

## Control of Bacterial Persister Cells by Trp/Arg-Containing Antimicrobial Peptides<sup>∇</sup>

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Received 14 October 2010/Accepted 16 May 2011

**Persister cells are dormant phenotypic variants inherent in a bacterial population. They play important roles in chronic infections and present great challenges to therapy due to extremely enhanced tolerance to antibiotics compared to that of normal cells of the same genotype. In this study, we report that cationic membrane-penetrating peptides containing various numbers of arginine and tryptophan repeats are effective in killing persister cells of *Escherichia coli* HM22, a hyper-persister producer. The activities of three linear peptides [(RW)<sub>n</sub>-NH<sub>2</sub>, where *n* is 2, 3, or 4] and a dendrimeric peptide, (RW)<sub>4D</sub>, in killing bacterial persisters were compared. Although the dendrimeric peptide (RW)<sub>4D</sub> requires a lower threshold to kill planktonic persisters, octameric peptide (RW)<sub>4</sub>-NH<sub>2</sub> is the most effective against planktonic persister cells at high concentrations. For example, treatment with 80 μM (RW)<sub>4</sub>-NH<sub>2</sub> for 60 min led to a 99.7% reduction in the number of viable persister cells. The viability of persister cells residing in surface-attached biofilms was also significantly reduced by (RW)<sub>4</sub>-NH<sub>2</sub> and (RW)<sub>4D</sub>. These two peptides were also found to significantly enhance the susceptibility of biofilm cells to ofloxacin. The potency of (RW)<sub>4</sub>-NH<sub>2</sub> was further marked by its ability to disperse and kill preformed biofilms harboring high percentages of persister cells. Interestingly, approximately 70% of the dispersed cells were found to have lost their intrinsic tolerance and become susceptible to ampicillin if not killed directly by this peptide. These results are helpful for better understanding the activities of these peptides and may aid in future development of more effective therapies of chronic infections.**

Cells within an isogenic bacterial population have been found to have different characteristic responses to high doses of antibiotics: the vast majority are rapidly eliminated, while a small fraction, known as persister cells (4, 5), are intrinsically tolerant to the antibiotic stress even with prolonged treatment (3). Persister cells are not genetic mutants capable of growing in the presence of an antibiotic but are dormant variants resulting from transitory phenotypic switches (3). The genetic basis of persister formation, though not fully understood, is frequently linked to either an increase in the intracellular (p)ppGpp level (34, 35) or the ectopic expression of toxin-antitoxin modules in bacteria (6, 7, 14, 24, 32, 33, 41, 42). The failure to identify any single mutant that completely lacks the ability to form persisters (13, 23, 28, 49) possibly points to the high level of redundancy in the mechanism of persister formation.

Persister cell formation has been implicated as an important cause of chronic infections by a number of clinical isolates, such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* isolates, that severely challenge antimicrobial therapy (16, 43–46). Further complications arise from attempting to treat bacterial cells within the sessile microbial consortia known as biofilms. Due to nutrient and oxygen limitation (19), cells within a biofilm structure are often found to be metabolically

inactive and highly tolerant to antimicrobials (32, 36). Thus, biofilms are considered a major cause of chronic bacterial infections with high mortality and morbidity, especially among immunocompromised individuals (1, 11, 12, 21).

Depending on the stage of growth of a culture, the frequency of persister formation of wild-type bacteria can range from 10<sup>-6</sup> to 10<sup>-4</sup> (31). Having survived antibiotic stress, these cells can reestablish the population, generating a similar percentage of persister cells once the stress is removed (31). Eradication of nonmultiplying bacteria has thus been thought to be a critical step in shortening the duration of antimicrobial treatment and decreasing the occurrence of antibiotic-resistant mutations (10). However, traditional drug screening based on MIC is not suitable for identifying effective compounds targeting this small percentage of dormant persister cells that neither grow nor die in the presence of an antibiotic (10, 18).

Antimicrobial peptides (AMPs) are active in the host defense of organisms from all orders of life, with a common ability to disrupt the membranes of bacterial cells (30). Some AMPs such as gramicidins, polymyxins, and bacitracin have been successfully applied clinically to treat topical infections (20, 50). However, most of the AMPs studied to date are not appropriate for clinical applications due to relatively low activities (high MICs), high manufacturing costs, and/or cytotoxicity to mammalian cells (22). Thus, it is important to design better AMPs with optimized structures.

It is also important to study the effects of AMPs on dormant persister cells and biofilms, which are highly tolerant to antibiotics. The lack of target specificity and absence of rapid resistance development make AMPs plausible candidates to

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<sup>∇</sup> Published ahead of print on 27 May 2011.

counter the dormancy of persister cells, which hinders most traditional antimicrobial therapies (17, 40). Recently, we reported that a series of synthetic linear AMPs containing various numbers of arginine and tryptophan repeats  $[(RW)_n\text{-NH}_2]$ , where  $n$  is 2, 3, or 4, and a dipeptide dendrimer,  $(RW)_{4D}$  can effectively kill both planktonic and biofilm cells of *Escherichia coli* in a concentration-dependent manner (26, 27). The octameric peptide  $(RW)_4\text{-NH}_2$  at 80  $\mu\text{M}$  was also found to disperse and kill 87% of *E. coli* cells in preformed biofilms on 316L stainless steel surfaces (26). These synthetic peptides are cationic and amphiphilic, as are most natural AMPs (51). Both properties allow these peptides to target the negatively charged lipopolysaccharide (LPS) component of the cell wall of Gram-negative bacteria (38).

In this work, we further evaluated this set of AMPs for their activities against persister cells of *E. coli* HM22 (41). This strain contains the *hipA7* allele, which maps to the *hipA* gene in the antitoxin-toxin module HipBA (41). The expression of the *hipA7* allele confers a 1,000-fold-higher frequency of persister formation (36, 37) due to a mutation that decreased the affinity of HipA7 to antitoxin HipB in comparison to the wild-type HipA (47). This strain is used here to obtain high densities of persister cells to study the activities of AMPs. Based on our best effort, we have not been successful in finding previous literature focusing on persister control with antimicrobial peptides.

#### MATERIALS AND METHODS

**Bacterial strain and growth media.** *Escherichia coli* HM22 (AT984 *dapA* zde-264::Tn10 *hipA7*) was kindly provided by Kim Lewis (Northeastern University) and used in this study. Luria-Bertani (LB) broth and 1.5% agar medium containing 10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl (48) supplemented with 25  $\mu\text{g}/\text{ml}$  of diaminopimelic acid (DPA) were used for growing *E. coli* HM22. Overnight cultures were made from glycerol stock ( $-80^\circ\text{C}$ ) and incubated with aeration at  $37^\circ\text{C}$ . Biofilms were grown in a petri dish with 316L stainless steel coupons (0.6 in. by 0.3 in., with a thickness of 0.02 in.) and 20 ml LB medium inoculated with an overnight culture of *E. coli* HM22 to a start optical density at 600 nm ( $\text{OD}_{600}$ ) of 0.05.

**Persister isolation.** Planktonic persister cells were isolated by following a protocol reported previously with slight modifications (32). *E. coli* HM22 cells from an overnight culture were used to inoculate 25 ml LB medium to an initial  $\text{OD}_{600}$  of 0.01 and was cultured for approximately 3.5 h until the  $\text{OD}_{600}$  reached 0.3 to 0.4. The culture was then dosed with 100  $\mu\text{g}/\text{ml}$  of ampicillin and incubated with 200 rpm of shaking for another 3 h at  $37^\circ\text{C}$  before it was washed and harvested by centrifugation at 8,000 rpm for 10 min at  $4^\circ\text{C}$  using an ultracentrifuge (LS-70 Beckman Coulter, Inc., CA). Prior to peptide treatments, cells were resuspended in the same volume of 0.85% NaCl buffer and divided into 2-ml aliquots.

**Antimicrobial peptides.** Four short cationic AMPs were prepared as described previously (37, 38), including a tetrameric peptide  $[(RW)_2\text{-NH}_2]$ , hexameric peptide  $[(RW)_3\text{-NH}_2]$ , octameric peptide  $[(RW)_4\text{-NH}_2]$ , and dendrimeric peptide  $[(RW)_{4D}]$  (Fig. 1), except that the  $(RW)_4\text{-NH}_2$  used in the biofilm test was purchased from RS Synthesis, LLC (Louisville, KY), through custom peptide synthesis. The molecular weight of each peptide was confirmed using matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry as described previously (37). Briefly, the peptide sequences were assembled on Rink amide resin obtained from Nova Biochem (San Diego, CA) with a RAININ Instrument PS3 solid-phase synthesizer (Woburn, MA) using Fmoc (9-fluorenylmethoxycarbonyl) chemistry. Fmoc-Trp(butoxycarbonyl [Boc])/Arg(2,2,4,6,7-pentamethylidihydrobenzofuran-5-sulfonyl [Pbf]), the coupling reagent HBTU [2-(1H-benzotriazol-1-yl) 1,1,3,3-tetramethyluroniumhexafluoro phosphate], and HOBt (*N*-hydroxybenzotriazol) were also purchased from Nova Biochem. Cleavage of the peptides from the resin was performed with 95% trifluoroacetic acid (TFA) in the presence of the scavenger, 2.5% triisopropylsilane (TIS), and 2.5%  $\text{H}_2\text{O}$ . After precipitation with cold ether, samples were purified on a reverse-phase high-performance liquid chromatography  $\text{C}_{18}$  preparative column (2.2 by 25 cm, 300  $\text{\AA}$ ; Grace Vydac Co., Hesperia, CA), with water and

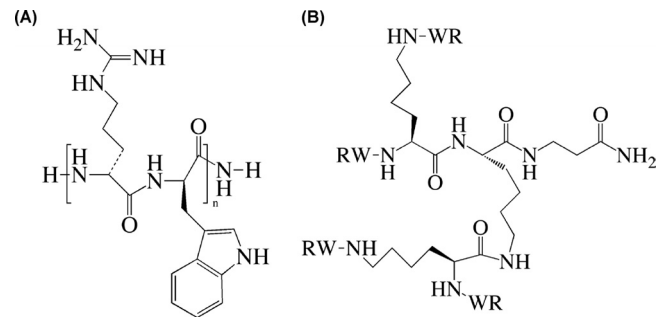


FIG. 1. Chemical structures of the linear antimicrobial peptides  $(RW)_n\text{-NH}_2$  (where  $n$  is 2, 3, or 4) (A) and the dendrimeric peptide  $(RW)_{4D}$  (B) used in this study.

acetonitrile as eluents. Fractions containing product were pooled and lyophilized. The molecular weight of each peptide was confirmed by a Bruker matrix-assisted laser desorption ionization–time of flight mass spectrometer (Billerica, MA). All peptides were dissolved in Tris buffer (12.1 g/liter Tris base and 8.8 g/liter sodium chloride, pH 7.2) to a final concentration of 2 mM as the stock solution and stored at  $4^\circ\text{C}$ .

**Antimicrobial activities against planktonic persister cells.** To investigate the antimicrobial activities of the four peptides, the viability of the above-described isolated persister cells incubated in the absence and presence of each peptide was examined. Three concentrations (20, 40, and 80  $\mu\text{M}$ ) and five durations of treatment (0, 10, 20, 40, and 60 min) were tested in triplicate for each peptide. The amount of Tris buffer was adjusted to be the same for all samples. The persister samples with or without peptide were incubated at  $37^\circ\text{C}$  with shaking at 200 rpm. A drop plate assay of CFU was adapted from the protocol of Chen et al. (9) to evaluate the viability of persister cells with and without peptide treatment. Briefly, six 100- $\mu\text{l}$  samples of control or treated persister cells were loaded into the first column of a 96-well plate using a multichannel pipette. A series of 10-fold dilutions was made into the subsequent columns of the same 96-well plate. CFU were counted after loading 10  $\mu\text{l}$  of each sample on a LB agar plate supplemented with 25  $\mu\text{g}/\text{ml}$  of DPA and incubation overnight at  $37^\circ\text{C}$ . The dilution that yielded 10 to 50 CFU per 10- $\mu\text{l}$  drop was used to quantify the number of CFU in each ml of the original sample. Each sample had at least 12 replicates.

**Antimicrobial activities against normal planktonic cells.** To compare with the effects on planktonic persister cells (isolated from exponential-phase cultures), we also tested the effects of peptides on normal cells in exponential phase (without ampicillin treatment). Briefly, an overnight culture of *E. coli* HM22 was diluted by 1:1,000 with LB medium to inoculate subcultures (25 ml each). The cultures were incubated for 3 h at  $37^\circ\text{C}$  to reach early exponential phase. The cells were then pelleted in 0.5-ml aliquots, and each was resuspended in 1 ml of 0.85% NaCl buffer. Peptides at 20, 40, and 80  $\mu\text{M}$  were added to the suspensions, and the amount of Tris buffer was adjusted to be the same for all the controls and treated samples. After 1 h of incubation at  $37^\circ\text{C}$ , the cells were plated on LB agar plates supplemented with 25  $\mu\text{g}/\text{ml}$  DPA to count CFU after incubation at  $37^\circ\text{C}$  overnight.

**Effects of AMPs on persister cells in preformed biofilms.** To understand the effects of AMPs on persister cells in biofilm, 24-h biofilms of *E. coli* HM22 formed on 316L stainless steel coupons in fresh LB medium with an inoculation  $\text{OD}_{600}$  of 0.05 were washed gently with 0.85% NaCl buffer three times to remove planktonic cells. The washed biofilms were then transferred to microcentrifuge tubes each containing 1 ml of 0.85% NaCl buffer supplemented with one of the four peptides at 0, 20, 40, or 80  $\mu\text{M}$ . The biofilm coupons were incubated at  $37^\circ\text{C}$  without shaking for 1 h before they were washed again and transferred to fresh 0.85% NaCl buffer. To release biofilm cells from the coupon surface and conduct a CFU count, the coupons soaked in 0.85% NaCl buffer were sonicated and vortexed for 1 min each. Microscopic examination of the coupon surfaces before and after sonication and vortexing and the resulted cell suspension confirmed that such treatment was sufficient to remove biofilms and break cell clusters (images not shown). Then the number of CFU of biofilm cells were counted in the same way as the planktonic CFU assay described above. A portion of each sample was also treated with 100  $\mu\text{g}/\text{ml}$  of ampicillin for 3 h to evaluate the persistence of biofilm cells. Compared to normal planktonic cells, biofilm cells are of a slow-growing nature (1, 8, 44). To allow a closer examination of the effects of the AMPs on biofilm-associated persister cells, we also conducted the

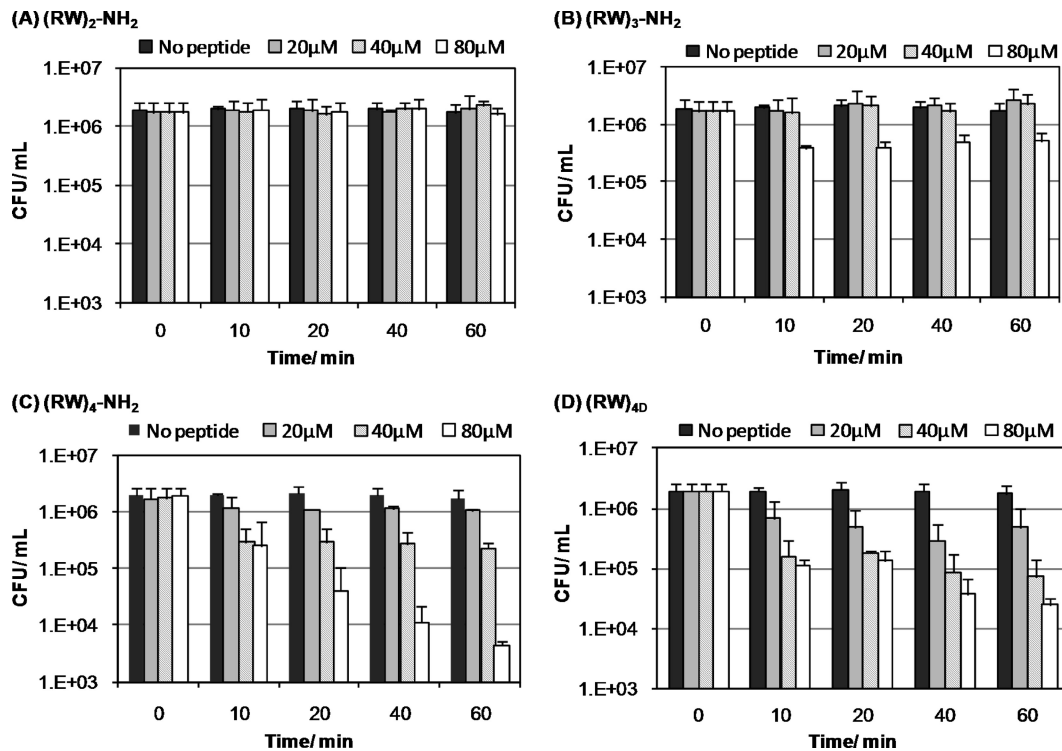


FIG. 2. Effects of  $(RW)_2-NH_2$  (A),  $(RW)_3-NH_2$  (B),  $(RW)_4-NH_2$  (C), and  $(RW)_{4D}$  (D) on the viability of planktonic persister cells of *E. coli* HM22. Bar graphs indicate the numbers of viable persister cells after incubation with or without AMPs. Means and standard deviations are shown. Statistical analyses were based on hierarchy model, two-way ANOVA adjusted by the Bonferroni method.

same experiments using 5  $\mu\text{g/ml}$  of ofloxacin (to isolate biofilm-associated persister cells), which is a more effective antibiotic against slow-growing cells than ampicillin (2).

To accommodate the heterogeneity in biofilm surface coverage, a bright line counting chamber (Hausser Scientific, Horsham, PA) was used to estimate the number of biofilm cells on each coupon and normalize the CFU data. Twelve microliters of the biofilm cells suspended in 0.85% NaCl buffer was loaded into the counting chamber, with a ruling area of 9  $\text{mm}^2$  and a depth of  $0.1 \pm 0.002$  mm. The number of bacterial cells within the chamber was counted using an AXIO Imager M1 microscope (Carl Zeiss, Inc., Germany) under a  $20\times$  phase contrast objective (N-Achroplan  $20\times/0.45$  phase 2) by focusing on the center square millimeter.

Significant dispersion was observed for biofilms treated with  $(RW)_4-NH_2$ . For these biofilm samples, in addition to the CFU assay and chamber counting mentioned above, we also collected the dispersed biofilm cells and conducted viability assays in a similar manner. All viability assays were done at least in duplicate, and consistent results were obtained.

The killing and/or dispersion of preformed biofilms by  $(RW)_4-NH_2$  was also investigated with biofilm imaging using the Live/Dead BacLight bacterial viability kit (Invitrogen Corporation, Carlsbad, CA). Briefly, biofilms were formed on 316L stainless steel coupons as described above. After 24 h of incubation, the coupons were washed gently with 0.85% NaCl buffer three times, transferred to 0.85% NaCl buffer supplemented with or without  $(RW)_4-NH_2$  (0, 40, or 80  $\mu\text{M}$ ), and incubated at  $37^\circ\text{C}$  without shaking for 1 h. The coupons were then washed and stained in 1 ml of fresh 0.85% NaCl buffer containing 1.5  $\mu\text{l}$  of 3.34 mM SYTO 9 (to stain live cells) and 1.5  $\mu\text{l}$  of 20 mM propidium iodide (to stain dead cells) in the dark for 15 min. Five spots on each coupon were randomly picked and imaged using an Axio Imager M1 microscope. The surface coverage of biofilms was calculated using COMSTAT software (25).

**Statistical analysis.** All statistical analysis was performed by using SAS 9.2, Windows version (SAS, Cary, NC). Statistical analyses of the planktonic cell data were based on hierarchy model, two-way analysis of variance (ANOVA). Data on biofilm-associated persister cells were analyzed with one-way ANOVA. Only *P* values are shown in Results.

## RESULTS

### Antimicrobial activities against planktonic persister cells.

In this study, the antimicrobial activities of four previously reported peptides,  $(RW)_2-NH_2$ ,  $(RW)_3-NH_2$ ,  $(RW)_4-NH_2$ , and  $(RW)_{4D}$  (Fig. 1) (26, 27, 37, 38), against planktonic persister cells of *E. coli* HM22 were compared. Consistent with a previous report by Keren et al. (31), we found that inoculation of LB medium with isolated *E. coli* HM22 persister cells (treatment with 100  $\mu\text{g/ml}$  ampicillin for 3 h) led to a new culture with the same percentage ( $\sim 1\%$ ) of persister cells (detailed CFU data not shown). This result confirmed that the tolerance of our isolated *E. coli* HM22 persister cells to ampicillin is due to persister formation rather than any ampicillin resistance gene(s). To test the effects of AMPs, the isolated persister cells were suspended in 0.85% NaCl to a density of approximately  $1 \times 10^6$  cells/ml. Each of the peptides was added to the persister cell suspension to a final concentration of 0, 20, 40, or 80  $\mu\text{M}$  and incubated at  $37^\circ\text{C}$  with shaking at 200 rpm. During incubation, 100  $\mu\text{l}$  culture was sampled at 0, 10, 20, 40, or 60 min after inoculation, diluted in 0.85% NaCl buffer, and plated on LB agar plates supplemented with 25  $\mu\text{g/ml}$  DPA to count CFU.

As shown in Fig. 2A, the peptide  $(RW)_2-NH_2$  had no apparent killing effect on *E. coli* HM22 planktonic persister cells. The numbers of viable persister cells with the 0-, 20-, 40-, and 80- $\mu\text{M}$   $(RW)_2-NH_2$  treatment for 1 h were all around  $1.6 \times 10^6$  cells/ml (*P* of  $>0.05$  for all tested conditions).

The peptide  $(RW)_3-NH_2$  was found to be effective; however,



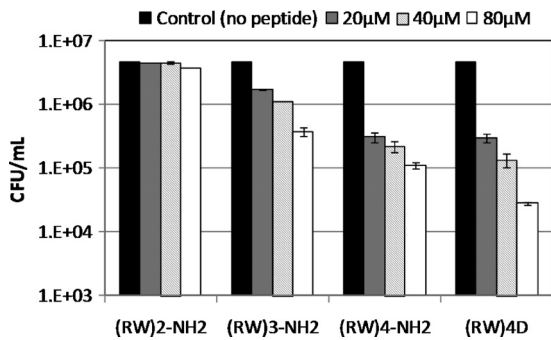


FIG. 3. Effects of (RW)<sub>2</sub>-NH<sub>2</sub>, (RW)<sub>3</sub>-NH<sub>2</sub>, (RW)<sub>4</sub>-NH<sub>2</sub>, and (RW)<sub>4D</sub> on the viability of regular *E. coli* HM22 cells in exponential phase. Bar graph indicates the number of viable cells after 1 h of incubation with or without AMPs. Means and standard deviations are shown. Data were analyzed with one-way ANOVA.

the killing required a threshold concentration to take effect. A half-log killing was observed when treating persister cells with (RW)<sub>3</sub>-NH<sub>2</sub> at 80 μM, but no significant killing was observed at 20 or 40 μM ( $P$  of >0.05 for both conditions) (Fig. 2B). Upon treatment with the (RW)<sub>3</sub>-NH<sub>2</sub> at 80 μM, most of the killing took place in the first 10 min after inoculation ( $P$  = 0.01), and no further killing in the following 50 min was found to be significant ( $P$  of >0.05 for all tested conditions).

Among the four AMPs, (RW)<sub>4</sub>-NH<sub>2</sub> showed the best killing efficacy (Fig. 2C). For example, treatment with 40 μM of this peptide for 10 min reduced the number of viable planktonic persister cells by nearly a log. At 80 μM, (RW)<sub>4</sub>-NH<sub>2</sub> showed nearly 3 logs of killing in 60 min, and the killing proceeded in a time-dependent manner ( $P$  = 0.0014). The number of viable persister cells within 10, 20, 40, and 60 min of treatment was reduced from  $1.91 \times 10^6$  to  $2.53 \times 10^5$ ,  $4.15 \times 10^4$ ,  $1.12 \times 10^4$ , and  $4.43 \times 10^3$ , respectively. In comparison, the peptide (RW)<sub>4D</sub> required a lower threshold to effectively kill persister cells. As shown in Fig. 2D, significant killing was observed for treatment with 20 and 40 μM of this peptide, reducing the number of viable persister cells by a half log and one log, respectively ( $P$  of <0.05 for both conditions). However, at 80 μM, only a 2-log killing by (RW)<sub>4D</sub> was observed in treated planktonic persister cells compared to an approximately 3-log killing by (RW)<sub>4</sub>-NH<sub>2</sub> at the same concentration.

Overall, the antimicrobial activity of peptides toward planktonic persister cells (isolated from exponential phase) follows this order: (RW)<sub>2</sub>-NH<sub>2</sub> < (RW)<sub>3</sub>-NH<sub>2</sub> < (RW)<sub>4D</sub> ≈ (RW)<sub>4</sub>-NH<sub>2</sub> (Fig. 2). (RW)<sub>4D</sub> is more effective than (RW)<sub>4</sub>-NH<sub>2</sub> at low concentrations, while the latter was more effective at a high concentration of 80 μM.

In addition to persister cells, we also tested the effects of these peptides on the normal cells of *E. coli* HM22 (harvested from exponential-phase culture but without ampicillin treatment). It was found that, similar to the planktonic persister killing, (RW)<sub>3</sub>-NH<sub>2</sub>, (RW)<sub>4</sub>-NH<sub>2</sub>, and (RW)<sub>4D</sub> can effectively kill normal cells of *E. coli* HM22 in a dose-dependent manner (Fig. 3). However, lower threshold concentrations were required for effective killing of normal cells than those required for the persister cells. For example, treatment with 20 μM (RW)<sub>3</sub>-NH<sub>2</sub> or (RW)<sub>4</sub>-NH<sub>2</sub> for 1 h led to effective killing ( $P$  < 0.0001 in both conditions), whereas when used to treat

against planktonic persisters, 20 μM was not an effective concentration for either peptide [ $P$  = 0.1046 for (RW)<sub>3</sub>-NH<sub>2</sub>;  $P$  = 0.2662 for (RW)<sub>4</sub>-NH<sub>2</sub>]. This finding suggests that persister cells are likely more tenacious to AMP treatment. However, it is interesting to note that at a higher concentration of 80 μM, (RW)<sub>4</sub>-NH<sub>2</sub> appeared to be more effective in killing persister cells than normal cells. As shown in Fig. 2C and 3, a 3-log reduction in the number of viable planktonic persisters after treatment was observed, but only a 2-log reduction in the number of normal cells isolated from the same growth (exponential) phase was observed.

**Effects on biofilm cells and associated persistence.** Due to the presence of polysaccharide matrix and slow growth, cells within a mature biofilm are often found to be highly tolerant to antimicrobials (15). To understand the effects of AMPs on persister cells residing in sessile biofilms, 24-h *E. coli* HM22 biofilms preformed on 316L stainless steel coupons were treated with and without AMP for 1 h. Viability and persistence levels of the biofilm cells were tested subsequently. To evaluate the persistence of biofilm cells, we treated the peptide-challenged biofilms with ampicillin or ofloxacin. Treatment with ampicillin allowed us to compare these results with our data of planktonic cells. However, while ampicillin is appropriate for isolating persister cells in planktonic cultures (32), it may not be appropriate for isolating persister cells from biofilms because biofilm cells are of a slow-growing nature (8). To allow a closer examination of the efficacy of the AMPs on biofilm-associated persister cells, we treated biofilms with ofloxacin, which is a more effective antibiotic against slow-growing cells than ampicillin (2). As described in Materials and Methods, all results were normalized with total biofilm cell numbers quantified using a counting chamber to eliminate the effects of heterogeneity in biofilm surface coverage.

As shown in Fig. 4A, (RW)<sub>2</sub>-NH<sub>2</sub> did not exhibit any significant killing or dispersion of biofilms ( $P$  = 0.3181). In addition, it did not significantly alter the susceptibilities of biofilm cells to ampicillin ( $P$  = 0.9366) and ofloxacin ( $P$  = 0.1800).

Comparable to the results of planktonic persister cells, (RW)<sub>3</sub>-NH<sub>2</sub> showed a threshold-based killing of biofilm cells, with a significant effect evidenced at 80 μM, reducing viable biofilm cells by 73% ( $P$  < 0.0001). Although (RW)<sub>3</sub>-NH<sub>2</sub>-treated biofilm cells did not show a significant change in susceptibility to ampicillin ( $P$  = 0.5097) (Fig. 4B), treatment with 80 μM (RW)<sub>3</sub>-NH<sub>2</sub> appeared to enhance the susceptibility to ofloxacin ( $P$  = 0.0403) (Fig. 4B). Since 20 and 40 μM (RW)<sub>3</sub>-NH<sub>2</sub> did not significantly alter the susceptibility to ofloxacin ( $P$  > 0.05), 80 μM also seems to be the threshold for synergy between this peptide and ofloxacin.

(RW)<sub>4D</sub> exhibited potent activities to eliminate biofilm cells and associated antibiotic tolerance in a dose-dependent manner (Fig. 4D). For example, after treatment for 1 h with 20, 40, and 80 μM (RW)<sub>4D</sub>, the total number of viable biofilm cells were reduced by  $77.1\% \pm 12.2\%$ ,  $98.8\% \pm 1.5\%$ , and  $99.3\% \pm 0.0\%$ , respectively, compared to the untreated control ( $P$  < 0.0001). The tolerance to ampicillin was also reduced by  $57.2\% \pm 7.9\%$ ,  $96.9\% \pm 0.2\%$ , and  $99.1\% \pm 0.2\%$ , respectively ( $P$  = 0.0012). Furthermore, no viable cells were found after the biofilms were treated with 40 or 80 μM (RW)<sub>4D</sub> followed by 5 μg/ml ofloxacin, suggesting all persister cells were eliminated.

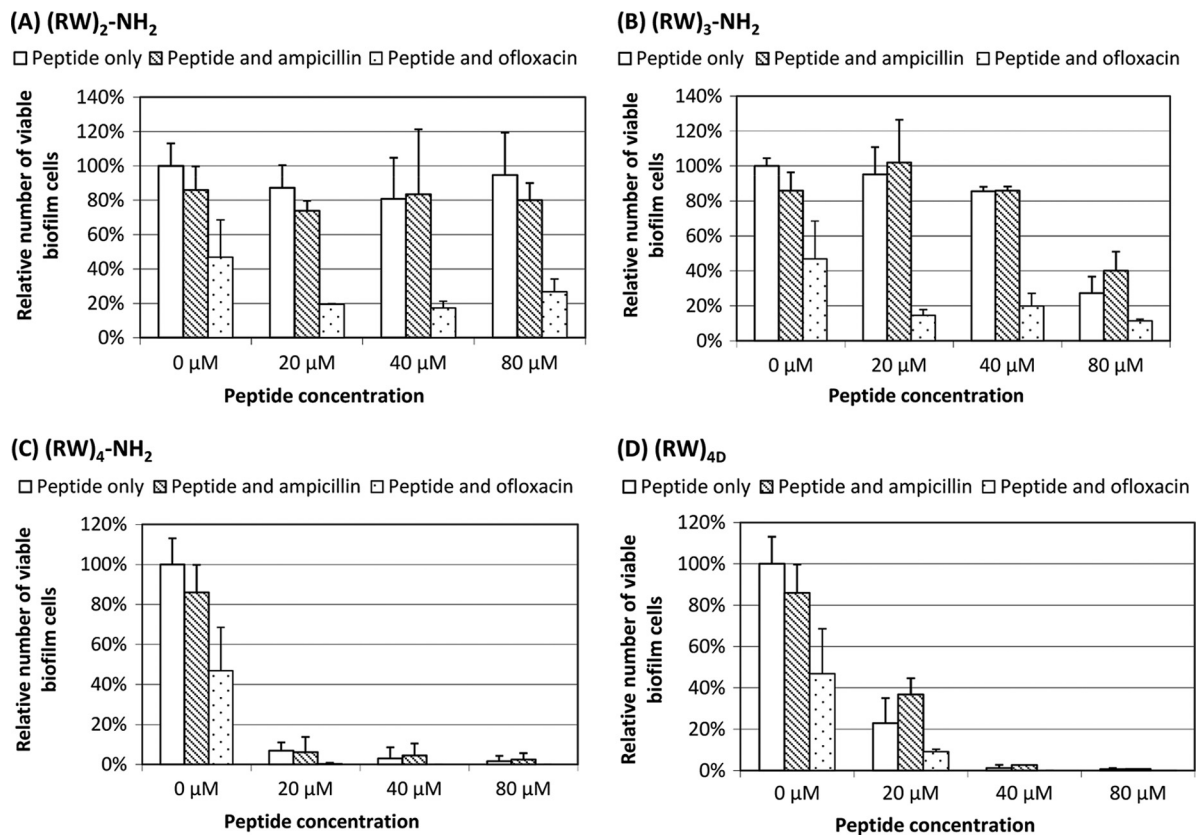


FIG. 4. Effects of (RW)<sub>2</sub>-NH<sub>2</sub> (A), (RW)<sub>3</sub>-NH<sub>2</sub> (B), (RW)<sub>4</sub>-NH<sub>2</sub> (C), and (RW)<sub>4D</sub> (D) on biofilm cells of *E. coli* HM22. The graphs show the effects of 1-h treatment of peptides on biofilms preformed on 316L biofilm coupons at three different drug concentrations (20, 40, and 80 μM). The first three bars in each graph represent the peptide-free control. White bars represent the relative numbers of total viable biofilm cells with or without peptide treatments; gray bars represent the relative numbers of viable biofilm cells after the above-described peptide treatment, followed by a 3-h treatment of 100 μg/ml ampicillin; and white dotted bars represent the relative numbers of total biofilm-associated persister cells after the above-described peptide treatment, followed by a 3-h treatment with 5 μg/ml ofloxacin. Means and standard deviations are shown. Data were analyzed with one-way ANOVA.

Among the four peptides studied, (RW)<sub>4</sub>-NH<sub>2</sub> was the most effective against biofilm cells at lower concentrations. Treatment with 20 μM (RW)<sub>4</sub>-NH<sub>2</sub> for 1 h reduced the number of viable biofilm cells by 93.1% ± 6.9% and that of ampicillin-tolerant cells by 92.8% ± 7.6%, in contrast to 77.1% ± 12.2% and 57.2% ± 7.9%, respectively, for (RW)<sub>4D</sub> at the same concentration (Fig. 4C and D). The effects were also dose dependent; e.g., at 40 and 80 μM, the killing efficacy of (RW)<sub>4</sub>-NH<sub>2</sub> was 97.0% ± 5.6% and 98.4% ± 2.6% for total biofilm cells and 94.8% ± 6.0% and 97.1% ± 3.1% for ampicillin-tolerant biofilm cells, respectively (Fig. 4C). Similar to (RW)<sub>4D</sub>, further treatment of ofloxacin led to no viable cells detected, indicating that all persister cells were eliminated (Fig. 4C).

**Synergistic effects between peptides and antibiotics.** To understand how effective AMPs are specifically toward biofilm-associated persister cells, we tested the persistence of biofilm cells to ampicillin and ofloxacin pretreated with or without an AMP. In the absence of peptides, control biofilms of *E. coli* HM22 showed high persistence levels, in which 85.9% ± 13.8% of the biofilm cells tolerated 100 μg/ml ampicillin and 46.8% ± 21.7% tolerated 5 μg/ml ofloxacin. Although (RW)<sub>3</sub>-NH<sub>2</sub>, (RW)<sub>4</sub>-NH<sub>2</sub>, and (RW)<sub>4D</sub> were able to kill biofilm cells, none of the four peptides tested was found to significantly

reduce the percentage of biofilm cells that are tolerant to ampicillin, e.g., all biofilm cells that were not killed by the peptides remained tolerant to ampicillin treatment (Fig. 4). This is probably because ampicillin is not effective against slow-growing cells. In comparison, biofilm cells treated with these peptides, especially high concentrations of (RW)<sub>4</sub>-NH<sub>2</sub> and (RW)<sub>4D</sub>, were rendered highly sensitive to ofloxacin (Fig. 4C and D). Such synergy may help develop more effective therapies.

**Octameric peptide disperses biofilm cells.** (RW)<sub>4</sub>-NH<sub>2</sub> at 200 μM has previously been shown to disperse and kill over 87% of the cells in preformed *E. coli* RP437 biofilms (24 h old) (26). To thoroughly evaluate the potency of this peptide and understand if the reduction in the number of viable biofilm-associated persister cells is partially due to the dispersion of *E. coli* HM22 biofilm cells, biofilm viability assays as well as biofilm imaging were performed.

As shown in the fluorescence images in Fig. 5A, treatment with 40 μM and 80 μM (RW)<sub>4</sub>-NH<sub>2</sub> markedly reduced the coverage of preformed biofilms to 29.1% ± 12.0% and 22.0% ± 11.3%, respectively, of the original peptide-free control (Fig. 5B) (*P* of <0.001 for both cases). In addition, the numbers of viable cells detached from the biofilms in the pres-

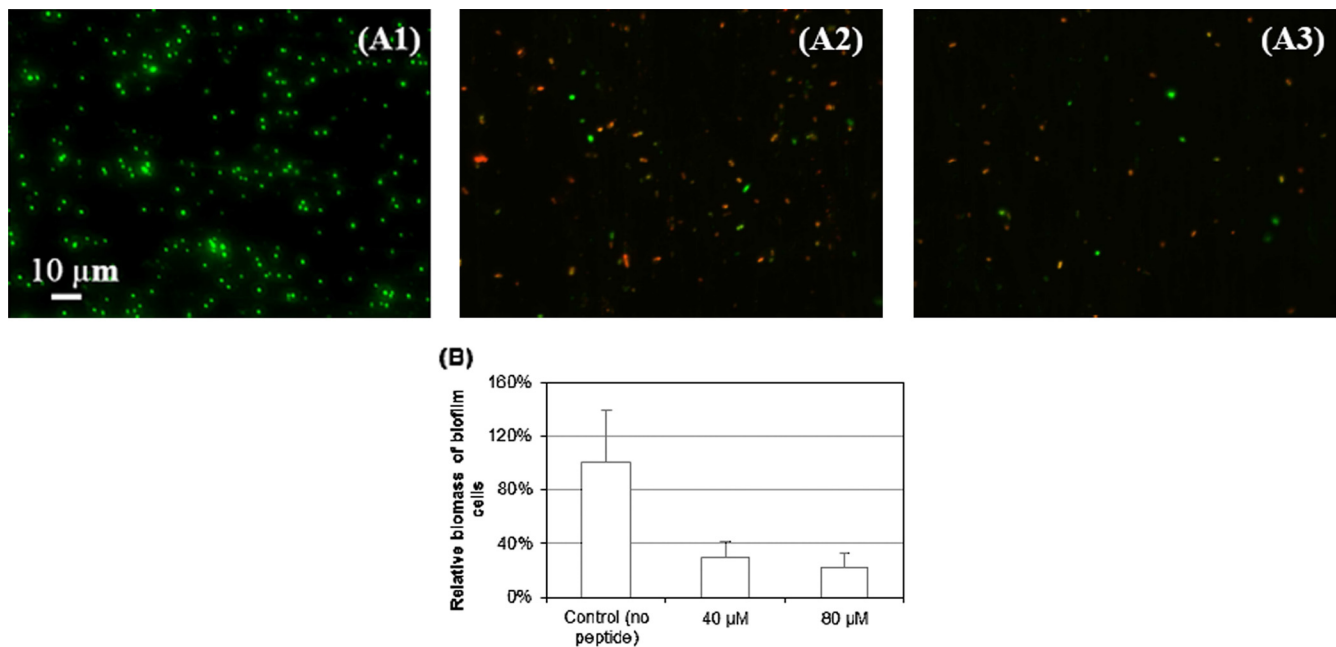


FIG. 5. Treatment with (RW)<sub>4</sub>-NH<sub>2</sub> caused detachment of preformed *E. coli* HM22 biofilms. The representative fluorescence images show 24-h *E. coli* HM22 biofilms after a 1-h treatment with no peptide (control) (A1) or with (RW)<sub>4</sub>-NH<sub>2</sub> at a concentration of 40 μM (A2) or 80 μM (A3). The live cells were stained green, and the dead cells were stained red. Bar = 10 μm. (B) The bars show surface coverage of biofilms after a 1-h treatment of (RW)<sub>4</sub>-NH<sub>2</sub> at concentrations of 0, 40, and 80 μM calculated from fluorescence images in panels A1 to A3 using COMSTAT. Means and standard deviations are shown.

ence of (RW)<sub>4</sub>-NH<sub>2</sub> at the concentration of 20, 40, or 80 μM were significantly reduced to 31.1% ± 18.0%, 22.2% ± 13.4%, and 16.3% ± 3.2%, respectively, compared to those of the peptide-free control (Fig. 6A) (*P* = 0.005). These data suggest that the dispersed cells were effectively killed by this peptide.

Intriguingly, after treatment with 80 μM (RW)<sub>4</sub>-NH<sub>2</sub>, only 28.4% ± 16.1% of dispersed biofilm cells stayed tolerant to ampicillin, if not killed directly by the peptide (Fig. 6B). Conversely, without peptide treatment, 94.5% ± 11.6% of dispersed cells remained tolerant to 100 μg/ml of ampicillin (Fig. 6B). Furthermore, the resumption of ampicillin susceptibility appeared to be dose dependent (Fig. 6B). This change in

antibiotic susceptibility suggests the possibility that treatment with the peptides caused damage to bacterial cell membranes and/or changes in cellular activities that increased the penetration and effectiveness of ampicillin.

DISCUSSION

In this study, the activities of three linear AMPs containing various numbers of Trp/Arg repeats and one dendrimeric peptide of the same amino acid composition but with a different branching structure against planktonic persister cells and biofilm-associated persister cells of *E. coli* HM22 were compared.

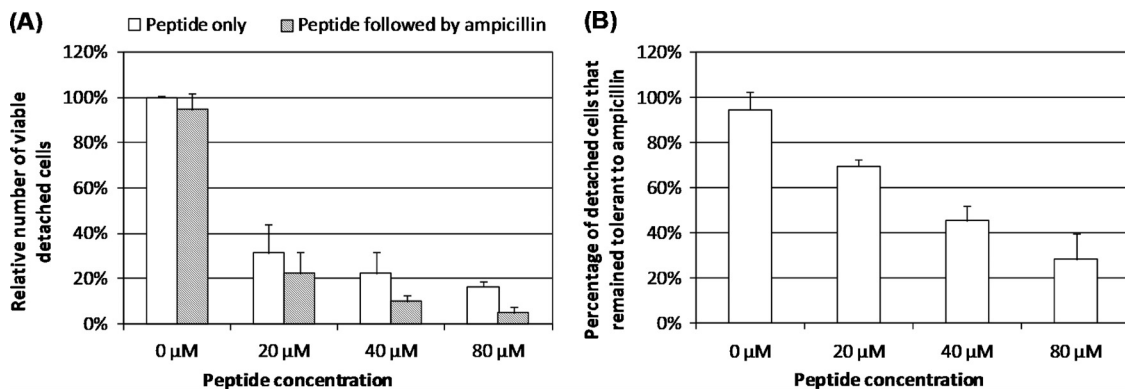


FIG. 6. Viability of detached biofilm cells of *E. coli* HM22 after treatment with (RW)<sub>4</sub>-NH<sub>2</sub> (A) and the percentage of viable cells that are ampicillin tolerant (B). For viability data, white bars represent the relative numbers of total viable biofilm cells with or without peptide treatments, and gray bars represent the relative numbers of detached biofilm cells that remained viable after a 3-h treatment with 100 μg/ml ampicillin. Means and standard deviations are shown.

The antimicrobial activities of the short cationic peptides followed this order:  $(RW)_2\text{-NH}_2 < (RW)_3\text{-NH}_2 < (RW)_{4D} \approx (RW)_4\text{-NH}_2$ . Specifically,  $(RW)_2\text{-NH}_2$  exhibited no observable effect on either planktonic or biofilm-associated persister cells. Compared to the peptide-free control, the  $(RW)_3\text{-NH}_2$  at 80  $\mu\text{M}$  can kill up to 70% of planktonic persister cells and 76% (ofloxacin-tolerant) biofilm-associated persister cells. The killing threshold for this peptide likely exists between 40  $\mu\text{M}$  and 80  $\mu\text{M}$  for planktonic persisters, and a concentration higher than 80  $\mu\text{M}$  is required for killing of biofilm-associated persisters.

Both  $(RW)_{4D}$  and  $(RW)_4\text{-NH}_2$  were potent against persister cells of *E. coli* HM22. For example, 1 h of  $(RW)_{4D}$  treatment at 80  $\mu\text{M}$  reduced the number of viable planktonic persister cells by 2 logs and reduced biofilm-associated persister cells by 99.98%. At 80  $\mu\text{M}$ , an up to 3-log killing for  $(RW)_4\text{-NH}_2$  against planktonic persisters was observed. Such killing appeared to be time dependent.  $(RW)_{4D}$ , in comparison, was more effective toward planktonic persister killing at 20  $\mu\text{M}$  but fell short of the linear octamers at 40 or 80  $\mu\text{M}$ .

Compared with their planktonic counterparts, biofilm cells were indeed more refractory to the action of AMPs (26). Almost all of the biofilm cells surviving AMP treatment remained tolerant to ampicillin. These results are consistent with the aforementioned hypothesis that normal cells in a biofilm are readily subject to elimination by antimicrobials, while biofilm-associated persisters are more refractory and thus likely responsible for the repopulation of biofilms and the consequent relapsing of biofilm-associated bacterial infections (33). However, the biofilm cells treated with  $(RW)_4\text{-NH}_2$  and  $(RW)_{4D}$  exhibited significantly enhanced susceptibility to ofloxacin. Such synergy between AMPs and antibiotics that are effective against slow-growing cells deserves further study and may help develop better therapies.

Most synthetic antimicrobial peptides are designed to mimic naturally occurring AMPs in the host defense system of multicellular organisms (39, 51). The shared features of these peptides are amphiphilicity and positive charge (38, 51). They are believed to function by clustering in hydrophobic patches and permeating the negatively charged lipid bilayers of Gram-negative bacteria to disrupt the membrane and ultimately lead to cell death.

The activity of AMPs against bacteria is dependent on the peptide-to-lipid (P/L) ratios, according to a two-state model previously proposed by Huang (29). The hexamer peptide used in this study exhibited such threshold-based killing of *E. coli* cells. The killing efficacy of short cationic peptides also depends on chain length. For the series of Arg/Trp repeats tested in this study, a chain length of four probably creates the optimal or near optimal antimicrobial activity against persister cells. Although  $(RW)_4\text{-NH}_2$  and  $(RW)_{4D}$  share the same amino acid composition (R and W), the specific mechanism of dispersion by  $(RW)_4\text{-NH}_2$ , but not  $(RW)_{4D}$  (26), is presently unclear.

The response such biofilm dispersion elicits is, nonetheless, promising.  $(RW)_4\text{-NH}_2$  was found to significantly disperse biofilm cells in their persistent state, consistent with our previous report that both killing and dispersion were observable in preformed *E. coli* RP437 biofilms (26). Interestingly, approximately 70% of the dispersed biofilm-associated persister cells

became sensitive to ampicillin treatment, if not killed directly by this peptide (Fig. 6B).

In conclusion, we systematically compared the activities of four cationic AMPs against planktonic persister cells as well as biofilm-associated persister cells of *E. coli* HM22.  $(RW)_{4D}$  and  $(RW)_4\text{-NH}_2$  were found to be the most potent, followed by the  $(RW)_3\text{-NH}_2$ , and killing by which was manifest only above a threshold concentration that is much higher than the other two peptides. Additionally, the linear peptide  $(RW)_4\text{-NH}_2$  was found to disperse preformed biofilms harboring  $85.9\% \pm 13.8\%$  of cells tolerant to ampicillin and  $46.8\% \pm 21.7\%$  tolerant to ofloxacin. We also showed that dispersed biofilm persister cells were rendered more susceptible to ampicillin by this treatment. This study focused on treatment of biofilms with AMPs followed by exposure to antibiotics to specifically understand the effects of AMPs on biofilm persistence. It will also be interesting to test cotreatment with AMPs and antibiotics or with antibiotics followed by AMPs. The effects of treatment time are also worth studying for *in vivo* applications. Such efforts can help identify the optimal treatment strategy.

While most conventional antibiotics specifically target growth-associated phenotypes, such as DNA replication, protein synthesis, and cell wall synthesis, AMPs cause physical damage to the cell membrane. Thus, agents that target membranes relatively nonspecifically may hold promise for activity against persister cells. Recently, it was demonstrated that  $(RW)_{4D}$  has a  $\text{MIC}_{50}$  against *Staphylococcus aureus* that is around 100 times lower than the concentration corresponding to 50% hemolysis ( $\text{HD}_{50}$ ) (38). Thus, further study of the mechanism of persister and biofilm control by these peptides can help design more effective persister targeting antimicrobial leads as well as specific therapeutic approaches for persister cell eradication.

#### ACKNOWLEDGMENTS

We thank the U.S. National Science Foundation (NSF-CMMI; grant 0826288) for partial support.

We thank Kim Lewis at Northeastern University for providing the *E. coli* HM22 strain and Arne Heydorn at the Technical University of Denmark for sharing the COMSTAT software.

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