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Review

Lights, Camera, Action! Antimicrobial Peptide Mechanisms Imaged in Space and Time

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Deeper understanding of the bacteriostatic and bactericidal mechanisms of antimicrobial peptides (AMPs) should help in the design of new antibacterial agents. Over several decades, a variety of biochemical assays have been applied to bulk bacterial cultures. While some of these bulk assays provide time resolution of the order of 1 min, they do not capture faster mechanistic events. Nor can they provide subcellular spatial information or discern cell-to-cell heterogeneity within the bacterial population. Single-cell, time-resolved imaging assays bring a completely new spatiotemporal dimension to AMP mechanistic studies. We review recent work that provides new insights into the timing, sequence, and spatial distribution of AMP-induced effects on bacterial cells.

Mechanistic Studies of Antimicrobial Peptides

In this era of multidrug-resistant bacterial infections, new antibacterial treatments are badly needed. Natural AMPs, also called host-defense peptides, serve as templates for the design of new antibacterial agents [1–3]. Deeper understanding of how AMPs kill bacterial targets should facilitate this design effort. Decades of intensive study have shown that the underlying mechanisms by which specific AMPs kill specific bacterial species exhibit remarkable variety from peptide to peptide, and remarkable specificity for particular AMP–bacteria pairings. Clear relationships between AMP structure and killing mechanisms have not yet emerged.

AMPs comprise an ancient class of short polypeptides (typically < 40 aa) that exhibit broad-spectrum antibacterial activity against both Gram-negative and Gram-positive bacteria [1]. They are secreted constitutively by epithelial cells within tissue repeatedly exposed to new microbes, such as in the lungs and the digestive tract. They are also found in vesicles within neutrophils and macrophages, cells that envelop and kill invading bacteria as part of the innate human immune response. One well studied class of cationic AMPs adopts amphipathic, α-helical conformations on binding to a membrane, often with a Pro-induced kink [4]. A second cationic class, the defensins, uses three disulfide bonds to enforce the three-β-strand defensin fold, a globular amphipathic structure [5,6]. Many other categories occur in nature. In addition, bacteria synthesize a wide variety of peptide-like agents that attack competing bacteria – the bacteriocins [7].

For a long time it was widely believed that most AMPs halt growth and kill bacterial cells by permeabilizing the cytoplasmic membrane (CM), thus destroying the proton-motive force (pmf)

Trends

Single-cell, real-time observations provide a remarkably detailed picture of the timing, sequence, and subcellular location of specific events during the attack of antimicrobial peptides (AMPs) on live bacteria.

In addition to permeabilizing membranes, AMPs induce a variety of 'downstream effects'. Specific peptides may interfere with cell wall synthesis; induce osmotic shock; disrupt synthesis of DNA, RNA, or proteins; destroy the proton-motive force; or induce oxidative stress.

Environmental factors can modulate potency by enabling specific bacteriostatic mechanisms or by altering the AMP structure. LL-37 is more effective against *Escherichia coli* in aerobic metabolism than in anaerobic conditions. In 2011, Schroeder and coworkers found that the reduced, unfolded form of human β-defensin-1 is much more potent against some intestinal bacteria that live in a naturally reducing environment.

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that drives ATP production [1]. Biophysical studies of AMP interactions with synthetic lipid bilayers have provided models for the mechanisms of bilayer disruption. These include 'barrel stave pores' made of contiguous amphiphilic AMPs inserted perpendicular to the bilayer surface; larger 'toroidal pores' lined by inserted AMPs and highly curved lipid surfaces; and 'carpet mechanisms' involving large-scale 'micellization' of the bilayer [1]. Molecular dynamics simulations of pore formation support a 'chaotic pore' model – a fluxional, localized permeabilization site comprising a time-varying number of peptide and lipid molecules [8]. The absence of apparent structure–function relationships has led to the suggestion that interfacial activity determines the ability of a peptide to permeabilize membranes [9]. This refers to a balance of hydrophobic and hydrophilic components with sufficiently imperfect amphiphilicity to facilitate disruption of normal lipid packing. The strength of the connection between AMP permeabilization of lipid vesicles and the killing mechanisms for real bacterial cells remains to be seen [10,11]. There is a correlation between an AMP's tendency to induce highly negatively curved phases in mixtures with lipids and its antibacterial activity [12].

Most mechanistic studies of the bacteriostatic and bactericidal effects of AMPs have focused on bulk, planktonic cultures. These bulk assays provide great insight into a variety of specific biophysical and biochemical mechanistic events, sometimes with time resolution of the order of several minutes. For example, they can distinguish disruption of the outer membrane (OM) from disruption of the CM using fluorogenic dyes; measure real-time release of K^+ from the cytoplasm [13]; and monitor dissipation of the pmf [14]. A remarkably thorough 2014 study of the effects of the synthetic hexapeptide RWRWRW-NH2 on *Bacillus subtilis* by Bandow and colleagues [15] applied some 15 different assays to the same system! However, the onset of damage mechanisms can occur within seconds, much faster than the response time of most bulk measurements. For example, a 2010 study from the Belcher laboratory used time-resolved atomic-force microscopy (AFM) to image the attack of CM15 on *Escherichia coli* [16]. Corrugation of the outer surface of live bacteria began within 13 s of AMP addition. In addition, the bulk assays provide no subcellular spatial information.

Recent work employs imaging methodologies such as immunotransmission electron microscopy (TEM), immunofluorescence, and AFM to directly observe the effects of AMPs on single cells. Most of these studies involve fixation and permeabilization of the cells, and they are typically carried out at a single time point after addition of the AMP. In a 2012 review, Munoz and Read surveyed studies that imaged the effects of AMPs on single, live microbes [17]. Most of the work reviewed involved yeast. Only two studies involved bacteria. The 2009 study of the interactions of the Sushi 1 peptide with *E. coli* used nanoparticle labeling of the peptide in both TEM and single-particle tracking methods [18].

The present review highlights a handful of recent studies in which spatial or temporal information gleaned from single-cell imaging experiments provides new insights into mechanisms of AMP attack on bacteria. The most common tool is fluorescence microscopy, either confocal or wide-field, in conjunction with phase-contrast microscopy (Box 1). A variety of one- and two-color fluorescent labeling schemes have proven useful, including labeling of the peptide itself. Single-cell imaging methods can provide a remarkably direct view of when and where key mechanistic events occur (Box 2). In our opinion, the most powerful mechanistic studies of the near future will augment time-resolved, live-cell imaging with increasingly powerful biochemical and genetic assays to provide a comprehensive picture of how AMPs halt growth and ultimately kill bacteria.

Survey of Recent Spatiotemporal Studies

AMPs That Bind Locally Near Cell Division Sites

Two recent studies illustrate how the distribution of binding sites of a fluorescently labeled AMP can provide clues to the bacterial targets of the attack, suggesting remarkably specific