Rodriguez, R.L., and Tait, R.C. (1983) *Recombinant DNA techniques: an introduction*. Menlo Park, CA: The Benjamin/Cummings Publiching Company, Inc.

Salem, A.M.H., Nakano, T., Takuwa, M., Matoba, N., Tsuboi, T., Terato, H. et al. (2009) Genetic analysis of repair and damage tolerance mechanisms for DNA-protein cross-links in *Escherichia coli*. *J Bacteriol* **191**: 5657-5668.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Sanders, C.C. (1988) Ciprofloxacin: in vitro activity, mechanism of action, and resistance. *Rev Infect Dis* **10**: 516-527.

Shields, C.L., Naseripour, M., and Shields, J.A. (2002) Topical mitomycin C for extensive, recurrent conjunctival-corneal squamous cell carcinoma. *Am J Ophthalmol* **133**: 601-606.

Sperandio, V., Torres, A.G., Girón, J.A., and Kaper, J.B. (2001) Quorum sensing is a global regulatory mechanism in enterohemorrhagic *Escherichia coli* O157:H7. *J Bacteriol* **183**: 5187-5197.

Sun, Y., Dowd, S.E., Smith, E., Rhoads, D.D., and Wolcott, R.D. (2008) *In vitro* multispecies Lubbock chronic wound biofilm model. *Wound Repair Regen* **16**: 805-813.

Szybalski, W., and Iyer, V.N. (1964) Crosslinking of DNA by enzymatically or chemically activated mitomycins and profiromycins, bifunctionally "alkylating" antibiotics. *Fed Proc* **23**: 946-957.

Tomasz, M. (1995) Mitomycin C: small, fast and deadly (but very selective). *Chem Biol* **2**: 575-579.

Vega, N.M., Allison, K.R., Khalil, A.S., and Collins, J.J. (2012) Signaling-mediated bacterial persister formation. *Nat Chem Biol* **8**: 431-433.

Wang, Y., and Wang, Y. (2003) Structure elucidation of DNA interstrand cross-link by a combination of nuclease P1 digestion with mass spectrometry. *Anal Chem* **75**: 6306-6313.

Weng, M.-w., Zheng, Y., Jasti, V.P., Champeil, E., Tomasz, M., Wang, Y. et al. (2010) Repair of mitomycin C mono- and interstrand cross-linked DNA adducts by UvrABC: a new model. *Nucleic Acids Res* **38**: 6976-6984.

Wolcott, R., and Dowd, S. (2011) The role of biofilms: are we hitting the right target? *Plast Reconstr Surg* **127**: 28S-35S.

Wood, T.K., Knabel, S.J., and Kwan, B.W. (2013) Bacterial persister cell formation and dormancy. *Appl Environ Microbiol* **79**: 7116-7121.

Zajdel, M., Wagrzynowicz, Z., and Jeljaszewicz, J. (1975) Action of staphylothrombin on bovine fibrinogen. *Thromb Res* **6**: 501-510.

Table 1. MICs for antibiotics used in this study.

Strain	MMC	Ciprofloxacin	Ampicillin	Gentamicin
E. coli K-12 BW25113	2 μg/mL	0.05 μg/mL	10 μg/mL	5 μg/mL
EHEC 86-24	1 μg/mL	0.05 μg/mL	5 μg/mL	
S. aureus ATCC29213	0.2 μg/mL	0.5 μg/mL	2 μg/mL	
P. aeruginosa PAO1	15 μg/mL	2 μg/mL	400 μg/mL	
P. aeruginosa PA14	2 μg/mL	0.1 μg/mL		

FIGURE LEGENDS

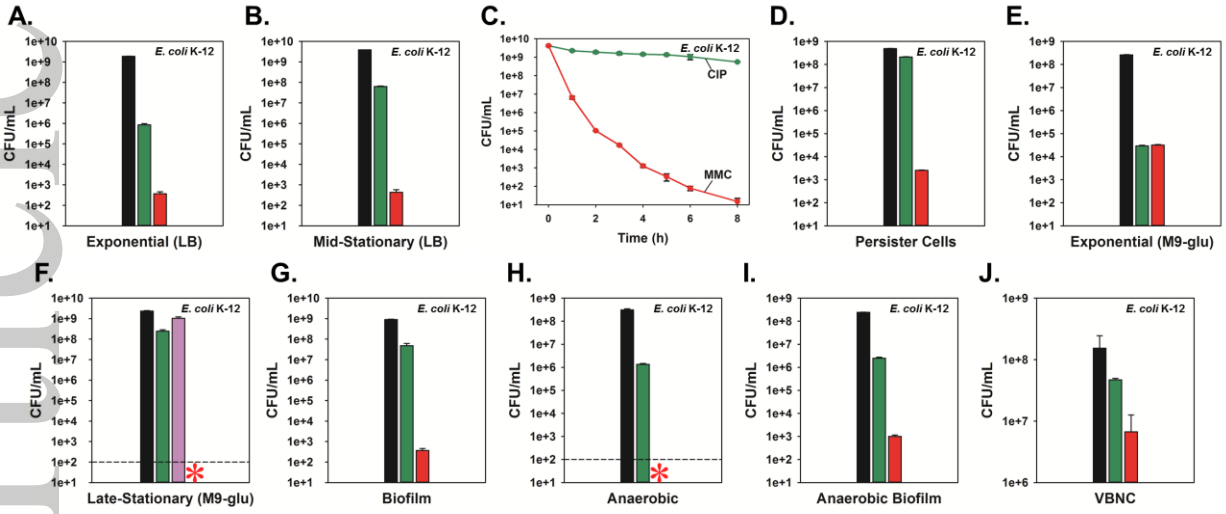
Fig. 1. MMC eradicates metabolically dormant *E. coli* K-12 cells in suspension and in biofilms. Cell viability for exponential (turbidity of 2 at 600 nm) **(A)** and mid-stationary phase (turbidity of 4 at 600 nm) (**B)** cultures in buffered LB. **(C)** Time course of killing of late stationary-phase cells (16 h of growth) in buffered LB. **(D)** Cell viability of rifampicin-induced persister cultures in buffered LB (30 min pretreatment with 100 μg/mL rifampicin followed by resuspension in fresh media). Cell viability of exponential (turbidity of 0.4 at 600 nm) **(E)** and late-stationary phase (24 h of growth) **(F)** cultures in M9-glucose. Cell viability of biofilm cultures (24 h of growth) in M9-glucose **(G)**, anaerobic late-stationary phase cultures (16 h of growth) in LB **(H)**, anaerobic biofilm cultures (24 h of growth) in M9-glucose **(I)**, and VBNC cultures (36 days of starvation in saline) **(J)**. Cell viability is shown before (black) and after treatment (3 h for planktonic cultures, 24 h for biofilm cultures, and 16 h for VBNC cultures) with 5 μg/mL ciprofloxacin (green), 10 μg/mL gentamicin (purple), and 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown throughout ($n \geq 2$). MMC is mitomycin C and CIP is ciprofloxacin.

Fig. 2. MMC crosslinks DNA in *E. coli* K-12 persister cells. (A) Cell viability of *E. coli* K-12 wild-type, Δ*uvrA*, Δ*uvrB*, and Δ*uvrC* mid-stationary phase cultures (turbidity of 3 at 600 nm) in buffered LB treated for 30 min with MMC. **(B)** Cell viability of *E. coli* K-12/pCA24N, Δ*uvrA*/pCA24N, and Δ*uvrA*/pCA24N-*uvrA* exponential phase cultures (turbidity of 2 at 600 nm) in buffered LB treated for 1 h with MMC. Cell viability is shown before (black) and after treatment with 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown for **A** and **B** ($n \geq 2$). **(C)** Denaturing gel electrophoresis for pDNA (4,518 nt) from *E. coli* K-12/pCA24N non-persisters (lanes 2 and 3) and rifampicin-induced persisters (lanes 4 and 5) before (lanes 2 and 4) and after (lanes 3 and 5) MMC treatment. Lane 1 is a positive control with *in vitro* crosslinked pDNA. "M" indicates the DNA ladder, the red arrow

indicates migration as double-stranded DNA, and the blue arrow indicates migration as single-stranded DNA.

Fig. 3. MMC eradicates pathogens in suspension and in biofilms. Cell viability of EHEC exponential (turbidity of 2 at 600 nm) **(A)** and mid-stationary phase (turbidity of 4 at 600 nm) **(B)** cultures in buffered LB, late-stationary phase cultures (24 h of growth) in M9-glucose **(C)**, and biofilm cultures (24 h of growth) in M9-glucose **(D).** Cell viability of *S. aureus* exponential (turbidity of 0.8 at 600 nm) **(E)** and mid-stationary phase (turbidity of 3 at 600 nm) cultures in tryptic soy broth (TSB) **(F)**, and biofilm cultures (24 h of growth) in modified M9-glucose **(G).** Cell viability of *P. aeruginosa* PA14 exponential phase cultures (turbidity of 2 at 600 nm) in buffered LB **(H)** and late-stationary phase cultures (24 h of growth) in M9-glucose **(I)**. Cell viability is shown before (black) and after treatment (3 h for planktonic cultures and 24 h for biofilm cultures) with 5 μg/mL ciprofloxacin (green) and 10 μg/mL MMC (red). * represents eradication beyond the limit of detection. Means ± s.d. are shown throughout ($n \geq 2$).

Fig. 4. MMC eradicates pathogens in clinically relevant wound and animal models. Cell viability of EHEC **(A)**, *S. aureus* **(B)**, and *P. aeruginosa* PAO1 **(C)** mono-cultures and *S. aureus* and *P. aeruginosa* PAO1 co-cultures **(D)** in an *in vitro* wound model (24 h of growth). Cell viability is shown before (black) and after 5 h treatment with ciprofloxacin (blue; 5 μg/mL for EHEC and *S. aureus* or 10 μg/mL for PAO1 mono- and co-cultures), ampicillin (green; 100 μg/mL for EHEC and *S. aureus* or 2 mg/mL for PAO1 mono- and co-cultures), and MMC (red; 10 μg/mL for EHEC and *S. aureus* or 15 μg/mL for *P. aeruginosa* PAO1 mono- and co-cultures). * represents eradication beyond the limit of detection. **(E)** Survival of *C. elegans* after infection with EHEC (days -2 to 0) followed by 6 h exposure to 5 μg/mL ciprofloxacin (green), 100 μg/mL ampicillin (blue), 10 μg/mL MMC (red), or no treatment (white). As a negative control, *C. elegans* was grown on OP50 without antibiotic treatment (black). Means ± s.d. are shown throughout ($n \geq 2$).



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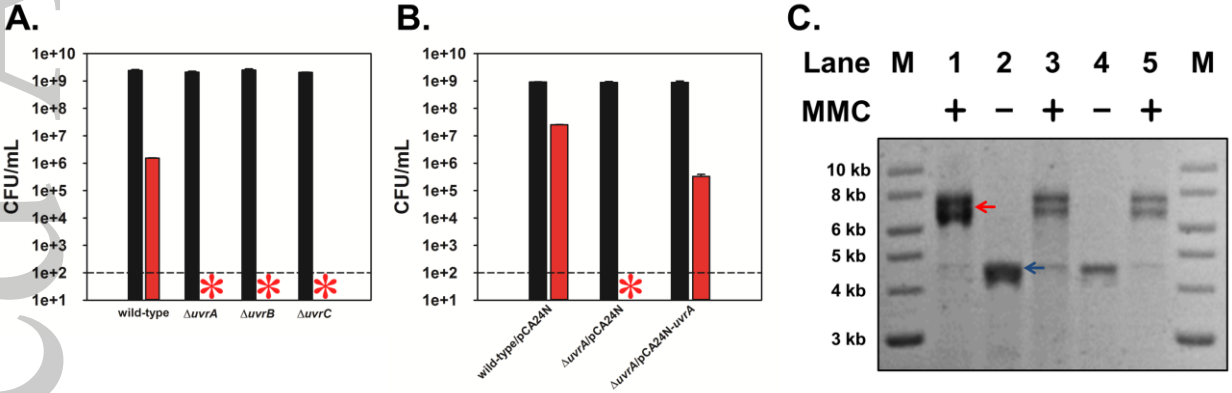

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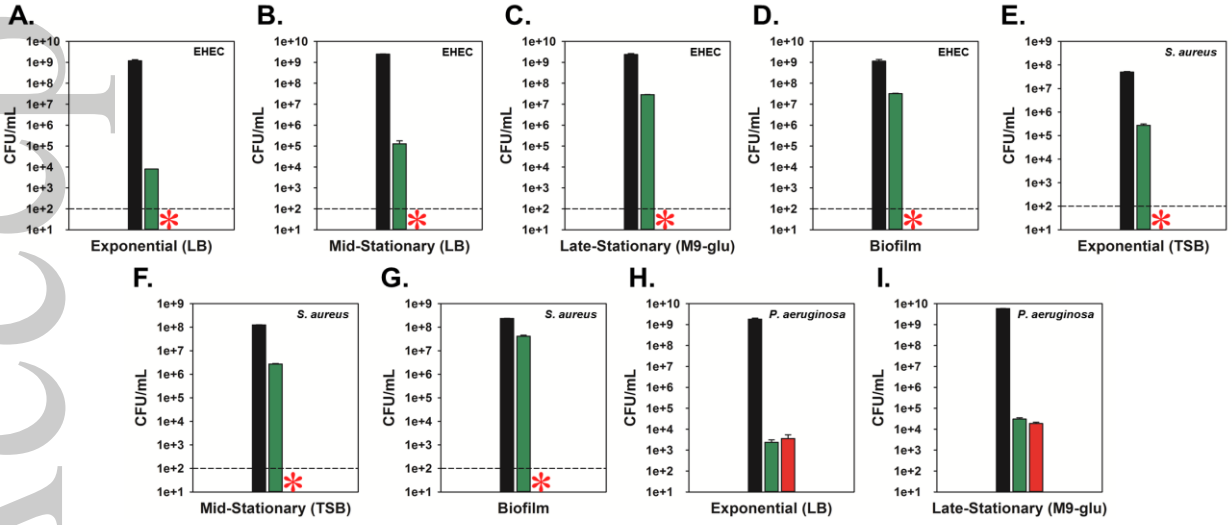

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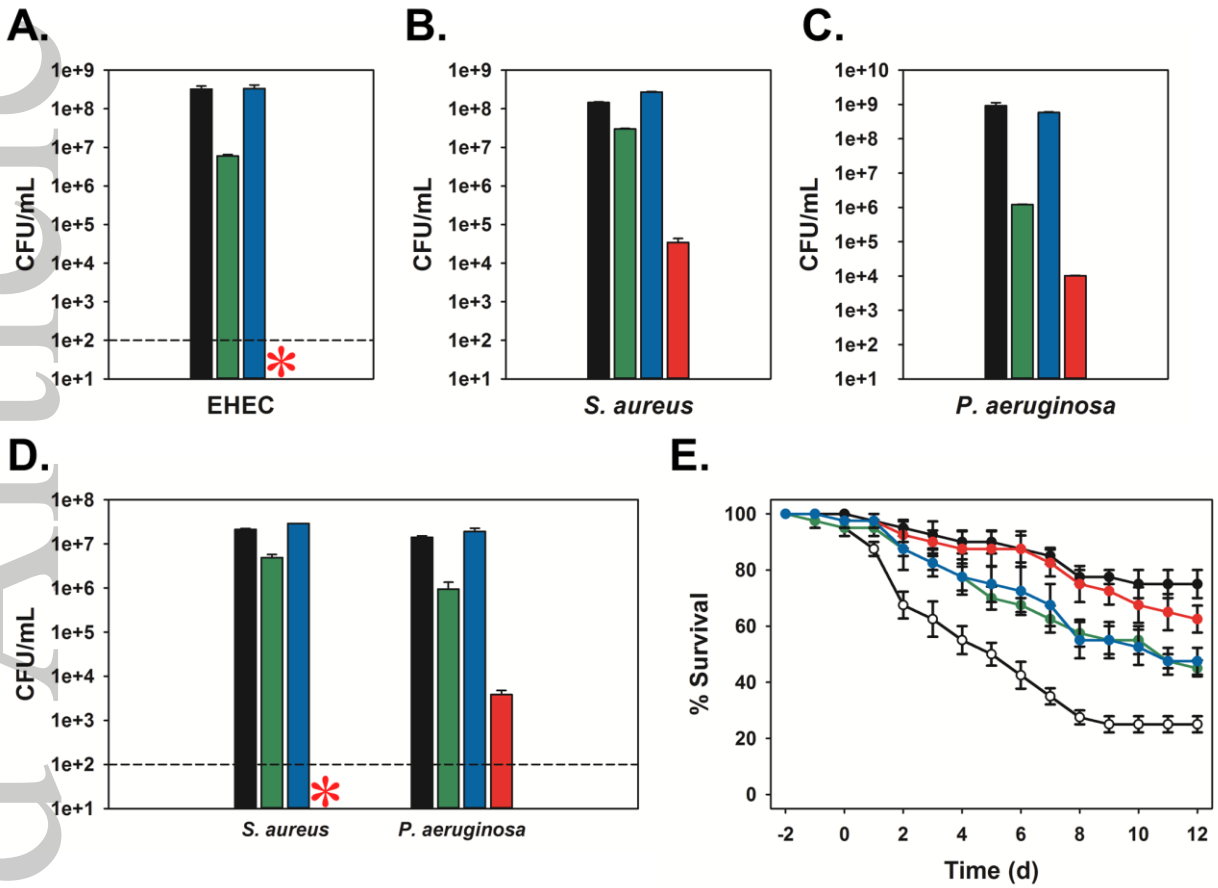

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