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Visualizing Attack of *Escherichia coli* by the Antimicrobial Peptide Human Defensin 5

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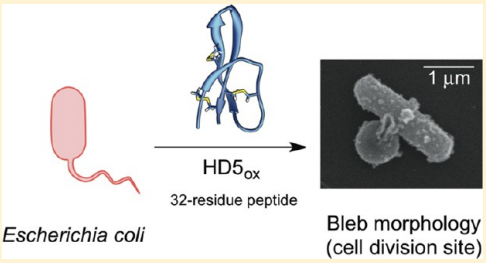
Supporting Information

ABSTRACT: Human α-defensin 5 (HD5) is a 32-residue cysteine-rich host-defense peptide that exhibits broad-spectrum antimicrobial activity and contributes to innate immunity in the human gut and other organ systems. Despite many years of investigation, its antimicrobial mechanism of action remains unclear. In this work, we report that $HD5_{ox}$, the oxidized form of this peptide that exhibits three regiospecific disulfide bonds, causes distinct morphological changes to *Escherichia coli* and other Gram-negative microbes. These morphologies include bleb formation, cellular elongation, and clumping. The blebs are up to ~1 μm wide and typically form at the site of cell division or cell poles. Studies with *E. coli* expressing cytoplasmic GFP reveal that $HD5_{ox}$ treatment causes GFP emission to localize in the bleb. To probe the cellular uptake of $HD5_{ox}$ and subsequent localization, we describe the design and characterization of a fluorophore−HD5 conjugate family. By employing these peptides, we demonstrate that fluorophore−$HD5_{ox}$ conjugates harboring the rhodamine and coumarin fluorophores enter the *E. coli* cytoplasm. On the basis of the fluorescence profiles, each of these fluorophore−$HD5_{ox}$ conjugates localizes to the site of cell division and cell poles. These studies support the notion that $HD5_{ox}$, at least in part, exerts its antibacterial activity against *E. coli* and other Gram-negative microbes in the cytoplasm.



Bacterial infections and the considerable rise in antibiotic resistance in hospital and community settings pose significant problems for global health initiatives.[1,2] The dearth of new antibiotics in the drug pipeline as well as an incomplete comprehension of human innate immunity and microbial pathogenesis further confound efforts to address these challenges.[3,4] Bacterial pathogens must overcome the innate immune system, which provides first-line defense against microbial invaders, to cause human disease. Fundamental investigations that address the molecular interworkings of the innate immune system are critical for improving our understanding of the host−microbe interaction and allowing the development of new therapeutic strategies for infectious disease. Antimicrobial peptides (AMPs) and/or host-defense peptides are important components of the innate immune system.[5−8] In humans, defensins[7,9] and the cathelicidin LL-37[10,11] are abundant host-defense peptides expressed and utilized by neutrophils and epithelial cells of the gastrointestinal tract, urogenital tract, airway, and skin.[12] Mammalian defensins are cysteine-rich peptides that are classified as α-, β-, and θ-defensins on the basis of regiospecific disulfide linkage patterns.[12] α-Defensins exhibit three disulfide bonds in the oxidized forms with Cys^{I}−Cys^{VI}, Cys^{II}−Cys^{IV}, and Cys^{III}−Cys^{V} linkages and have three-stranded β-sheet structures.[7] In humans, six α-defensins are known.[7] The enteric α-defensin HD5 (Figure 1), the focus of this work, is an abundant constituent of small intestinal Paneth cell granules.[13−16] Although human defensins are established contributors to immunity, and many exhibit broad-spectrum *in vitro* antimicrobial activity, details pertaining to the physiological function of each peptide are often unclear.[12,17,18] In this work, we focus on the fundamental and outstanding question of how HD5 kills bacteria.

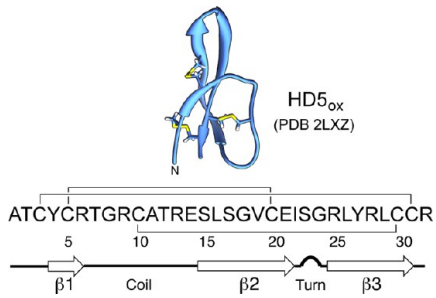


Figure 1. Structure of the $HD5_{ox}$ monomer determined by solution NMR (PDB entry 2LXZ)[19] and amino acid sequence. The regiospecific disulfide bond linkages and secondary structure are indicated.

How defensins kill bacteria as well as how *in vitro* antibacterial activity relates to the physiological milieu are questions of current interest and debate.[17] The oxidized α-defensins display remarkable similarity in their tertiary

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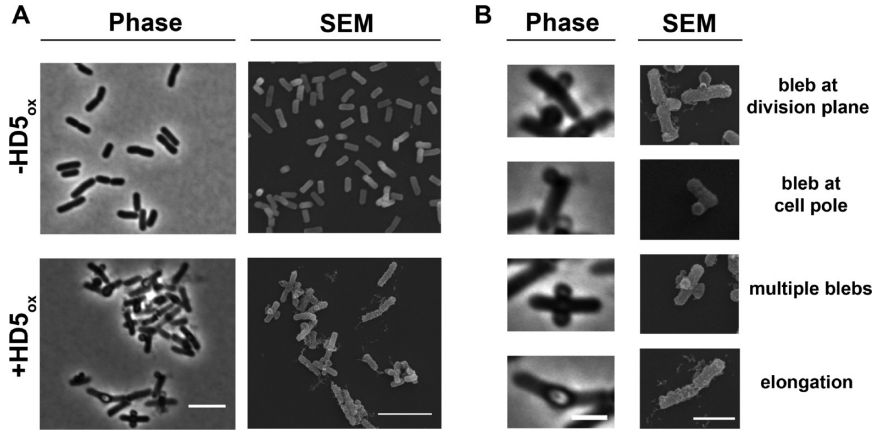

Figure 2. $HD5_{ox}$ causes distinct morphological changes in *E. coli* that include bleb formation, cellular elongation, and clumping. (A) Phase-contrast and SEM images of *E. coli* ATCC 25922 (1×10^8 CFU/mL) in the absence ($-HD5_{ox}$) or presence ($+HD5_{ox}$) of 20 μM $HD5_{ox}$ (scale bar of 5 μm). (B) Phase-contrast and SEM images of single cells illustrate the various morphologies caused by $HD5_{ox}$ (scale bar of 2 μm).

structure, and most characterized to date are cationic and amphipathic.[20] Moreover, defensins from various organisms have the capacity to disrupt bacterial cell membranes.[21−23] On the basis of early investigations, including seminal structural studies of HNP3,[24] a working model whereby defensins kill bacteria by nonspecific membrane destabilization was presented.[21,24] Over the years, this type of model was generalized for many defensins and other antimicrobial peptides.[17] Nevertheless, defensins exhibit remarkable diversity in primary sequence, and recent studies support alternative mechanisms of action for some family members. Fungal plectasin,[25] oyster defensin,[26] and fungal copsin[27] bind lipid II and block cell wall biosynthesis. The human defensins human neutrophil peptide 1 (HNP1, α-defensin)[28] and human β-defensin 3 (HBD3)[29] are also reported to bind lipid II to varying degrees.[30] Studies of HBD3 attributed the *in vitro* antibacterial activity against *Staphylococcus aureus* to lipid II binding and subsequent cell wall lysis.[29] Recently, human β-defensin 2 (HBD2) was found to localize at septal foci of *Enterococcus faecalis* and disrupt virulence factor assembly.[31] Human α-defensin 6 (HD6) lacks antimicrobial activity *in vitro* and is proposed to serve a host-defense function by self-assembling into a weblike structure termed a "nanonet" that captures bacteria in the intestinal lumen.[32,33] Taken together, these investigations highlight tremendous variation in defensin mechanism of action despite similar tertiary structures and demonstrate that membrane permeabilization is only one of the many factors that contribute to the *in vitro* antimicrobial activities demonstrated by this vast peptide family.

Our laboratory has initiated a research program focused on understanding the biophysical properties and biological functions of human defensins that are produced and released in the small intestine. Paneth cells,[16] located in the crypts of Lieberkühn, contain granules that store two α-defensins, HD5 and HD6, as well as other antimicrobial peptides, proteases, and a labile zinc pool of unknown function.[34,35] HD5 is the most abundant Paneth cell antimicrobial peptide,[36] and it exhibits broad-spectrum antimicrobial activity *in vitro*.[37−40] Moreover, transgenic mice expressing HD5 are more resistant to *Salmonella* challenge than wild-type mice,[41] and studies of the resident intestinal microbiota suggest that HD5 contributes to controlling its composition.[41,42] In humans suffering from ileal Crohn's disease, an inflammatory disorder of the small bowel, a deficiency in Paneth cell defensins has been reported.[36] Despite these compelling observations from animal models and clinical studies, the antimicrobial mechanism of action of HD5 is not well understood.

HD5 is a 32-residue peptide with an overall charge of +4 at neutral pH (Figure 1). Over the past decade, structure−activity relationship studies of $HD5_{ox}$ evaluated how quaternary structure,[40,43] disulfide linkages,[44,45] cationic residues,[46] the canonical α-defensin salt bridge between [Arg^6] and [Glu^{14}],[47] and chirality[48] contribute to its *in vitro* bactericidal activity. Results from several recent studies probing interactions between $HD5_{ox}$ and *Escherichia coli* support a model whereby (i) the Gram-negative outer membrane serves as a permeability barrier for $HD5_{ox}$[49,50] and (ii) the inner membrane becomes damaged as a result of $HD5_{ox}$ exposure.[44]

In this work, we utilize microscopy to investigate the attack of $HD5_{ox}$ on Gram-negative bacteria. We establish that *E. coli* cells treated with native $HD5_{ox}$ exhibit distinct morphologies that include clumping, cell elongation, and formation of one or more cellular blebs typically at the cell poles or division site. We demonstrate that similar morphological changes occur for other Gram-negative bacteria, including the opportunistic human pathogens *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, following $HD5_{ox}$ exposure. Through the design, characterization, and utilization of a fluorophore−HD5 conjugate family, we report that rhodamine- and coumarin-modified $HD5_{ox}$ enter the *E. coli* cytoplasm. Moreover, these fluorophore−$HD5_{ox}$ conjugates preferentially localize at cell poles and cell division sites in *E. coli*, suggesting locales of a possible intracellular target.

RESULTS AND DISCUSSION

HD5 Causes Distinct Morphological Changes in *E. coli*.

For morphology studies, we chose to image Gram-negative *E. coli*. This organism is a commensal microbe of the human gut as well as a pathogen of the gut and urogenital tract, and a number of studies have probed the antibacterial activity of $HD5_{ox}$ against this strain.[37,44,47] In antimicrobial activity (AMA) assays, the concentration of $HD5_{ox}$ required to kill *E. coli* (e.g., lethal dose 99.99% or 4-fold log reduction in colony-forming units per milliliter) depends on the number of colony-forming units (CFU). Our standard AMA assay for evaluating $HD5_{ox}$ activity employs mid log-phase bacteria at $\approx 1 \times 10^6$ CFU/mL cultured in an AMA buffer [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB without

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dextrose]. Under these conditions, the concentration of $HD5_{ox}$ required to kill 99.99% of *E. coli* is ≈4 μM depending on the precise starting number of colony-forming units per milliliter. Treatment of mid log-phase *E. coli* ATCC 25922 (1×10^6 CFU/mL) with $HD5_{ox}$ (2 and 4 μM) under standard AMA assay conditions resulted in marked changes to the bacterial morphology that could be observed by phase-contrast microscopy. The morphological changes included the formation of large bulges, hereafter called blebs (Figure S1 of the Supporting Information). To facilitate the visualization of more cells per experiment, we modified the standard AMA conditions and employed a greater number of cells (1×10^8 CFU/mL) and higher concentrations of $HD5_{ox}$ (0−80 μM). Under these conditions, $HD5_{ox}$ displays AMA, and an ≈2-fold log reduction in the number of CFU/mL is observed following treatment of the *E. coli* with 40 μM $HD5_{ox}$ (Figure S2 of the Supporting Information). Moreover, the *E. coli* displayed distinct morphological changes as observed in the preliminary experiment (Figure S1 of the Supporting Information). On the basis of this similarity, we employed the modified conditions with greater cell density and higher $HD5_{ox}$ concentration for further imaging experiments. The morphologies observed under these conditions included cellular elongation and the formation of blebs (Figure 2). Clumping of *E. coli* was also observed. Bacterial cells with lengths of ≥5 μm were categorized as elongated, and cells with lengths in the 10−15 μm range were periodically observed following $HD5_{ox}$ treatment. The blebs were typically localized at the cell division sites and cell poles; however, some bacteria displayed blebs along the cell body, and some bacteria exhibited multiple blebs per cell. The blebs remained intact during the centrifugation and wash steps required for scanning electron microscopy (SEM) sample preparation. Indeed, the blebs as well as what appeared to be outer membrane vesicles (Figure S3 of the Supporting Information) and cellular debris (*vide infra*) were markedly apparent in SEM images (Figure 2).

Neither blebs nor elongation was observed for untreated cells, and the number of cells exhibiting these morphological changes increased with an increasing $HD5_{ox}$ concentration (0−80 μM) (Figure 3). Following exposure to 20 μM $HD5_{ox}$, ≈17% of cells exhibited blebs and 2% were elongated (n = 212 cells). These values increased to ≈30% and ≥4%, respectively, at ≥40 μM $HD5_{ox}$ (n = 203 cells). We performed time-course experiments in which *E. coli* cells were exposed to 20 μM $HD5_{ox}$ on the microscope stage and imaged over time (≈2 h). Many of the treated cells displayed blebs, and the blebbing cells did not lyse over the course of this experiment (Figure S4 of the Supporting Information). Propidium iodide (PI) uptake was therefore employed to evaluate the viability of $HD5_{ox}$-treated cells. The cells exhibiting blebs were labeled with PI, which indicated that bleb formation correlated with cell death (Figure S5 of the Supporting Information). The morphological changes observed for *E. coli* ATCC 25922 were comparable to those we recently reported for *E. coli* K-12 and select mutants from the Keio Collection.[50]

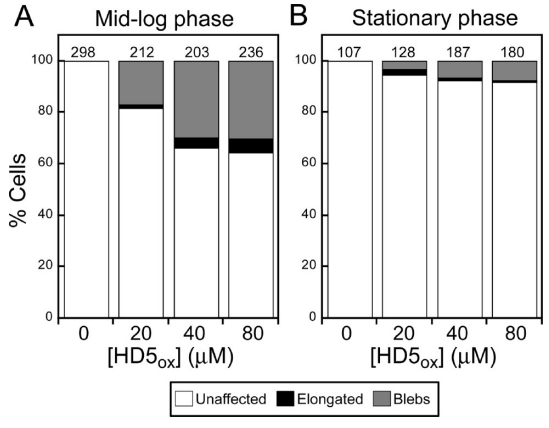

Figure 3. Effect of $HD5_{ox}$ treatment on *E. coli* ATCC 25922 morphology. The cells (1×10^8 CFU/mL) were treated with varying concentrations of $HD5_{ox}$ for 1 h at 37 °C [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB]. (A) Quantification of unaffected as well as elongated and bleb morphologies for mid log-phase *E. coli*. (B) Quantification for stationary-phase *E. coli*. The number above each bar indicates the number of cells counted.

The susceptibility of *E. coli* to $HD5_{ox}$ depends on the growth phase, and stationary-phase cultures exhibit resistance to $HD5_{ox}$ relative to mid log-phase cultures as observed for other defensins.[21] Under the standard AMA assay conditions employed in this work, treatment of mid log-phase *E. coli* with 4 μM $HD5_{ox}$ resulted in an ≈4-fold log reduction in the number of colony-forming units per milliliter, whereas only an ≈2-fold log reduction was observed for stationary-phase *E. coli* (Figure S6 of the Supporting Information). In agreement with this trend, fewer morphological changes were observed for stationary-phase cells treated with $HD5_{ox}$ (Figure 3 and Figure S6 of the Supporting Information). Taken together, the microscopy and AMA assays with $HD5_{ox}$ and *E. coli* provided a correlation between bacterial susceptibility and altered cellular morphology.

Treatment of *E. coli* with Other AMPs Does Not Result in the Bleb Morphology. We questioned whether other antimicrobial peptides confer the same morphological changes observed for $HD5_{ox}$ under the experimental conditions used in this work. Prior biophysical studies proposed the ability of the murine Paneth cell defensin cryptdin-4 to induce negative curvature on bacterial membranes, resulting in structures called blebs or pores.[51] To visualize the effect of cryptdin-4 on *E. coli* morphology, we first obtained cryptdin-4 by overexpression in *E. coli* and confirmed its antimicrobial activity (Figure S9 of the Supporting Information). The *E. coli* treated with cryptdin-4 (20 μM) did not form large blebs as observed with $HD5_{ox}$ (Figure 4). Likewise, *E. coli* did not exhibit blebs following exposure to the pore-forming antimicrobial peptide melittin[52] (20 μM), the LPS-associated membrane-destabilizing peptide colistin[53] (20 μM), or the pore-forming antimicrobial peptide human LL-37[54] (20 μM) (Figure 4). The bacteria treated with LL-37 were somewhat elongated relative to the untreated control cells. Small membrane protrusions (<100 nm wide) and surface roughness observed for bacteria treated with AMPs such as magainin 2[55] and Bac8c[56] have been described as blebs. To the best of our knowledge, larger blebs (∼1 μm wide), formed preferentially at poles and cell division sites as observed for $HD5_{ox}$, have not been reported for a human host-defense peptide. The most similar bleb morphologies we identified in the literature are the bulges that result from β-lactam treatment.[57] Moreover, a knockout mutant of elyC,[58] an inner membrane protein involved in peptidoglycan synthesis from the Keio Collection, and some Tol-Pal mutants of *Caulobacter crescentus*[59] are reported to display large blebs.

On the basis of this modest AMP screen, we concluded that $HD5_{ox}$ affects *E. coli* differently compared to the other AMPs considered in this work, including the murine α-defensin

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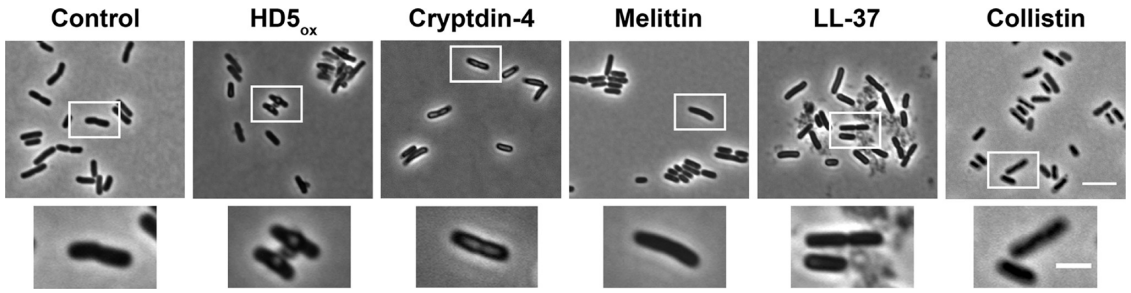

Figure 4. Consequences of various antimicrobial peptides on *E. coli* morphology. *E. coli* ATCC 29522 (1×10^8 CFU/mL, mid log phase) were exposed to each peptide (20 μM) for 1 h at 37 °C [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB] prior to imaging. In the top panels, the scale bar is 5 μm; in the bottom panels, the scale is 2 μm.

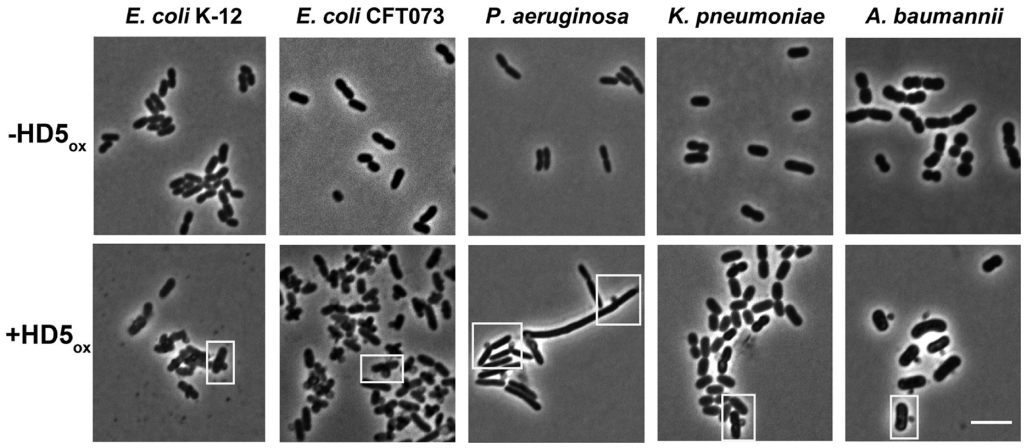

Figure 5. Effect of $HD5_{ox}$ exposure on the morphology of Gram-negative bacteria. Each microbe (1×10^8 CFU/mL, mid log phase) was exposed to $HD5_{ox}$ (20 μM except for *P. aeruginosa*, for which 40 μM was used) for 1 h at 37 °C [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB] prior to imaging. The scale bar is 5 μm. Additional images are provided in Figure S8 of the Supporting Information.

cryptdin-4. Although morphology comparisons alone do not provide detailed insight into antibacterial mechanisms, our imaging results suggest that the mechanism of action of $HD5_{ox}$ against *E. coli* cannot be explained fully by membrane permeabilization.

$HD5_{ox}$ Causes Similar Morphological Changes in Other Gram-Negative Organisms. $HD5_{ox}$ exhibits broad-spectrum antibacterial activity,[37,38,40] and the question of whether its mechanism of action is general or strain-specific remains unclear. Several structure−activity relationship studies indicated that $HD5_{ox}$ operates by different mechanisms for Gram-negative and -positive organisms, but these studies were limited to comparisons between *E. coli* and *S. aureus*.[44,45,48] To delineate whether $HD5_{ox}$ perturbs the morphologies of other Gram-negative strains, four human pathogens, *A. baumannii* 17978, *Klebsiella pneumoniae* 13883, *P. aeruginosa* PAO1, and *E. coli* CFT073, were evaluated along with the laboratory strain *E. coli* K-12 (Figure 5). Similar to nonpathogenic *E. coli* ATCC 25922 and K-12, bleb formation, elongation, and clumping were observed for the pathogenic strains. We previously reported that *A. baumannii* exhibits a relatively high sensitivity to $HD5_{ox}$.[40] In accordance with this observation, *A. baumannii* displayed blebs at 10 μM $HD5_{ox}$ (Figure S8 of the Supporting Information). *P. aeruginosa* is less susceptible to $HD5_{ox}$ killing,[40] and relatively high concentrations (40 μM) of $HD5_{ox}$ were required to elicit bleb formation for this strain (Figure S8 of the Supporting Information).

Morphological Changes Are Attenuated by Salt and Divalent Metal Ions. The *in vitro* antimicrobial activity of many defensins is attenuated by the presence of salt and divalent cations.[60] This phenomenon is commonly attributed to a disruption of electrostatic interactions between the cationic defensin and anionic bacterial cell membrane.[17] Like many defensins, the antibacterial activity of $HD5_{ox}$ is attenuated by millimolar concentrations of sodium chloride.[38] We observed that NaCl prevents bleb formation and other morphological changes associated with $HD5_{ox}$ activity. Indeed, *E. coli* cotreated with $HD5_{ox}$ (40 μM) and NaCl (200 mM) displayed a smooth morphology similar to that of the untreated control (Figure S7 of the Supporting Information). The divalent cations Ca(II), Mg(II), and Zn(II) also blocked $HD5_{ox}$ activity (Figure S7 of the Supporting Information). This effect was most potent for Zn(II), for which a 2:1 Zn(II):$HD5_{ox}$ molar ratio resulted in a loss of antibacterial activity and $HD5_{ox}$-associated morphologies. This observation is of broad interest because the Paneth cell granules, which harbor HD5, also contain a labile zinc pool of unknown function.[34]

Disulfide Bonds Are Necessary for the Bleb Morphology. The canonical α-defensin disulfide array provides a three-stranded β-sheet fold to each $HD5_{ox}$ monomer. This compact structure orients the positively charged residues on one face of the peptide and the hydrophobic residues on the opposite, rendering HD5 amphipathic.[19] To investigate the structural requirements for $HD5_{ox}$ activity, we evaluated the consequences of treating *E. coli* with three HD5 derivatives, HD5-TE, HD5-CD, and $HD5[E21S]_{ox}$, selected to probe the disulfide array as well as quaternary structure. HD5-TE is a linear disulfide-null analogue where the six cysteines are carbox-

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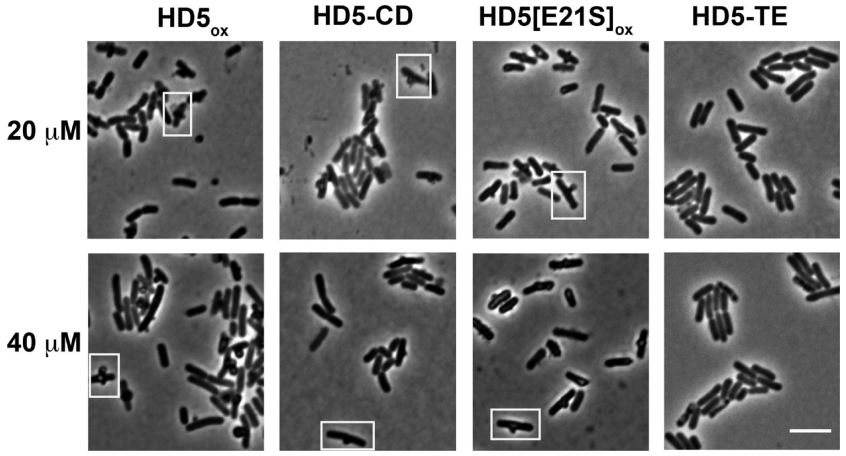

Figure 6. *E. coli* morphologies in the presence of HD5 derivatives reveal that the disulfide bonds are necessary for bleb formation. *E. coli* ATCC 25922 (1×10^8 CFU/mL, mid log phase) was exposed to each peptide for 1 h at 37 °C [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB] prior to imaging. The scale bar is 5 μm.

ymethylated with 2-iodoacetamide.[61] Defensins have the propensity to self-associate, and in prior work, we reported that $HD5_{ox}$ forms tetramers at neutral pH.[19] HD5-CD is a C_2-symmetric covalent dimer of $HD5_{ox}$ with a cationic surface that results from intermolecular disulfide exchange between the Cys^5–Cys^{20} disulfide bonds (canonical Cys^{II}–Cys^{IV}) of two HD5 monomers.[40] We also prepared and characterized $HD5[E21S]_{ox}$, a new $HD5_{ox}$ mutant that forms a noncovalent dimer, but not a tetramer, at neutral pH (Table S3 and Figure S23 of the Supporting Information).

E. coli cells treated with HD5-CD or $HD5[E21S]_{ox}$ were indistinguishable from those treated with $HD5_{ox}$ (Figure 6), in agreement with antimicrobial activity assays in which both $HD5[E21S]_{ox}$ and HD5-CD killed *E. coli* (Figure S10 of the Supporting Information). In contrast, the antimicrobial activity of linear and unstructured HD5-TE against *E. coli* was attenuated (2-fold log reduction in the CFU/mL at 16 μM) relative to $HD5_{ox}$. HD5-TE did not cause bleb formation (Figure 6); however, SEM revealed that the cells treated with HD5-TE were corrugated and frequently elongated (Figure S11 of the Supporting Information). These results highlight the importance of cysteine residues housed in disulfide linkages in the overall antimicrobial activity of HD5 and the induced morphological changes.

Cytoplasmic GFP is Observed in the Blebs. The blebs observed in this work are reminiscent of the cellular morphologies that result from treatment of *E. coli* with β-lactams, and *E. coli* strains expressing GFP have proven to be useful in imaging studies of β-lactam action.[57,62] Guided by this work, we investigated the contents as well as time-dependent formation of the blebs by employing an *E. coli* strain that expresses cytoplasmic GFP (*E. coli* cyto-GFP). Mid log-phase *E. coli* cyto-GFP formed blebs following exposure to $HD5_{ox}$. The blebs displayed GFP emission, and the GFP emission from the cell body was markedly reduced, suggesting that cytoplasmic contents localized to the blebs (Figure 7). Some additional phenotypes were observed for dividing cells. In several instances in which a dividing cell exhibited a bleb at the cell division site, the GFP localization differed between the daughter cells (Figure S12 of the Supporting Information). Moreover, for cells with blebs at the cell division site, the GFP intensity in the region between the two daughter cells was relatively weak, indicating the presence of a membrane at the

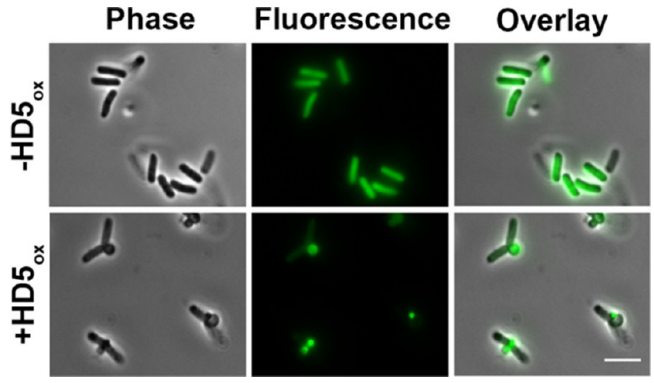

Figure 7. Treatment of *E. coli* cyto-GFP with $HD5_{ox}$ reveals that the cytoplasmic contents leak into the blebs. *E. coli* cyto-GFP (1×10^7 CFU/mL, mid log phase) were exposed to 4 μM $HD5_{ox}$ for 1 h at 37 °C [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB] prior to imaging. The scale bar is 5 μm.

division plane. In agreement with this observation, labeling $HD5_{ox}$-treated *E. coli* ATCC 25922 with the membrane-binding dye FM4-64 confirmed the presence of this membrane (Figure S13 of the Supporting Information).

Cell viability, as observed by PI uptake, was inversely correlated to the overall GFP fluorescence intensity of the bacteria (Figure S14 of the Supporting Information). When *E. coli* cyto-GFP cells (1×10^8 CFU/mL) were treated with $HD5_{ox}$ (20 μM) and subsequently stained as a result of PI uptake (5 μg/mL), the cells with brighter GFP emission and fewer morphological defects exhibited less PI labeling than cells that were affected by $HD5_{ox}$.

In agreement with studies using *E. coli* ATCC 25922 (Figure 3), negligible changes in morphology and GFP localization were observed for $HD5_{ox}$-treated *E. coli* cyto-GFP in the stationary phase. To obtain quantitative comparisons, the GFP intensity and morphological changes for both mid log-phase and stationary-phase *E. coli* cyto-GFP were analyzed (Figure S15 of the Supporting Information). The mean cell length for the mid log-phase cells increased from 2.8 ± 0.8 to 3.4 ± 1.0 μm (Student's *t* test, *t* value = 6.32, *t* probability < 0.0001) with an increasing $HD5_{ox}$ concentration (0–80 μM), and the mean GFP fluorescence intensity decreased accordingly (from 155 ± 48 to 93 ± 47 units). Only minor changes in both cell lengths

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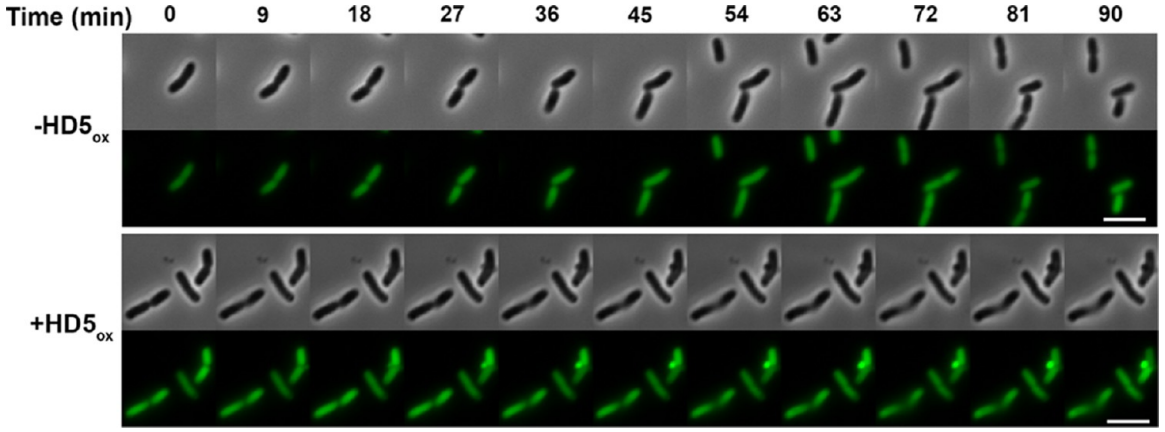

Figure 8. Time-lapse imaging of *E. coli* cyto-GFP (1×10^7 CFU/mL, mid log phase) treated with 20 μM $HD5_{ox}$ at 37 °C [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB]. The scale bar is 5 μm.

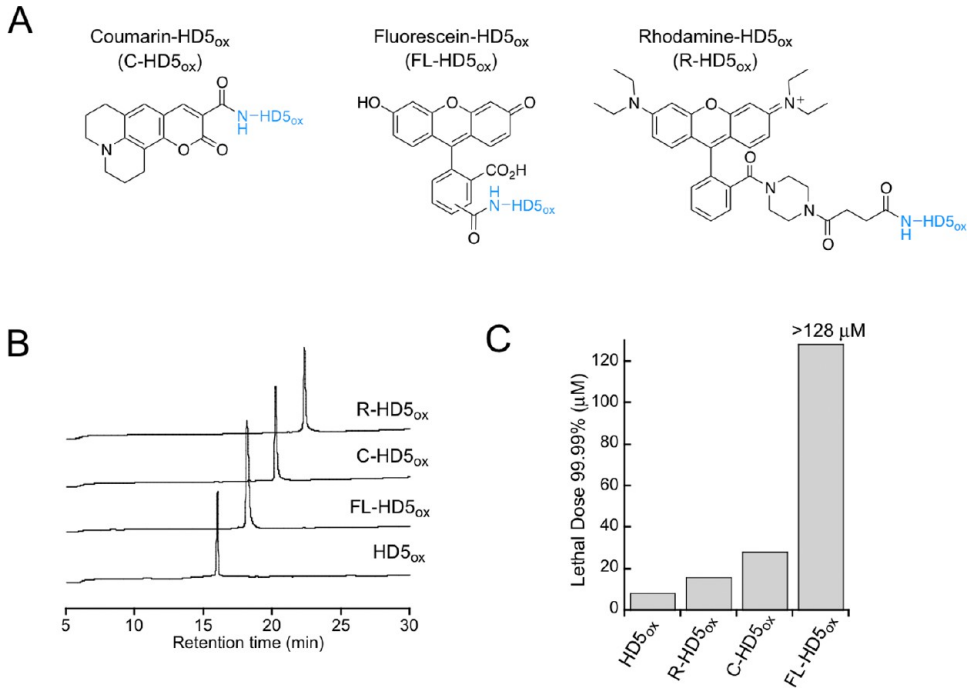

Figure 9. Functionalization of the N-terminus of $HD5_{ox}$ affords three fluorophore–$HD5_{ox}$ conjugates. (A) Structures of coumarin-, fluorescein-, and rhodamine-derivatized $HD5_{ox}$. The $HD5_{ox}$ primary sequence is presented in Figure 1. (B) Analytical HPLC traces (220 nm absorption, 10–60% B over 30 min, 1 mL/min) of purified peptides. (C) Antimicrobial activity of the peptides against *E. coli* ATCC 25922 (n = 3).

(from 3.0 ± 0.8 to 2.9 ± 0.7 μm) (Student's t test, t value = 1.97, t probability = 0.049) and mean intensities (from 112 ± 38 to 139 ± 53 units) were found when stationary-phase cells were treated with $HD5_{ox}$.

To obtain temporal information about bleb formation and GFP redistribution, we performed time-course experiments in which *E. coli* cyto-GFP cells were treated with $HD5_{ox}$ on the microscope stage and collected images over a 2 h period (Figure 8). The replication time for *E. coli* in the standard AMA buffer [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB without dextrose] on a MatTek plate ranged from 90 to 120 min at 37 °C. These cultures were unsynchronized, and bleb formation occurred at different time points depending on the cell. Blebs were observed immediately for some cells, whereas others formed blebs after exposure to $HD5_{ox}$ for ≈30 min. After the appearance of one or more blebs, the GFP intensity in the cell body diminished in all cases observed. Although dividing *E. coli* cells were observed for untreated cells (Figure 8, top panels), cells that were affected by $HD5_{ox}$ no longer divided (Figure 8, bottom panels).

Membrane Composition of the Blebs and Observation of Outer Membrane Vesicles. The composition of the membrane surrounding the blebs of $HD5_{ox}$-treated *E. coli* was investigated by fluorescence imaging of an *E. coli* strain harboring a plasmid encoding periplasmic GFP (*E. coli* peri-GFP) as well as transmission electron microscopy (TEM) of *E. coli* ATCC 25922. These studies afforded several phenotypes and suggested that two different types of blebs form (Figure S16 of the Supporting Information). Some cells exhibited a ring of GFP emission around the blebs, which indicated that both the outer and inner membranes surrounded the bleb and were intact. Other cells presented uniform GFP emission throughout the bleb. Possible explanations for this phenotype include the following: (i) only the outer membrane surrounded the blebs

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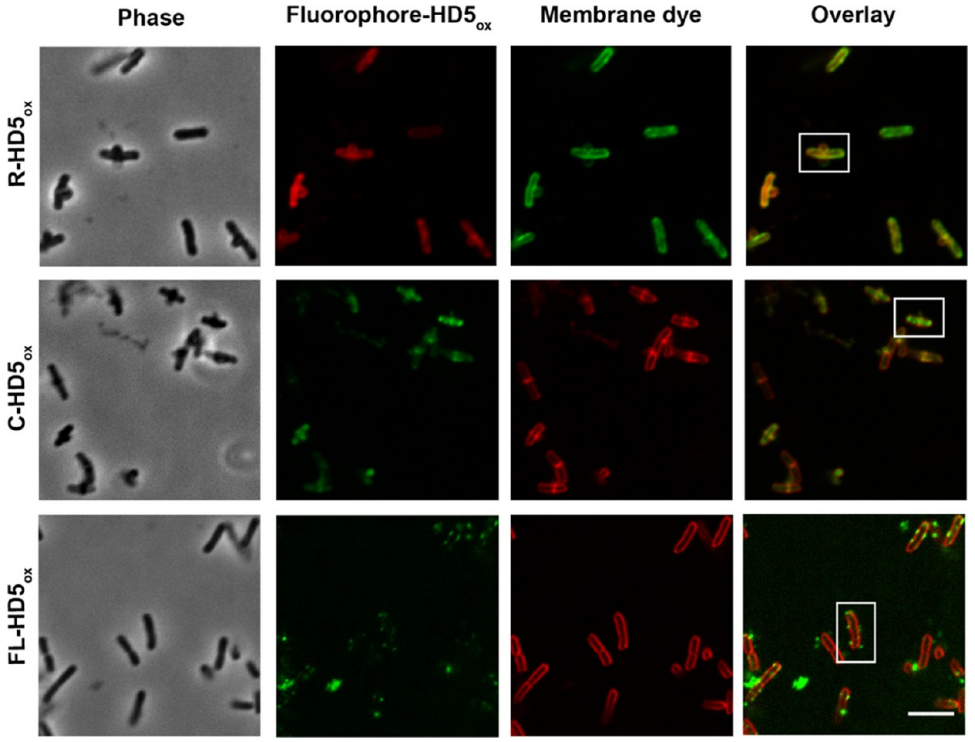

Figure 10. Fluorescence imaging of *E. coli* treated with fluorophore−$HD5_{ox}$ conjugates and membrane dyes. *E. coli* ATCC 25922 (1×10^8 CFU/mL, mid log phase) treated with R−$HD5_{ox}$ (20 μM), C−$HD5_{ox}$ (20 μM), and FL−$HD5_{ox}$ (8 μM) at 37 °C [10 mM sodium phosphate buffer (pH 7.4) and 1% (v/v) TSB] and incubated with 2 μg/mL FM1-43 for the R−$HD5_{ox}$ sample and 2 μg/mL FM4-64 for both C−$HD5_{ox}$ and FL−$HD5_{ox}$ samples prior to imaging. The boxed cells are depicted in Figure 11. The scale bar is 5 μm.

or (ii) the inner membrane surrounded the bleb but was damaged and leaked the periplasmic contents into the bleb. TEM imaging revealed that the blebs are membrane-bound; however, it was difficult to confirm whether one or both membranes surround each bleb (Figure S17 of the Supporting Information).

TEM also revealed profound changes to the *E. coli* cell surface upon $HD5_{ox}$ treatment, including vesicles surrounded by a membrane that often clustered in chainlike arrangements (Figure S17F of the Supporting Information). During phase-contrast imaging of $HD5_{ox}$-treated bacteria, we frequently observed surface-appended structures resembling debris. We attribute these structures, at least in part, to outer membrane vesicles (OMVs) on the basis of TEM and SEM studies (Figure S3 of the Supporting Information). Formation of OMVs (≈20−200 nm wide) is an important bacterial stress response pathway, and OMVs contribute to pathogenesis.[63] Thus, we speculate that *E. coli* may attempt to evade $HD5_{ox}$ by generating and shedding OMVs, along with $HD5_{ox}$, into the extracellular space.

Design and Synthesis of Fluorophore−HD5 Conjugates for Visualizing Peptide Localization. To probe the cellular localization of $HD5_{ox}$, we designed, prepared, and characterized a family of fluorophore−HD5 conjugates (Figure 9A,B, Figure S18, and the Supporting Information). Because addition of a fluorophore constitutes a substantial modification to a 32-residue peptide that may perturb the function and cellular localization of the native peptide, we evaluated the behavior of fluorophore−HD5 conjugates harboring different fluorophores as well as peptides harboring rhodamine attached to different positions (Table S1 of the Supporting Information). First, we modified the N-terminus of HD5 with three different fluorophores that afford variable photophysical properties as well as overall charge. We selected coumarin 343 (C), fluorescein (FL), and rhodamine B 4-(3-carboxypropionyl)piperazine amide (R)[64] as fluorophores to achieve emission properties spanning the range from ≈490 to ≈590 nm. Moreover, we reasoned that the overall charge of the fluorophore-modified peptide may influence its antimicrobial activity. Coumarin 343 is neutral following coupling to an α-amino group, fluorescein anionic, and rhodamine B cationic. This selection allowed us to probe the effect of fluorophore charge on the antimicrobial activity and cellular localization of fluorophore−$HD5_{ox}$ conjugates. We prepared R−$HD5[R9K]_{ox}$ and R−$HD5[R13K]_{ox}$ to evaluate the consequences of fluorophore positioning within the peptide sequence. We selected these positions because (i) prior structure−activity relationship studies reported that R9K and R13K mutants retained some antimicrobial activity[46] and (ii) the crystal structure (PDB entry 1ZMP)[20] and NMR solution structure (PDB entry 2LXZ)[19] of $HD5_{ox}$ revealed that the side chains of R9 and R13 are solvent-exposed and directed away from the dimer interface of native $HD5_{ox}$.

The syntheses of the fluorophore−HD5 conjugates were achieved using Fmoc-based solid-phase peptide synthesis (SPPS) as described previously.[40] Analytical and photophysical characterization of the peptides is detailed in the Supporting Information (Tables S1 and S2 and Figure S18). To determine whether the absence of disulfide bonds influences cellular localization, two disulfide-null mutants, R−HD5-TE and FL−HD5-TE, were synthesized by capping the cysteines of the reduced peptides, R−$HD5_{red}$ and FL−$HD5_{red}$, respectively, with 2-iodoacetamide (Supporting Information).

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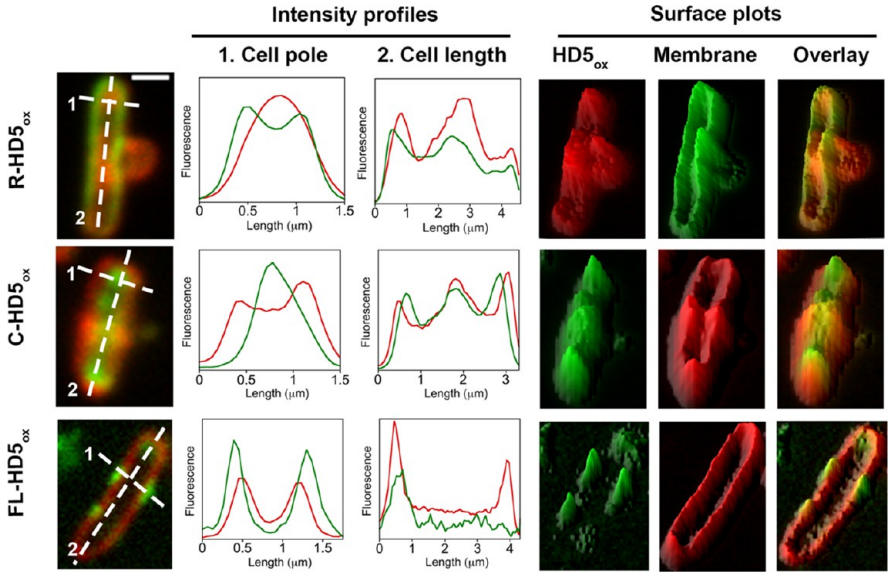

Figure 11. Intensity profiles and surface plots of the boxed cells depicted in Figure 10. Fluorescence intensities along the cell poles (dashed line 1) and across cell (dashed line 2) are plotted. Surface plots were generated using ImageJ. The scale bar is 1 μm.

Fluorophore Modifications Influence the Antibacterial Activity of HD5. To assess the impact of fluorophore modifications on $HD5_{ox}$ function, the AMAs of the conjugates against *E. coli* ATCC 25922 (1×10^6 CFU/mL) were first evaluated and compared to that of native $HD5_{ox}$ (Figure 9C and Figure S18C of the Supporting Information). Fluorophore modification attenuated the cell killing ability of $HD5_{ox}$ to varying degrees. The AMA (reported as the lethal dose for 99.99% killing, $LD_{99.99}$) of $HD5_{ox}$ and the analogues harboring N-terminal fluorophores followed a trend: $HD5_{ox}$ (8 μM) > R−$HD5_{ox}$ (16 μM) > C−$HD5_{ox}$ (28 μM) > FL−$HD5_{ox}$ (>128 μM) (Figure 9C). This trend suggests that the charge of the fluorophore may influence AMA. Additional factors, such as the physiochemical properties of the fluorophores and linkers (e.g., succinic acid spacer for R−$HD5_{ox}$), may also contribute to such changes in the AMA of modified $HD5_{ox}$. R−$HD5[R9K]_{ox}$ and R−$HD5[R13K]_{ox}$ were active against *E. coli*, and the linear derivatives were less active than their respective folded peptides, as expected (Figure S18C of the Supporting Information).

$HD5_{ox}$ Enters the *E. coli* Cytoplasm and Localizes at the Cell Poles and Division Site. On the basis of the AMA assay results described above, we first investigated the cellular localization of R−$HD5_{ox}$. We observed $HD5_{ox}$-like morphological changes, including bleb formation, for *E. coli* ATCC 25922 (Figure 10) and *E. coli* CFT073 (Figure S19 of the Supporting Information). Intracellular fluorescence was also observed. Co-labeling studies with the membrane-binding dye FM1-43 revealed that the FM1-43 emission profile enclosed a significant portion of the R−$HD5_{ox}$ fluorescence intensity profile (Figures 10 and 11), which indicates that the peptide penetrated the outer and inner membranes and entered the cytoplasm. In most of the cells examined by fluorescence microscopy, the rhodamine emission was most intense at the poles and division plane. This type of labeling pattern was not observed for R−HD5-TE or rhodamine-modified LL-37 (Figure S20 of the Supporting Information), both of which provided uniform cytoplasmic fluorescence.

When *E. coli* cells were treated with R−$HD5_{ox}$ under conditions that result in attenuated $HD5_{ox}$ activity (*vide supra*), different labeling patterns were observed. For instance, the presence of NaCl (200 mM) resulted in rhodamine emission only at the cell surface, indicating that R−$HD5_{ox}$ did not enter *E. coli* (Figure S22 of the Supporting Information). Stationary-phase *E. coli* treated with R−$HD5_{ox}$ exhibited diminished intracellular fluorescence relative to that of mid log-phase cells (Figure S22 of the Supporting Information). Taken together, these observations support a model whereby $HD5_{ox}$ must overcome the outer membrane permeability barrier and enter the cytosol to exert its full capacity to kill bacteria.

Co-incubation of *E. coli* with a 1:1 molar ratio of rhodamine B and $HD5_{ox}$ resulted in uniform cytoplasmic staining, whereas treatment of *E. coli* with rhodamine B alone resulted in negligible cellular fluorescence (Figure S21 of the Supporting Information). This result suggested that $HD5_{ox}$ treatment allowed for rhodamine B entry as a result of membrane permeabilization and confirmed that covalent attachment of rhodamine to $HD5_{ox}$ is essential for observing fluorescence localized to the cell poles and division site. The R−$HD5[R13K]_{ox}$ conjugate provided a labeling pattern similar to that of R−$HD5_{ox}$ and thereby indicated that localization of the R−$HD5_{ox}$ conjugate is not an artifact resulting from a particular site of rhodamine attachment (Figure S20 of the Supporting Information). Moreover, C−$HD5_{ox}$ preferentially labeled the cell poles and cell division sites, and co-labeling with the membrane-binding dye FM4-64 confirmed its cytoplasmic localization (Figures 10 and 11). The fact that the same labeling pattern was observed for $HD5_{ox}$ modified with either rhodamine or coumarin, taken with these modified peptides causing the same morphological changes as observed for unmodified $HD5_{ox}$, provides a strong indication that the localization is a result of $HD5_{ox}$, and not the fluorophore.

It should be noted that FL−$HD5_{ox}$, which provided no *in vitro* antibacterial activity against *E. coli* at the highest concentration evaluated (128 μM), did not enter *E. coli*. Rather, the peptide afforded a punctate labeling pattern on and around the bacterial surface (Figures 10 and 11). We attribute this phenomenon to the overall negative charge of the fluorescein moiety.

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The fluorescence imaging studies of $HD5_{ox}$ may be considered in the context of other AMPs that have been examined by similar approaches to probe uptake and mechanism of action. For instance, fluorescence microscopy revealed that buforin II entered the bacterial cytoplasm without permeabilizing the inner membrane.[65] A rhodamine derivative of the only human cathelicidin, LL-37, attacked septating *E. coli* more readily than nonseptating cells and permeabilized the bacteria via a carpet model of membrane destabilization.[66,67] A fluorophore-labeled lantibiotic was shown to interact with lipid II of *Bacillus subtilis* 168.[68] Recent imaging studies of Cy3-derivaitzed HBD2 indicated that HBD2 preferentially localizes to the nascent poles and cell division site of *E. faecalis*.[31] This focal labeling pattern was attributed to the binding of the HBD2 to anionic lipids in these regions. The cell poles and division site of *E. coli* are also enriched in negatively charged phospholipids that include cardiolipin and phosphatidylglycerol.[69] Whether $HD5_{ox}$ also binds to anionic lipids at the cell poles of *E. coli* and/or another target in these locales is currently unknown and a topic for future investigation.

SUMMARY AND OUTLOOK

In this study, we defined how the antimicrobial peptide $HD5_{ox}$ affects the morphology of *E. coli* and other Gram-negative bacteria. We established that $HD5_{ox}$ causes distinct morphological changes to *E. coli* as well as *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* that include bleb formation, cellular clumping, and elongation. We also demonstrated that other AMPs, including human LL-37 and murine α-defensin cryptdin-4, do not cause such morphologies. Taken together, these observations indicate that $HD5_{ox}$ kills *E. coli* by a mechanism different from those of the other peptides examined in this work. Our results highlight the importance of treating host-defense peptides individually and not generalizing cell killing mechanisms.

Defensins are often described as membrane-disrupting peptides. Our prior investigations indicate that $HD5_{ox}$ traverses the outer membrane and subsequently damages the inner membrane of *E. coli*.[44,50] In this work, the insights gained from utilizing fluorophore−$HD5_{ox}$ conjugates modified with rhodamine or coumarin support a model in which $HD5_{ox}$ enters the *E. coli* cytoplasm. Because the cytoplasm is a reducing environment, intracellular reduction of the $HD5_{ox}$ disulfide array to liberate free cysteine residues may occur. Deciphering whether such redox chemistry contributes to altered cellular morphologies and cell killing warrants exploration. The cytoplasmic localization also supports the possibility that $HD5_{ox}$ (or the reduced form) has an as yet undiscovered intracellular target. Indeed, the intracellular staining pattern at the cell poles and cell division site routinely observed for *E. coli* treated with R− and/or C−$HD5_{ox}$ suggest the locale of a possible target. On the basis of this localization, coupled with the elongation phenotype observed for *E. coli* and other Gram-negative microbes, we reason that $HD5_{ox}$ may exert antimicrobial activity by affecting cell division. Efforts are underway to further investigate this notion.

ASSOCIATED CONTENT

Supporting Information

Complete experimental methods and additional data. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

AMA, antimicrobial activity; C, coumarin 343; CFU, colony-forming unit; CFU/mL, colony-forming units per milliliter; Crp-4, cryptdin-4 (a murine α-defensin); FL, 5(6)-carboxyfluorescein; FM1-43, *N*-(3-triethylammoniumpropyl)-4-[4-(dibutylamino)styryl]pyridinium dibromide; FM4-64, *N*-(3-triethylammoniumpropyl)-4-{6-[4-(diethylamino)phenyl]-hexatrienyl}pyridinium dibromide; HD5, human α-defensin 5; $HD5_{red}$, reduced form of human α-defensin 5; $HD5_{ox}$, oxidized form of human α-defensin 5; HD5-TE, linear form of human α-defensin 5 (iodoacetamide-capped); HD5-CD, human α-defensin 5 covalent dimer; NMR, nuclear magnetic resonance; PDB, Protein Data Bank; PI, propidium iodide; R, rhodamine B 4-(3-carboxypropionyl)piperazine amide; SEM, scanning electron microscopy; TEM, transmission electron microscopy; TSB, trypticase soy broth.

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