ACS Infectious Diseases

pubs.acs.org/journal/aidcbc

Article

Combined Efficacy of an Antimicrobial Cationic Peptide Polymer with Conventional Antibiotics to Combat Multidrug-Resistant Pathogens

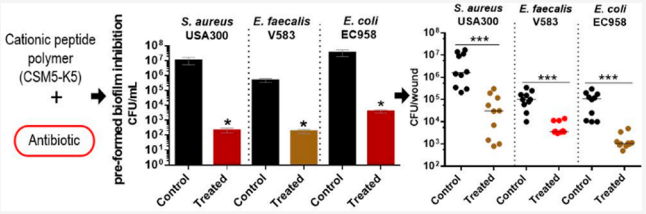
Kishore R. V. Thappeta, Yogesh S. Vikhe,[‡] Adeline M. H. Yong,[‡] Mary B. Chan-Park, and Kimberly A. Kline*

Cite This: *ACS Infect. Dis.* 2020, 6, 1228−1237

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: Antibiotic-resistant infections are predicted to kill 10 million people worldwide per year by 2050 and to cost the global economy 100 trillion USD. Novel approaches and alternatives to conventional antibiotics are urgently required to combat antimicrobial resistance. We have synthesized a chitosan-based oligolysine antimicrobial peptide, CSM5-K5 (where CSM denotes chitosan monomer repeat units and K denotes lysine amino acid repeat units), that targets multidrug-resistant (MDR) bacterial species. Here, we show that CSM5-K5 exhibits rapid bactericidal activity against methicillin-resistant *Staphylococcus aureus* (MRSA), MDR *Escherichia coli*, and vancomycin-resistant *Enterococcus faecalis* (VRE). Combinatorial therapy of CSM5-K5 with antibiotics to which each organism is otherwise resistant restores sensitivity to the conventional antibiotic. CSM5-K5 alone significantly reduced preformed bacterial biofilm by 2−4 orders of magnitude and, in combination with conventional antibiotics, reduced preformed biofilm by more than 2−3 orders of magnitude at subinhibitory concentrations. Moreover, using a mouse excisional wound infection model, CSM5-K5 treatment reduced bacterial burdens by 1−3 orders of magnitude and acted synergistically with oxacillin, vancomycin, and streptomycin to clear MRSA, VRE, and MDR *E. coli*, respectively. Importantly, little to no resistance against CSM5-K5 arose for any of the three MDR bacteria during 15 days of serial passage. Furthermore, low level resistance to CSM5-K5 that did arise for MRSA conferred increased susceptibility (collateral sensitivity) to the β-lactam antibiotic oxacillin. This work demonstrates the feasibility and benefits of using this synthetic cationic peptide as an alternative to, or in combination with, traditional antibiotics to treat infections caused by MDR bacteria.

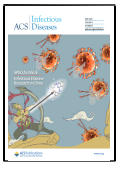


KEYWORDS: *biofilm therapeutics, biofilm-associated infection, MRSA, VRE, E. coli, antimicrobial peptide*

Antibiotic resistance is a threat to global public health and sustainability.[1] Antibiotic resistance currently accounts for an estimated 70,000 annual deaths globally, and in the absence of new therapeutics, infections caused by resistant "superbugs" could kill an additional 10 million people each year worldwide by 2050, surpassing cancer.[2] Moreover, by 2050, antibiotic-resistant infections are estimated to cost up to 3.5% of the global GDP, equivalent to 100 trillion USD.[2] Because corporate antibiotic development pipelines have progressively declined over the past 20 years,[3] there is substantial interest in seeking alternative therapeutic approaches to combat these multidrug-resistant (MDR) pathogens.

Host-derived antimicrobial peptides (CAMPs), typically composed of cationic and hydrophobic domains, have garnered interest as alternative therapies for MDR infections.[4−6] CAMPs are electrostatically attracted to anionic bacterial cell surfaces, followed by peptide insertion into the lipid bilayer via their hydrophobic residues.[5,7,8] Chitosan is a polysaccharide composed of repeating N-glucosamine, with a structure similar to that of bacterial peptidoglycan. This unique feature of chitosan renders it potentially compatible with the bacterial cell wall. Numerous studies have examined the efficacy of chitosan−CAMP hybrids containing quaternary ammonium,[9,10] pyridinium,[11] piperazinium,[12] phosphonium,[13] or sulfonamide[14] derivatives. However, many of these derivatives possess high cationicity and rely on hydrophobicity for improved bacterial interaction, which often lead to mammalian hemolysis and toxicity. To overcome these limitations, we have synthesized a low molecular weight copolymer CSM5-K5 hydrochloride salt (where CSM denotes chitosan monomer repeat units and K denotes lysine amino acid repeat units) with a controlled molecular weight of 1450 Da.[15] CSM5-K5 displays low hemolysis and toxicity, is

Received: January 13, 2020
Published: March 5, 2020



https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

antibacterial against a variety of MDR bacteria, and reduces methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial burden in a murine wound infection model by 4 orders of magnitude.[15]

Combinational therapy is also a promising approach to overcome and prevent antimicrobial resistance.[16] In combination treatment, a combination of conventional antibiotics is used together with other antibiotics[17,18] or non-antibiotic drugs[19,20] to increase the treatment efficacy compared to single drug therapy. Combination treatment can extend the lifetime of drugs, inhibits aftereffects, and suppresses the emergence of resistance.[21−23] One study reports that combination therapy with conventional antibiotics is less likely to result in synergy, and antagonism or indifference are more likely to arise.[24] While there have been several reports of synergy between conventional antibiotics and other drugs, very few have examined synthetic antimicrobial polymers in combination with conventional antibiotics.[25−27]

In this study, we examine the ability of CSM5-K5 to reduce preformed methicillin-resistant *Staphylococcus aureus* (MRSA), MDR *Escherichia coli*, and vancomycin-resistant *Enterococcus faecalis* (VRE) biofilm *in vitro* and *in vivo*. We show that CSM5-K5 alone and in synergy with clinically useful antibiotics demonstrates bactericidal activity and antibiofilm activity both *in vitro* and *in vivo* in a mouse excisional wound infection model. Taken together, these results demonstrate the feasibility and benefits of using the synthetic cationic peptide CSM5-K5 as an alternative to, or in combination with, conventional antibiotics to treat difficult to treat infections caused by MDR bacteria.

RESULTS AND DISCUSSION

CSM5-K5 Displays Broad-Spectrum Killing against MDR Bacterial Strains. To extend our previous studies[15] demonstrating CSM5-K5 (a cationic peptidopolysaccharide) (Figure 1A) displays broad-spectrum antimicrobial activity, we tested the efficacy of CSM5-K5 against a panel of MDR bacterial strains. Using minimum inhibitory concentration (MIC) assays, we found that CSM5-K5 displayed potent antibacterial activity against both Gram-positive and Gram-negative MDR pathogens including methicillin-resistant *S. aureus* USA300, vancomycin-resistant *E. faecalis* V583, and a highly virulent globally disseminating MDR and extended β-lactamase expressing strain of *E. coli* EC958. For all MDR strains tested, exposure to CSM5-K5 resulted in >98% killing within 5 h (Figure 1B).

Combination Treatment of CSM5-K5 with Conventional Antibiotics against MDR Clinical Isolates Restores Drug Sensitivity. To determine whether the potency of CSM5-K5 could be further improved if used in combination with conventional antibiotics, we performed combinatorial MIC assays with clinically relevant antibiotics for MDR strains of *S. aureus* USA300, *E. faecalis* V583, and *E. coli* EC958. We first determined the MIC for CSM5-K5 and a panel of antibiotics against each MDR clinical isolate (Table S1) and then tested for synergy between CSM5-K5 and the antibiotics for which each organism was resistant to using a checkerboard assay containing 2-fold dilutions for each compound. CSM5-K5 displayed partial synergy with oxacillin, Meropenem, and other antibiotics against *S. aureus* USA300 with a fractional inhibitory concentration index (FICI) of >0.5 to ≤0.1 (Table S2 and Figure 1C). Further, the reduction of CSM5-K5 to 0.5× MIC (8 μg/mL) in combination with oxacillin and

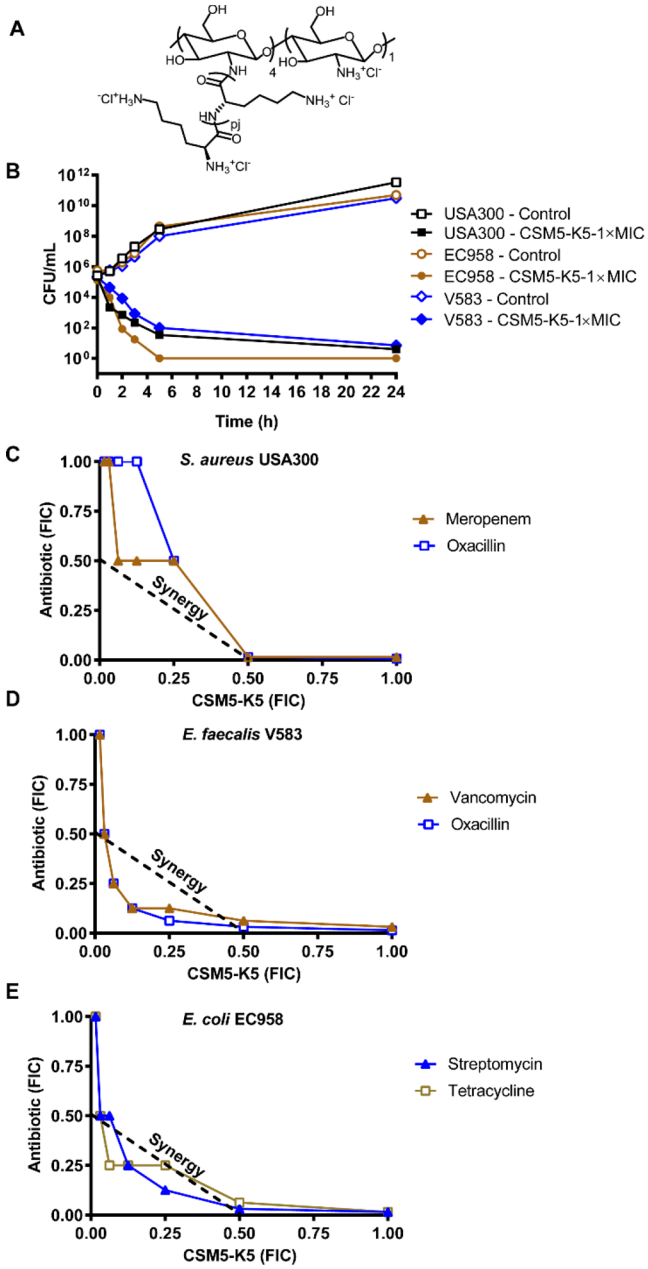


Figure 1. Synergistic combination treatment of MDR clinical isolates with CSM5-K5 and conventional antibiotics *in vitro*. (A) Structure of CSM5-K5. Adapted from ref 15. Copyright 2017 American Chemical Society. (B) CSM5-K5 time-killing activity on the three MDR strains, *S. aureus* USA300, *E. coli* EC958, and *E. faecalis* V583. (C−E) The FIC of each antibiotic−CSM5-K5 pair for (C) *S. aureus* USA300, (D) *E. faecalis* V583, and (E) *E. coli* EC958 are plotted. Synergy is concluded when the sum of individual FIC values is <0.5 (indicated by the diagonal dashed line). FIC values ≥0.5 indicate additive or partial synergistic activity. The data shown are derived from two independent biological experiments.

Meropenem reduced the respective MICs to 0.5 and 1.0 μg/mL, representing 64- and 8-fold reductions from their stand-alone MICs (32 and 8 μg/mL), respectively (Table S1, data not shown). Similarly, *E. faecalis* V583 is resistant to vancomycin and oxacillin, respectively, at concentrations of 32 μg/mL (Table S1). However, the exposure of *E. faecalis* V583 to CSM5-K5 at 0.25× MIC (16 μg/mL) demonstrated synergistic activity with vancomycin and oxacillin and restored

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

drug sensitivity to MICs of 4 and 2 μg/mL, respectively (Figure 1D, data not shown). Furthermore, CSM5-K5 exhibited potent synergy with streptomycin and tetracycline against *E. coli* EC958 (FICI <0.5) (Figure 1E, data not shown). The MICs for *E. coli* EC958 for both streptomycin and tetracycline are 32 μg/mL (Table S1). However, CSM5-K5 (0.25× MIC) is synergistic with streptomycin and tetracycline at concentrations of ≤8 μg/mL, bringing the MICs to 8 and 4 μg/mL, respectively (data not shown).

For all the antibiotics we tested in combination with CSM5-K5, we observed a reduction of the antibiotic MIC into the clinical therapeutic range for each bacterial species.[28] We confirmed synergistic interactions using time-killing curve assays and demonstrated that combinatorial bactericidal action occurred within synergistic concentrations of CSM5-K5 and respective antibiotics against each bacterial species. For *E. coli* EC958, we observed a more than 2-log reduction within 5 h of CSM5-K5 exposure at sub-MIC concentrations of 0.25× MIC and 0.37× MIC with aminoglycosides streptomycin or tetracycline at 0.125× MIC or 0.25× MIC (Figure S1A,B). In contrast, higher concentrations of 0.5× MIC for either streptomycin or tetracycline alone resulted in an initial drop in CFU followed by recovery by 5 h and growth of the culture (Figure S1A,B; see 0.5× MIC). Similarly, we observed a more than 2-log reduction of *E. faecalis* V583 within 5 h with combinatorial sub-MIC concentrations of CSM5-K5 and vancomycin or oxacillin (Figure S1C,D). Moreover, we observed bactericidal action against *S. aureus* USA300 at sub-MIC concentrations of CSM5-K5 of 0.5× with 0.03× MIC oxacillin and 0.06× MIC Meropenem (Figure S1E,F). CSM5-K5 alone and in combination with antibiotics effectively eradicates preformed biofilms of MDR pathogens *in vitro*.

Many of the most difficult to treat nosocomial and chronic infections are biofilm associated.[29] The intrinsic antibiotic tolerance of biofilms coupled with genetic antibiotic resistance of MDR strains often renders these infections recalcitrant to treatment. Therefore, we next addressed whether CSM5-K5 alone and in combination with antibiotics had synergistic bactericidal activity against biofilm, similar to that observed for planktonic bacteria. We therefore performed a minimum biofilm eradication concentration (MBEC) assay in which we exposed preformed 27 to 28 h biofilms to CSM5-K5 in the absence or presence of a conventional antibiotic for 3 to 4 h. Exposure of *S. aureus* USA300, *E. faecalis* V583, or *E. coli* EC958 to 1× MIC of CSM5-K5 resulted in an approximately 3-, 2, or 4-log reduction, respectively, for each of the organisms, representing >99% reduction in biofilm bacteria after just 4 h of treatment compared to the untreated controls (Figure 2A).

We observed for biofilms that, as for planktonic bacteria, CSM5-K5 was synergistic with conventional antibiotics at concentrations well below the MIC of each resistant strain. For example, CSM5-K5 displayed an antibiotic-enhancing effect for oxacillin and Meropenem against *S. aureus* USA300 preformed biofilms at subinhibitory concentrations of each. CSM5-K5 increased oxacillin biofilm reduction by 3−4.5 orders of magnitude compared with untreated biofilm and 1.9−3.3 orders of magnitude compared to either of their single agent treatment alone (Figure 2B). CSM5-K5 in combination with Meropenem displayed an even higher efficacy for biofilm reduction with 3.5−5.3 and 2−4 orders of magnitude reduction in CFU compared to untreated controls and single agent treatment, respectively (Figure 2B). Similarly, we

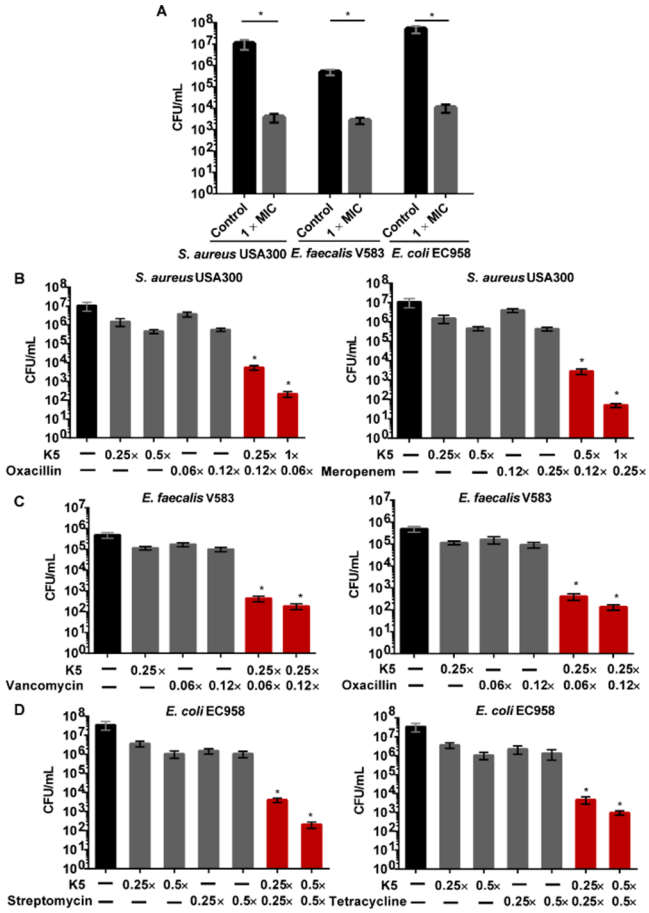


Figure 2. CSM5-K5 treatment reduces biomass of preformed MDR pathogen biofilms alone and synergistically in combination with traditional antibiotics *in vitro*. (A) *In vitro* biofilm assay upon exposure to CSM5-K5 (1× MIC) for *S. aureus* USA300, *E. faecalis* V583, or *E. coli* EC958. *In vitro* biofilm assay upon exposure to sub-MIC concentrations of CSM5-K5 in combination with (B) oxacillin or Meropenem against *S. aureus* USA300; (C) vancomycin or oxacillin against *E. faecalis* V583; (D) streptomycin or tetracycline against *E. coli* EC958. Data shown are combined from two independent experiments, each composed of 3 biological replicates, with mean values ± the standard error of the mean plotted. Significant differences between groups analyzed by one-way ANOVA using GraphPad (*$P < 0.05$).

observed synergy of CSM5-K5 with vancomycin and oxacillin against *E. faecalis* V583 preformed biofilms. Combinatorial treatment resulted in more than 3- and 2.4-log reductions compared to untreated controls and single agent treatment, respectively. At a subinhibitory concentration of CSM5-K5 at 0.25× MIC (16 μg/mL) with 0.06× MIC (2 μg/mL) and 0.12× MIC (4 μg/mL) of either of the two antibiotics, >99.8% of killing efficacy was achieved (Figure 2C). Finally, we applied CSM5-K5 in combination with streptomycin and tetracycline to preformed *E. coli* EC958 biofilms. The combination of CSM5-K5 (at 0.25× to 0.5× MIC) with either antibiotic at subinhibitory concentrations (0.25× and 0.5×) resulted in a biofilm reduction of >2.5−4 and >3.8−5.2 orders of magnitude compared with single agent treated and untreated controls, respectively. Combination therapy killed >99% of *E. coli* EC958 biofilm (Figure 2D).

***In Vivo* Combined Efficacy of CSM5-K5 with Clinically Relevant Antibiotics.** We next tested the ability of CSM5-K5

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

alone and in combination with conventional antibiotics to treat biofilm infections in a murine excisional wound infection model. Excisional wounds were infected with ~10^3 CFU of each bacterial species, and then, the wound site was treated 24 h post-infection with CSM5-K5 at 1× MIC alone or in combination with subinhibitory concentrations of antibiotics to which each strain is resistant. After 4−5 h of antimicrobial treatment, CFU was enumerated from the infection site. Treatment of each bacterial species with 1× MIC CSM5-K5 alone (16 and 64 μg/mL for *S. aureus* USA300 and *E. faecalis* V583, respectively) resulted in greater than 90% reduction of USA300 and V583 CFU compared to untreated controls (Figure 3A). Treatment of *E. coli* EC958 with 1× MIC of

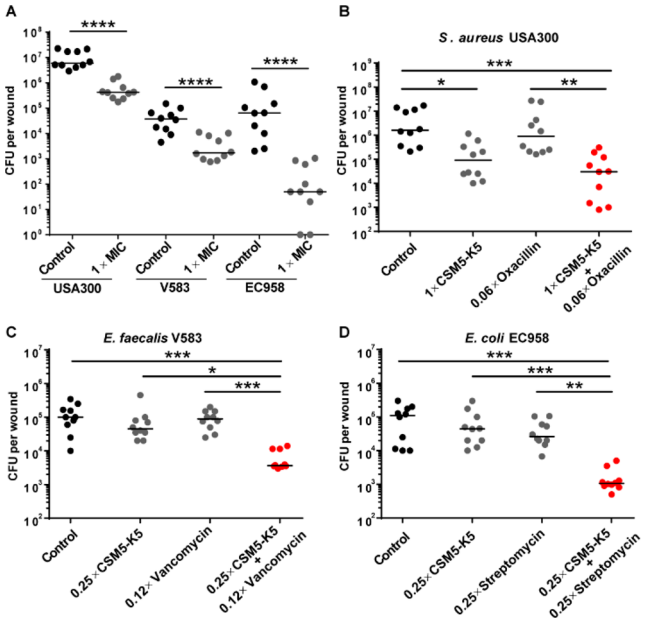


Figure 3. CSM5-K5 and conventional antibiotics synergistically attenuate MDR infection in a mouse model of biofilm-associated wound infection. Excisional wounds in mice were infected with the indicated pathogen for 24 h followed by treatment with CSM5-K5 (1× MIC) alone (A) or in combination with antibiotics (B−D) for 5 h. Each circle represents a single mouse. Horizontal lines represent the median of each group. Significant differences between groups were determined by the Kruskal−Wallis test using GraphPad (*$P < 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$). Data are combined from 2 biological experiments, each containing 5 mice per group for each infection.

CSM5-K5 resulted in >99% killing and a >3-log reduction in CFU compared to the untreated control (Figure 3A). While combinatorial treatment did not further reduce *S. aureus* CFU at the concentrations tested in this experiment compared to CSM5-K5 alone (Figure 3B), the treatment of *E. faecalis* and *E. coli* infected wounds with subinhibitory concentrations of CSM5-K5 together with subinhibitory concentrations of antibiotics significantly improved bacterial killing compared to either single agent treated or untreated control (Figure 3C,D). Importantly, combination treatment of each MDR strain with CSM5-K5 rendered them susceptible to antibiotic concentrations that fall below the clinical break point (0.06× MIC oxacillin = 2 μg/mL, 0.012× MIC vancomycin = 4 μg/mL, 0.25× MIC streptomycin = 8 μg/mL), although the effect was less pronounced for *S. aureus* where the addition of oxacillin did not result in an additive effect with CSM5-K5, as observed for *E. faecalis* and *E. coli*. Hence, CSM5-K5 sensitized MDR strains of these wound-associated pathogens to antibiotics to which they are otherwise resistant.

CSM5-K5 Exposure Does Not Result in Antimicrobial Resistance. The development of antimicrobial resistance is defined by greater than a 4-fold change to their initial MIC.[30−33] To investigate if long-term use of CSM5-K5 can lead to resistance in *S. aureus* USA300, *E. faecalis* V583, and *E. coli* EC958, we subjected these three MDR strains to continuous serial passaging at subinhibitory concentrations of CSM5-K5 over 15 days. However, *S. aureus* USA300, *E. faecalis* V583, and *E. coli* EC958 did not display more than a 2-fold increase in MIC after 15 days of culture with subinhibitory concentrations of CSM5-K5 (Figure 4). In contrast, serial passaging of *S. aureus* USA300 at subinhibitory concentrations of rifampicin, vancomycin, or gentamicin resulted in antibiotic resistance that was more than 4-fold higher than the initial

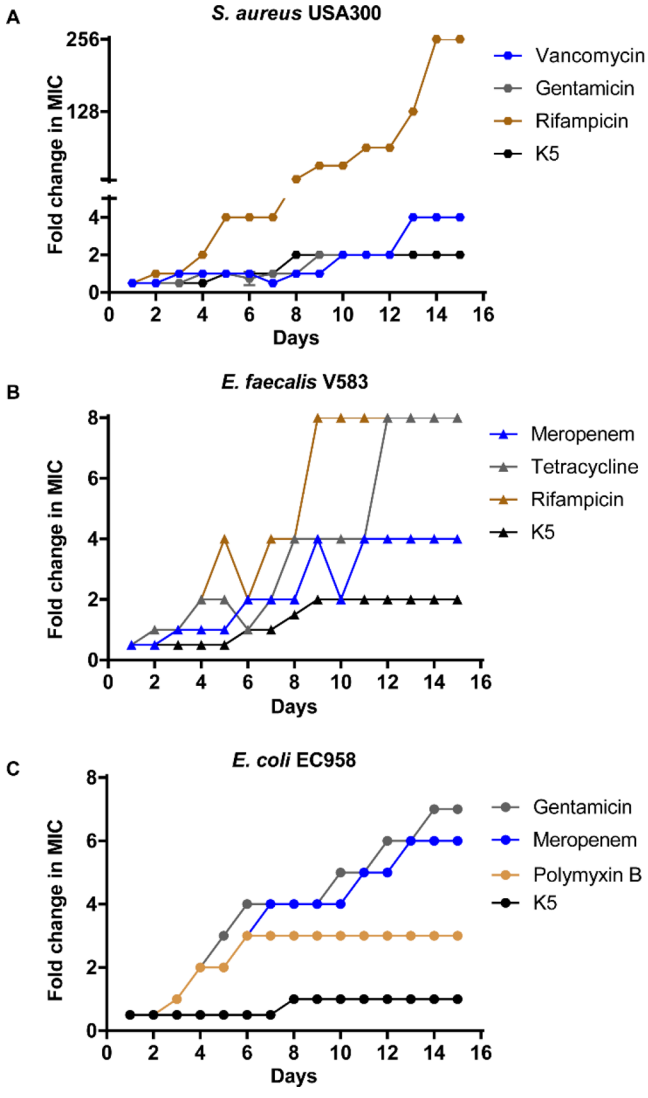


Figure 4. Prolonged CSM5-K5 exposure does not result in antimicrobial resistance. Continuous serial passaging of (A) *S. aureus* USA300, (B) *E. faecalis* V583, (C) and *E. coli* EC958 at subinhibitory concentrations of CSM5-K5 or antibiotics over 15 days. *Y*-axes represent the lowest drug concentration at which bacterial growth was observed, plotted as the fold change in comparison to the predetermined MIC for the respective antibiotic (Table S1).

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

MIC (Figure 4A). Similarly, MICs of rifampicin, tetracycline, and Meropenem were more than 4-fold higher in *E. faecalis* V583 after 15 days of coculture with the antibiotic (Figure 4B). In *E. coli* EC958, a resistance of 7, 6, and 3 times higher than the original MIC was observed for gentamicin, Meropenem, and polymyxin B, respectively (Figure 4C). The failure to obtain CSM5-K5 resistance levels more than 2-fold higher than their initial MIC values suggests a nonspecific mode of action.

Genetic Basis of CSM5-K5^R in MDR *E. coli*, *S. aureus*, and *E. faecalis*. To identify the genetic basis for low level CSM5-K5^R, we performed whole-genome sequencing analysis of CSM5-K5^R isolates of *E. coli* EC958, *S. aureus* USA300, and *E. faecalis* V583. With the aim of detecting mutations related to CSM5-K5^R in *E. faecalis* V583, we sequenced 5 isolates from each of two independent serial passage experimental evolution assays (10 isolates in total) isolated at day 6 when the CSM5-K5 MIC was 64 μg/mL (1× MIC). Using a threshold variant frequency cutoff of >35%, we found that resistant mutants contained a diverse set of mutations (Table S3). At day 6, we observed independent mutations in genes encoding ATP synthase machinery in 6 of 10 mutants (Table S3, Figure 4B). We validated this finding by assessing the MIC for *E. faecalis* transposon mutants in the respective genes in strain OG1RF (*atpE*, *atpE2*, and *atpG*), which all displayed a 2-fold higher MIC for CSM5-K5 (Table S4). CSM5-K5^R *E. faecalis* mutants isolated at day 15 when the MIC reached 2× (128 μg/mL) appeared to be siblings and displayed mutations in *guaB* and genes encoding a TetR family regulator and a HAD superfamily hydrolase in all the isolates. The examination of a transposon mutant in the TetR family regulator, OG1RF_11670, which displays >99% sequence identity with V583 EF2066 demonstrated a 2-fold higher MIC for CSMK5-K5. Together, these results indicate that mutations in ATP synthase and a TetR family regulator can contribute to CSM5-K5 resistance in *E. faecalis*.

Similarly, we sequenced nine isolates of CSM5-K5^R *S. aureus* USA300 from two independent serial passage evolution experiments at day 5 at which the MIC was 16 μg/mL (1× MIC) (Table S3, Figure 4A). We identified a diverse array of mutations, including substitution mutations in the MFS transporter encoded by *narK* and in an ABC transporter permease (FtsX-like permease family protein), both of which are known to confer antimicrobial peptide resistance[34−36] and which may contribute to the observed low level CSM5-K5 resistance in *S. aureus*. Nine CSM5-K5^R isolates of *E. coli* EC958 from two independent experiments were also isolated and sequenced at day 8, where the MIC was 32 μg/mL (1× MIC) (Table S3, Figure 4C). All mutants contained a nonsense mutation in the membrane associated peptidase, encoded by *pepP*, which cleaves peptide bonds between any amino acid and proline[37] and is involved in outer membrane vesicle production,[38] both of which could be contributing to the mechanism of CSM5-K5 resistance.

Restored Oxacillin Susceptibility in CSM5-K5^R USA300 Isolates. In an attempt to understand why we observed enhanced oxacillin susceptibility after *S. aureus* coincubation with CSM5-K5 and oxacillin, we reanalyzed the same CSM5-K5^R mutants with a reduced threshold variant frequency of <35% and observed mutations in the *ebh* gene encoding hyperosmolarity resistance protein Ebh in nine isolates, which is associated with susceptibility to the β-lactam antibiotic oxacillin[39] (Table S5). We therefore examined whether *S. aureus* USA300 CSM5-K5^R isolates display oxacillin sensitivity and found that all *S. aureus* isolates showed reduced susceptibility to oxacillin (MIC ≤ 2 μg/mL) compared to the wild-type (MIC 32 μg/mL) (Table S6). *S. aureus* USA300 CSM5-K5^R isolates also displayed 2- to 4-fold greater susceptibility to carbenicillin and piperacillin but no change in susceptibility to other antibiotic classes tested (Table S6). Similarly, CSM5-K5^R did not confer cross resistance to any other antibiotic for either *E. faecalis* or *E. coli* (Table S6). Finally, we selected and evaluated whether CSM5-K5^R mutant 11 conferred oxacillin susceptibility to this otherwise oxacillin-resistant strain both *in vitro* and *in vivo* models. We observed that CSM5-K5^R mutant 11 exhibited oxacillin susceptibility at an MIC of 2 μg/mL (Figure 5A). Similarly, we observed a significant difference between mutant and wild-type growth when treated with oxacillin as well as with CSM5-K5 (Figure

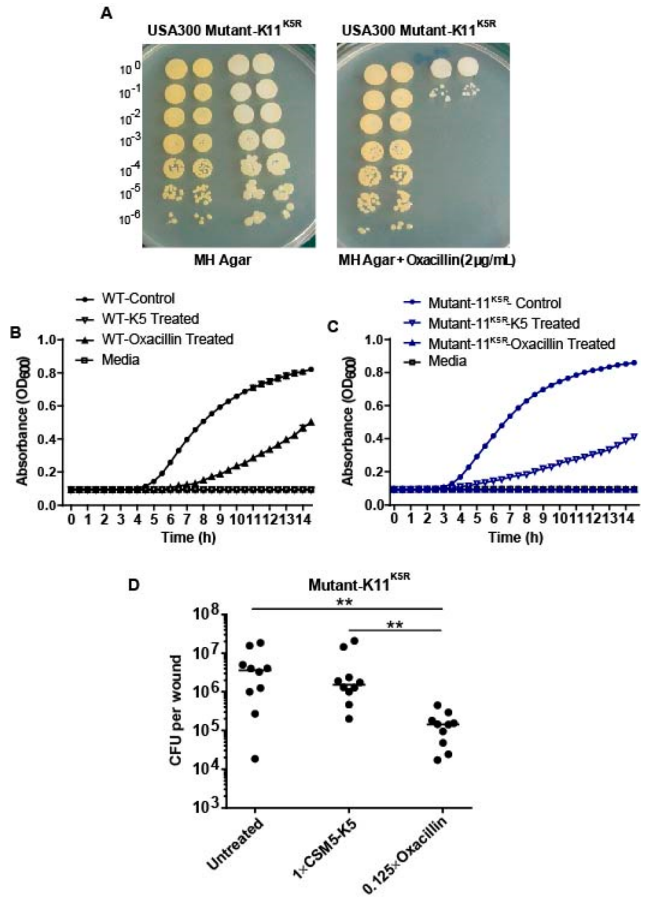


Figure 5. Collateral sensitivity in *S. aureus* USA300 CSM5-K5^R mutants to oxacillin. (A) Serial dilutions of WT USA300 and CSM5-K5^R mutant-11 were spotted onto MH agar alone or MH agar containing 2 μg/mL oxacillin. Growth curves of (B) *S. aureus* USA300 WT parent strain and (C) CSM5-K5^R mutant-11 in the presence of oxacillin and CSM5-K5 at concentrations of 2 and 16 μg/mL, respectively. Bacteria without any treatment as well as media only were used as negative controls. Error bars represent the standard deviation. (D) Excisional wounds in mice were infected with CSM5-K5^R mutant-11 for 24 h followed by 5 h of treatment with CSM5-K5 16 μg/mL (1× MIC) or oxacillin (4 μg/mL). Each circle represents a single mouse. Horizontal lines represent the median of each group. Statistical analysis was performed by the Kruskal−Wallis test using GraphPad (**$P \leq 0.01$). Data shown are combined from two independent experiments, each containing 5 mice per group for each infection.

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

5B,C). Finally, in the murine excisional wound infection model, we observed that oxacillin alone was now effective in reducing infection by a CSM5-K5^R mutant 11 at 0.125× MIC (4 μg/mL) determined for this resistant strain (Figure 5D).

CONCLUSION

Multidrug-resistant (MDR) bacterial infections are a growing and significant threat to public health and are caused by species including methicillin-resistant *S. aureus*, vancomycin-resistant *E. faecalis*, and the globally disseminated CTX-M type ESBL-expressing strains of *E. coli*.[40,41] Antimicrobial peptides (AMPs) are of interest as alternatives to traditional antibiotics because of their broad spectrum antimicrobial properties and efficacy against MDR bacteria; however, to date, they have met limited success due to their often nonselective toxicity.[42−46] We previously reported that chitosan-based cationic polymers showed antimicrobial activity against a variety of MDR pathogens.[15,30−32] In this study, we demonstrate that CSM5-K5 is synergistically active with traditional antibiotics against three antimicrobial-resistant pathogens growing as biofilms *in vitro* and *in vivo*. Moreover, CSM5-K5 restores sensitivity of MRSA USA300 to oxacillin, *E. faecalis* V583 to vancomycin, and MDR and ESBL producing *E. coli* EC958 to streptomycin. Significantly, antibiotic sensitivity is restored to concentrations equal or below the clinical break point value for each antibiotic. Finally, prolonged exposure to CSM5-K5 did not give rise to clinically significant levels of resistance. Moreover, remarkably, the low level CSM5-K5 resistance that did arise in *S. aureus* USA300 exhibited collateral sensitivity to β-lactam antibiotics including oxacillin, piperacillin, and carbenicillin, restoring oxacillin and carbenicillin sensitivity to clinical breakpoint values.

CSM5-K5 is a cationic nanoparticle that is self-assembled from chitosan-*graft*-oligolysine chains with ultralow molecular weight (1450 Da) that selectively kills bacteria with minimal toxicity toward mammalian cells.[15] Hydrogen bonding within CSM5-K5 causes the polymer chains to aggregate into small nanoparticles to concentrate the cationic charge of the lysine. Upon contact with the bacterial membrane, these cationic nanoparticles synergistically cluster anionic membrane lipids and produce a greater membrane perturbation and antibacterial effect than would be achievable by the same quantity of charge if dispersed in individual copolymer molecules in solution.[15] We observed partial or full antimicrobial synergy, against both Gram-negative and Gram-positive pathogens, between CSM5-K5 and nearly every antibiotic that we tested. We propose that the membrane-perturbing action of CSM5-K5 enables increased uptake and/or access of each antibiotic to its target. In this study, this synergy could be achieved at antibiotic concentrations to which the MDR pathogens were otherwise resistant. These observations suggest that this may be true for any antimicrobial-resistant organism, regardless of the resistance mechanism or antibiotic mechanism of action. Together, the data in this manuscript present a viable combinatorial treatment strategy for difficult to treat antibiotic tolerant biofilm-associated infections, as well as those with genetically encoded resistance to traditional antibiotics, with minimal risk of antimicrobial resistance.

METHODS

Bacterial Strains and Growth Conditions. Bacterial strains used in this study are listed in Table 1. *E. coli* strains were grown overnight in Luria−Bertani (LB) broth with shaking or on agar at 37 °C under static conditions. *E. faecalis* strains were grown statically in brain heart infusion (BHI) broth or agar at 37 °C under static conditions. *S. aureus* strains were grown overnight in tryptic soy broth (TSB) or agar at 37 °C under static conditions. We used Muller Hinton (MH) broth with shaking or with 1.5% agar to perform antibiotic susceptibility assays. All inoculations were cultured for 16−18 h at 37 °C, unless stated otherwise. Overnight cultures of bacteria were centrifuged at 6000*g* for 5 min and resuspended in 1× PBS at an optical density (OD600) of 0.7 for all MIC assays, unless stated otherwise.

Table 1. Bacterial Strains Used in This Study

strains	references or source
E. faecalis	
OG1RF	33
V583	47
E. coli	
UTI89	48
EC958	49
S. aureus	
USA300 (Strain LAC)	50
ATCC BAA-40	ATCC
E. faecalis OG1RF Tn mutants[a]	51

[a]Refer to Table S4 for the list of Tn mutants used in this study.

Polymer Synthesis. CSM5-K5 was synthesized and characterized and has the same properties as the identical preparation of CSM5-K5 described by Hou et al.[15] Briefly, 5 g of low molecular weight chitosan (MW 200 kDa) was first dispersed in 100 mL of anhydrous DMF and sonicated at 80 °C for 1 h under argon protection. Protection of the amine group on chitosan was carried out by further adding 13.8 g of phthalic anhydride under 130 °C and reacting for 24 h. Further protection of the 6-hydroxyl group was carried out by reacting 8 g of phthalic protected chitosan with 24 g of trityl chloride in 100 mL of anhydrous pyridine at 100 °C for 24 h. Then, the chitosan macroinitiator was obtained by deprotection of the phthalic group using hydrazine. Typically, 5 g of protected chitosan was deprotected by 100 mL of 50% hydrazine at 100 °C for 24 h. The protected chitosan-grafted-polylysine was synthesized by ring-opening polymerization of lysine *N*-carboxyanhydride (NCA) monomer initiated from the chitosan macroinitiator. Briefly, 1.32 g of lysine−NCA monomer was dissolved in 8 mL of anhydrous DMF, and 112 mg of chitosan macroinitiator was dissolved in 3 mL of anhydrous DMF; the chitosan macroinitiator solution was added into lysine−NCA monomer solution under argon protection to initiate polymerization. The polymerization was carried out at room temperature for 3 days. Ultrashort CSM5-K5 cationic peptidopolysaccharide was obtained by acidic deprotection and hydrolysis of 1 g of protected chitosan-grafted-polylysine with 10 mL of concentrated hydrochloride solution (37%) at 60 °C for 100 min. The crude deprotected product was neutralized by NaOH solution (1 M) and dialyzed with a 1000 Da cutoff cellulose membrane against deionized water for 5 days. The residue was lyophilized to obtain a white solid with a molecular weight of 1450 Da (determined by MALDI-TOF analysis).

Determination of Minimum Inhibitory Concentration (MIC). MIC values were determined using a broth micro dilution method as previously described.[52] Bacterial cells were

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

grown to mid-log phase and an optical density (600 nm) of 0.5 for each organism and normalized to 10^6 CFU/mL. We dissolved the peptide polymer in water to a stock concentration of 10 mg/mL. The stock concentrations of antibiotics were prepared according to CLSI guidelines.[28] Fifty microliters of the 1−5 × 10^5 CFU/mL bacterial cultures were aliquoted into 96-well microtiter plates and mixed with 50 μL of media without or with 2-fold dilutions of the peptide polymer or antibiotics and incubated for 16−18 h at 37 °C with shaking at 200 rpm. Growth inhibition was determined by measuring the optical density (OD600) of each well using a microplate reader (Infinite M200 Pro, Tecan, Switzerland). We determined the MIC of each bacterial strain by the lowest peptide concentration that inhibits more than 90% bacterial growth.

Time-Dependent Killing Assay. Bacteria were grown, diluted, and aliquoted into 96-well microtiter plates as described above and mixed with 50 μL of either 0.5× or 1× MIC of the peptide polymer with or without antibiotics added. The plates were sealed with parafilm and incubated at 37 °C with shaking at 200 rpm. Twenty microliters of the culture was extracted at time intervals of 0, 0.5, 1, 2, 3, 5, and 24 h for measurement of the OD and colony-forming units, determined by dilution plating. Five microliters of each dilution was spotted on the BHI agar plates and incubated at 37 °C for 24 h prior to enumeration.

Antimicrobial Synergy Assay. We measured the synergetic effects between the peptide polymer CSM5-K5 and the antibiotics using the fractional inhibitory concentration index (FICI) method.[53] We performed checkerboard susceptibility assays to measure the MICs of antimicrobial combinations as previously described.[54] The fractional inhibitory concentration (FIC) indices were calculated according to the following formulas:

$$\text{FICA} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}}$$

$$\text{FICB} = \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

$$\text{FIC index} = \text{FICA} + \text{FICB}$$

FIC index = FICA + FICB, where FICA = (MIC of drug A in combination)/(MIC of drug A alone) and FICB = (MIC of drug B in combination)/(MIC of drug B alone). A conservative interpretation of the FICI defines synergy as a FIC index of ≤0.5.[53]

Minimum Biofilm Eradication Concentration (MBEC) Assay. MBEC biofilm assays were carried out according to published Innovotech methods (https://www.astm.org/Standards/E2799.htm?A). Briefly, overnight grown cultures of bacteria were diluted to between 10^5 and 10^6 CFU/mL in fresh MHB (for *E. faecalis*, TSB with 0.25% glucose was used to achieve optimal growth), and 150 μL was transferred to the wells of a MBEC microtiter plate (Innovotech, Canada); the MBEC lid was placed on top of the wells. Biofilms were grown on the MBEC pegs at 37 °C with shaking at 200 rpm for 24 h. The pegs were washed gently with 200 μL of 1× PBS, and the lid was transferred to a new plate in which wells contained CSM5-K5 and/or antibiotic in MHB and incubated at 37 °C for 4 h. The pegs were gently washed twice with 200 μL of 1× PBS to remove nonadherent cells. Adherent biofilms on the pegs were placed in 200 μL of 1× PBS and in a sonicating water bath for 30 min to disrupt the biofilm, prior to serial dilution in 1× PBS and CFU enumeration on BHI agar plates after growth at 37 °C. Experiments were carried out in triplicate, and two independent experiments were executed for each of these assays.

Antimicrobial Resistance Evolution Assay. We assessed resistance development of the bacterial strains by sequential passaging of each strain in the presence of subinhibitory concentrations of the cationic peptide polymer, CSM5-K5, or conventional antibiotics, essentially as previously described.[55] In brief, bacterial cells were grown at 37 °C in MH broth with shaking to mid-log phase, diluted to 1−5 × 10^5 CFU/mL in MH broth containing 0.2×, 0.5×, 1×, 2×, and 4× MIC concentrations of the peptide polymer or antibiotic, and incubated at 37 °C with shaking for EC958 and USA300 and statically for V583. At 24 h intervals, the cultures from the second highest concentration of polymer or antibiotics that allowed growth (OD600 of 0.1−0.2) were diluted 1:100 into fresh media containing the same set of MIC concentrations on the basis of the most recently visually observed MIC. The serial passaging was therefore repeated with increasing concentrations of peptide polymer or antibiotics over a period of 15 days from two independent starter cultures per bacterial strain. To test the stability of the resistant mutations, cultures that grew at the MIC or higher were streaked onto peptide polymer-free MH agar plates; individual colonies were selected and passaged daily in MH broth for 5 days, and a MIC was determined by broth micro dilution.

Whole-Genome Sequencing. Genomic DNA was extracted from overnight bacteria cultures of resistant and wild-type parental strains using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) and quantified and measured for DNA quality by the Qubit High Sensitive dsDNA assay (Invitrogen, Carlsbad, CA, USA) and NanoDrop. The genomic DNA samples were sequenced on an Illumina MiSeq v3 platform. Whole genome sequencing data was analyzed using CLC Genomics Workbench 9.5 compared with reference genomes of *E. faecalis* V583 (Gene bank accession number NC_004668), *S. aureus* USA300 _FPR3757 (Gene bank accession number NC_007793), and *E. coli* EC958 (Gene bank accession number NZ_HG941718) from NCBI for mapping and annotation. Threshold variant frequency was set at >35% for all bacterial species. To detect variants, all mappings were analyzed with the basic variant detection with regions of no coverage compared to the sequences of the respective wild-type parental strains at the particular time point.

Murine Excisional Wound Model. The murine wound infection model was carried out as described with minor modifications.[56] Briefly, we grew the bacterial strains in 15 mL of TSB supplemented with 0.25% glucose for 16−18 h at 37 °C with continuous shaking at 200 rpm. Cells were collected, washed twice with 1× sterile PBS, diluted to an OD of 0.5, and normalized to 1−3 × 10^7 CFU/mL. We isoflurane-anesthetized groups of five male wild-type C57BL/6 mice (7−8 weeks old, 22 to 25 g; InVivos, Singapore) with their dorsal hair trimmed. Following trimming, Nair cream (Church and Dwight Co, Charles Ewing Boulevard, USA) was applied and the fine hair was removed via shaving with a scalpel. We then disinfected the skin with 70% ethanol. A 6 mm biopsy punch (Integra Miltex, New York, USA) was used to create a full-thickness wound, and an inoculum of ~10^5 CFU of the bacteria in a 10 μL volume was applied. We sealed the wound

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

site with a Finn chamber on a Scanpor tape (Smart Practice, Phoenix, AZ, USA), and the chamber was fixed to the skin via Fixomull stretch plasters (BSN medical GmbH, Hamburg, Germany). After 24 h post-infection, the Finn chambers were detached and discarded. We treated the infected wound with 10 μL of 1× MIC CSM5-K5 polymer prior to sealing of the wound site with new Finn chambers and Scanpor tape and allowed treatment for another 5 h. After 5 h of application, mice were euthanized and a 1 cm × 1 cm squared piece of skin surrounding the wound site was excised and collected in sterile 1× PBS. We used a homogenizer (Pro200, SPD scientific, Singapore) for approximately 10 s at high speed to homogenize the skin samples, and the viable bacteria were enumerated by plating dilutions onto both BHI plates and antibiotic selection plates (ciprofloxacin for EC958, vancomycin for V583, and oxacillin for USA300) to ensure all recovered colony forming units corresponded to the inoculating strain. For synergy studies, we treated the infected wound with 5 μL of CSM5-K5 polymer and 5 μL of the respective antibiotics. For each experiment, two independent biological replicates were performed containing 5 mice per group. Statistical analysis was performed by the Mann−Whitney test using Prism software (GraphPad). We performed all approved procedures in accordance with the Institutional Animal Care and Use Committee (IACUC) at Nanyang Technological University, School of Biological Sciences, (ARFSBS/NIEA0198Z) for the murine wound infection model.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.0c00016.

> Additional experimental data, including time-dependent killing assays, FIC indices of antibiotics, MIC data, transposon screening assay, cross-sensitivity/resistance assay, and SNP identification shown in this study (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Kimberly A. Kline − *Singapore Centre for Environmental Life Science Engineering and School of Biological Sciences, Nanyang Technological University, Singapore 637551;* orcid.org/0000-0002-5472-3074; Email: KKLINE@ntu.edu.sg

Authors

Kishore R. V. Thappeta − *Singapore Centre for Environmental Life Science Engineering, Nanyang Technological University, Singapore 637551*

Yogesh S. Vikhe − *School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459*

Adeline M. H. Yong − *Singapore Centre for Environmental Life Science Engineering and School of Biological Sciences, Nanyang Technological University, Singapore 637551*

Mary B. Chan-Park − *School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637459;* orcid.org/0000-0003-3761-7517

Complete contact information is available at: https://pubs.acs.org/10.1021/acsinfecdis.0c00016

Author Contributions

‡Y.S.V. and A.M.H.Y. contributed equally. K.R.V.T., A.M.H.Y., and K.A.K. wrote the manuscript with input from the other authors. K.R.V.T. performed all the experiments with assistance with the animal infections from A.M.H.Y. Y.S.V. and M.B.C.P. provided CSM5-K5. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Jenny Dale and Gary Dunny for supplying us with the *E. faecalis* OG1RF transposon mutants used in this study. This work was supported by the National Research Foundation and Ministry of Education Singapore under its Research Centre of Excellence Program and by a Singapore Ministry of Education Tier 3 grant (MOE2013-T3-1-002).

■ ABBREVIATIONS

AMPs, antimicrobial peptides; BHI, brain heart infusion; CAMPs, cationic antimicrobial peptides; CFU, colony forming units; CSM5-K5, five chitosan monomer repeat units and five lysine amino acid repeat units; DMF, dimethylformamide; ESBL, extended spectrum β-lactamase; FICI, fractional inhibitory concentration index; LB, Luria−Bertani; MBEC, minimum biofilm eradication concentration; MDR, multidrug resistant; MIC, minimum inhibitory concentration; MH, Muller Hinton; MRSA, methicillin-resistant *Staphylococcus aureus*; NCA, *N*-carboxyanhydride; TSB, tryptic soy broth; VRE, vancomycin-resistant *Enterococcus faecalis*

■ REFERENCES

(1) World Health Organization, Food and Agriculture Organization of the United Nations, and World Organization for Animal Health (2018) *Monitoring global progress on addressing antimicrobial resistance: analysis report of the second round of results of AMR country self-assessment survey 2018*, World Health Organization.

(2) Piddock, L. J. V. (2016) Reflecting on the final report of the O'Neill Review on Antimicrobial Resistance. *Lancet Infect. Dis. 16* (7), 767−768.

(3) Cooper, M. A., and Shlaes, D. (2011) Fix the antibiotics pipeline. *Nature 472* (7341), 32.

(4) Chen, S., Shao, X., Xiao, X., Dai, Y., Wang, Y., Xie, J., Jiang, W., Sun, Y., Cong, Z., Qiao, Z., Zhang, H., Liu, L., Zhang, Q., Zhang, W., Zheng, L., Yu, B., Chen, M., Cui, W., Fei, J., and Liu, R. (2020) Host Defense Peptide Mimicking Peptide Polymer Exerting Fast, Broad Spectrum, and Potent Activities toward Clinically Isolated Multidrug-Resistant Bacteria. *ACS Infect. Dis.*, DOI: 10.1021/acsinfecdis.9b00410.

(5) Zhang, S., Xiao, X.-M., Qi, F., Ma, P.-C., Zhang, W.-W., Dai, C.-Z., Zhang, D.-F., and Liu, R.-H. (2019) Biofilm Disruption Utilizing α/β Chimeric Polypeptide Molecular Brushes. *Chin. J. Polym. Sci. 37* (11), 1105−1112.

(6) Zhang, K., Du, Y., Si, Z., Liu, Y., Turvey, M. E., Raju, C., Keogh, D., Ruan, L., Jothy, S. L., Reghu, S., Marimuthu, K., De, P. P., Ng, O. T., Mediavilla, J. R., Kreiswirth, B. N., Chi, Y. R., Ren, J., Tam, K. C., Liu, X. W., Duan, H., Zhu, Y., Mu, Y., Hammond, P. T., Bazan, G. C., Pethe, K., and Chan-Park, M. B. (2019) Enantiomeric glycosylated cationic block co-beta-peptides eradicate Staphylococcus aureus biofilms and antibiotic-tolerant persisters. *Nat. Commun. 10* (1), 4792.

(7) Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol. 3* (3), 238−50.

(8) He, Y., and Lazaridis, T. (2013) Activity determinants of helical antimicrobial peptides: a large-scale computational study. *PLoS One 8* (6), No. e66440.

(9) Sahariah, P., Gaware, V. S., Lieder, R., Jonsdottir, S., Hjalmarsdottir, M. A., Sigurjonsson, O. E., and Masson, M. (2014) The effect of substituent, degree of acetylation and positioning of the cationic charge on the antibacterial activity of quaternary chitosan derivatives. *Mar. Drugs 12* (8), 4635−58.
(10) Li, P., Poon, Y. F., Li, W., Zhu, H. Y., Yeap, S. H., Cao, Y., Qi, X., Zhou, C., Lamrani, M., Beuerman, R. W., Kang, E. T., Mu, Y., Li, C. M., Chang, M. W., Leong, S. S., and Chan-Park, M. B. (2011) A polycationic antimicrobial and biocompatible hydrogel with microbe membrane suctioning ability. *Nat. Mater. 10* (2), 149−156.
(11) Sajomsang, W., Tantayanon, S., Tangpasuthadol, V., and Daly, W. H. (2009) Quaternization of N-aryl chitosan derivatives: synthesis, characterization, and antibacterial activity. *Carbohydr. Res. 344* (18), 2502−11.
(12) Vinsova, J., and Vavrikova, E. (2011) Chitosan derivatives with antimicrobial, antitumour and antioxidant activities–a review. *Curr. Pharm. Des. 17* (32), 3596−3607.
(13) Cai, J., Zhang, W., Xu, J., Xue, W., and Liu, Z. (2017) Evaluation of N-phosphonium chitosan as a novel vaccine carrier for intramuscular immunization. *J. Biomater. Appl. 32* (5), 677−685.
(14) Dragostin, O. M., Samal, S. K., Dash, M., Lupascu, F., Panzariu, A., Tuchilus, C., Ghetu, N., Danciu, M., Dubruel, P., Pieptu, D., Vasile, C., Tatia, R., and Profire, L. (2016) New antimicrobial chitosan derivatives for wound dressing applications. *Carbohydr. Polym. 141*, 28−40.
(15) Hou, Z., Shankar, Y. V., Liu, Y., Ding, F., Subramanion, J. L., Ravikumar, V., Zamudio-Vazquez, R., Keogh, D., Lim, H., Tay, M. Y. F., Bhattacharjya, S., Rice, S. A., Shi, J., Duan, H., Liu, X. W., Mu, Y., Tan, N. S., Tam, K. C., Pethe, K., and Chan-Park, M. B. (2017) Nanoparticles of Short Cationic Peptidopolysaccharide Self-Assembled by Hydrogen Bonding with Antibacterial Effect against Multidrug-Resistant Bacteria. *ACS Appl. Mater. Interfaces 9* (44), 38288−38303.
(16) Worthington, R. J., and Melander, C. (2013) Combination approaches to combat multidrug-resistant bacteria. *Trends Biotechnol. 31* (3), 177−184.
(17) Paul, M., Lador, A., Grozinsky-Glasberg, S., and Leibovici, L. (2014) Beta lactam antibiotic monotherapy versus beta lactam-aminoglycoside antibiotic combination therapy for sepsis. *Cochrane Database Syst. Rev.* No. 1, CD003344.
(18) Tamma, P. D., Cosgrove, S. E., and Maragakis, L. L. (2012) Combination therapy for treatment of infections with gram-negative bacteria. *Clin Microbiol Rev. 25* (3), 450−70.
(19) Ejim, L., Farha, M. A., Falconer, S. B., Wildenhain, J., Coombes, B. K., Tyers, M., Brown, E. D., and Wright, G. D. (2011) Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. *Nat. Chem. Biol. 7* (6), 348−50.
(20) Schneider, E. K., Reyes-Ortega, F., Velkov, T., and Li, J. (2017) Antibiotic-non-antibiotic combinations for combating extremely drug-resistant Gram-negative 'superbugs'. *Essays Biochem. 61* (1), 115−125.
(21) Tyers, M., and Wright, G. D. (2019) Drug combinations: a strategy to extend the life of antibiotics in the 21st century. *Nat. Rev. Microbiol. 17* (3), 141−155.
(22) Elphick, H. E., and Scott, A. (2016) Single versus combination intravenous anti-pseudomonal antibiotic therapy for people with cystic fibrosis. *Cochrane Database Syst. Rev. 12*, CD002007.
(23) Mouton, J. W. (1999) Combination therapy as a tool to prevent emergence of bacterial resistance. *Infection 27* (Suppl 2), S24−S28.
(24) Brochado, A. R., Telzerow, A., Bobonis, J., Banzhaf, M., Mateus, A., Selkrig, J., Huth, E., Bassler, S., Zamarreno Beas, J., Zietek, M., Ng, N., Foerster, S., Ezraty, B., Py, B., Barras, F., Savitski, M. M., Bork, P., Gottig, S., and Typas, A. (2018) Species-specific activity of antibacterial drug combinations. *Nature 559* (7713), 259−263.
(25) Tian, J., Zhang, J., Yang, J., Du, L., Geng, H., and Cheng, Y. (2017) Conjugated Polymers Act Synergistically with Antibiotics to Combat Bacterial Drug Resistance. *ACS Appl. Mater. Interfaces 9* (22), 18512−18520.
(26) Ng, V. W., Ke, X., Lee, A. L., Hedrick, J. L., and Yang, Y. Y. (2013) Synergistic co-delivery of membrane-disrupting polymers with commercial antibiotics against highly opportunistic bacteria. *Adv. Mater. 25* (46), 6730−6.
(27) Namivandi-Zangeneh, R., Sadrearhami, Z., Dutta, D., Willcox, M., Wong, E. H. H., and Boyer, C. (2019) Synergy between Synthetic Antimicrobial Polymer and Antibiotics: A Promising Platform To Combat Multidrug-Resistant Bacteria. *ACS Infect. Dis. 5* (8), 1357−1365.
(28) Clinical and Laboratory Standards Institute (2018) *Performance Standards for Antimicrobial Susceptibility Testing*, 29th ed.
(29) Bjarnsholt, T. (2013) The role of bacterial biofilms in chronic infections. *APMIS Suppl 121* (136), 1−51.
(30) Sader, H. S., Fedler, K. A., Rennie, R. P., Stevens, S., and Jones, R. N. (2004) Omiganan pentahydrochloride (MBI 226), a topical 12-amino-acid cationic peptide: spectrum of antimicrobial activity and measurements of bactericidal activity. *Antimicrob. Agents Chemother. 48* (8), 3112−8.
(31) Gottler, L. M., and Ramamoorthy, A. (2009) Structure, membrane orientation, mechanism, and function of pexiganan–a highly potent antimicrobial peptide designed from magainin. *Biochim. Biophys. Acta, Biomembr. 1788* (8), 1680−6.
(32) Mygind, P. H., Fischer, R. L., Schnorr, K. M., Hansen, M. T., Sonksen, C. P., Ludvigsen, S., Raventos, D., Buskov, S., Christensen, B., De Maria, L., Taboureau, O., Yaver, D., Elvig-Jorgensen, S. G., Sorensen, M. V., Christensen, B. E., Kjaerulff, S., Frimodt-M?ller, N., Lehrer, R. I., Zasloff, M., and Kristensen, H. H. (2005) Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. *Nature 437* (7061), 975−980.
(33) Dunny, G. M., Brown, B. L., and Clewell, D. B. (1978) Induced cell aggregation and mating in Streptococcus faecalis: evidence for a bacterial sex pheromone. *Proc. Natl. Acad. Sci. U. S. A. 75* (7), 3479−83.
(34) Nawrocki, K. L., Crispell, E. K., and McBride, S. M. (2014) Antimicrobial Peptide Resistance Mechanisms of Gram-Positive Bacteria. *Antibiotics (Basel, Switz.) 3* (4), 461−92.
(35) Joo, H. S., Fu, C. I., and Otto, M. (2016) Bacterial strategies of resistance to antimicrobial peptides. *Philos. Trans. R. Soc., B 371* (1695), 20150292.
(36) Cole, J. N., and Nizet, V. (2016) Bacterial Evasion of Host Antimicrobial Peptide Defenses. *Microbiol. Spectrum 4* (1), VMBF-0006-2015.
(37) Bazan, J. F., Weaver, L. H., Roderick, S. L., Huber, R., and Matthews, B. W. (1994) Sequence and structure comparison suggest that methionine aminopeptidase, prolidase, aminopeptidase P, and creatinase share a common fold. *Proc. Natl. Acad. Sci. U. S. A. 91* (7), 2473−7.
(38) McBroom, A. J., Johnson, A. P., Vemulapalli, S., and Kuehn, M. J. (2006) Outer membrane vesicle production by Escherichia coli is independent of membrane instability. *J. Bacteriol. 188* (15), 5385−92.
(39) Cheng, A. G., Missiakas, D., and Schneewind, O. (2014) The giant protein Ebh is a determinant of Staphylococcus aureus cell size and complement resistance. *J. Bacteriol. 196* (5), 971−81.
(40) Brolund, A. (2014) Overview of ESBL-producing Enterobacteriaceae from a Nordic perspective. *Infect Ecol Epidemiol 4*, 24555.
(41) Schembri, M. A., Zakour, N. L., Phan, M. D., Forde, B. M., Stanton-Cook, M., and Beatson, S. A. (2015) Molecular Characterization of the Multidrug Resistant Escherichia coli ST131 Clone. *Pathogens 4* (3), 422−30.
(42) Wu, X., Li, Z., Li, X., Tian, Y., Fan, Y., Yu, C., Zhou, B., Liu, Y., Xiang, R., and Yang, L. (2017) Synergistic effects of antimicrobial peptide DP7 combined with antibiotics against multidrug-resistant bacteria. *Drug Des., Dev. Ther. 11*, 939−946.
(43) Kim, H., Jang, J. H., Kim, S. C., and Cho, J. H. (2014) De novo generation of short antimicrobial peptides with enhanced stability and cell specificity. *J. Antimicrob. Chemother. 69* (1), 121−32.

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237

(44) Steckbeck, J. D., Deslouches, B., and Montelaro, R. C. (2014) Antimicrobial peptides: new drugs for bad bugs? *Expert Opin. Biol. Ther. 14* (1), 11−4.
(45) Deslouches, B., Steckbeck, J. D., Craigo, J. K., Doi, Y., Burns, J. L., and Montelaro, R. C. (2015) Engineered cationic antimicrobial peptides to overcome multidrug resistance by ESKAPE pathogens. *Antimicrob. Agents Chemother. 59* (2), 1329−33.
(46) Hancock, R. E. W., and Sahl, H. G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nat. Biotechnol. 24* (12), 1551−1557.
(47) Paulsen, I. T., Banerjei, L., Myers, G. S., Nelson, K. E., Seshadri, R., Read, T. D., Fouts, D. E., Eisen, J. A., Gill, S. R., Heidelberg, J. F., Tettelin, H., Dodson, R. J., Umayam, L., Brinkac, L., Beanan, M., Daugherty, S., DeBoy, R. T., Durkin, S., Kolonay, J., Madupu, R., Nelson, W., Vamathevan, J., Tran, B., Upton, J., Hansen, T., Shetty, J., Khouri, H., Utterback, T., Radune, D., Ketchum, K. A., Dougherty, B. A., and Fraser, C. M. (2003) Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. *Science 299* (5615), 2071−4.
(48) Chen, S. L., Hung, C. S., Xu, J., Reigstad, C. S., Magrini, V., Sabo, A., Blasiar, D., Bieri, T., Meyer, R. R., Ozersky, P., Armstrong, J. R., Fulton, R. S., Latreille, J. P., Spieth, J., Hooton, T. M., Mardis, E. R., Hultgren, S. J., and Gordon, J. I. (2006) Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a comparative genomics approach. *Proc. Natl. Acad. Sci. U. S. A. 103* (15), 5977−82.
(49) Totsika, M., Beatson, S. A., Sarkar, S., Phan, M. D., Petty, N. K., Bachmann, N., Szubert, M., Sidjabat, H. E., Paterson, D. L., Upton, M., and Schembri, M. A. (2011) Insights into a multidrug resistant Escherichia coli pathogen of the globally disseminated ST131 lineage: genome analysis and virulence mechanisms. *PLoS One 6* (10), No. e26578.
(50) Miller, L. G., Perdreau-Remington, F., Rieg, G., Mehdi, S., Perlroth, J., Bayer, A. S., Tang, A. W., Phung, T. O., and Spellberg, B. (2005) Necrotizing fasciitis caused by community-associated methicillin-resistant Staphylococcus aureus in Los Angeles. *N. Engl. J. Med. 352* (14), 1445−53.
(51) Dale, J. L., Beckman, K. B., Willett, J. L. E., Nilson, J. L., Palani, N. P., Baller, J. A., Hauge, A., Gohl, D. M., Erickson, R., Manias, D. A., Sadowsky, M. J., and Dunny, G. M. (2018) Comprehensive Functional Analysis of the Enterococcus faecalis Core Genome Using an Ordered, Sequence-Defined Collection of Insertional Mutations in Strain OG1RF. *mSystems 3* (5), e00062-18.
(52) Wiegand, I., Hilpert, K., and Hancock, R. E. (2008) Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc. 3* (2), 163−75.
(53) Jenkins, S. G., and Schuetz, A. N. (2012) Current concepts in laboratory testing to guide antimicrobial therapy. *Mayo Clin. Proc. 87* (3), 290−308.
(54) Elion, G. B., Singer, S., and Hitchings, G. H. (1954) Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. *J. Biol. Chem. 208* (2), 477−488.
(55) Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schaberle, T. F., Hughes, D. E., Epstein, S., Jones, M., Lazarides, L., Steadman, V. A., Cohen, D. R., Felix, C. R., Fetterman, K. A., Millett, W. P., Nitti, A. G., Zullo, A. M., Chen, C., and Lewis, K. (2015) A new antibiotic kills pathogens without detectable resistance. *Nature 517* (7535), 455−9.
(56) Chong, K. K. L., Tay, W. H., Janela, B., Yong, A. M. H., Liew, T. H., Madden, L., Keogh, D., Barkham, T. M. S., Ginhoux, F., Becker, D. L., and Kline, K. A. (2017) Enterococcus faecalis Modulates Immune Activation and Slows Healing During Wound Infection. *J. Infect. Dis. 216* (12), 1644−1654.

https://dx.doi.org/10.1021/acsinfecdis.0c00016
ACS Infect. Dis. 2020, 6, 1228−1237