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The RTX pore-forming toxin α -hemolysin of uropathogenic *Escherichia coli*: progress and perspectives

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Abstract

Members of the RTX family of protein toxins are functionally conserved among an assortment of bacterial pathogens. By disrupting host cell integrity through their pore-forming and cytolytic activities, this class of toxins allows pathogens to effectively tamper with normal host cell processes, promoting pathogenesis. Here, we focus on the biology of RTX toxins by describing salient properties of a prototype member, α -hemolysin, which is of ten encoded by strains of uropathogenic *Escherichia coli*. It has long been appreciated that RTX toxins can have distinct effects on host cells aside from outright lysis. Recently, advances in modeling and analysis of host–pathogen interactions have led to novel findings concerning the consequences of pore formation during host–pathogen interactions. We discuss current progress on longstanding questions concerning cell specificity and pore formation, new areas of investigation that involve toxin-mediated perturbations of host cell signaling cascades and perspectives on the future of RTX toxin investigation.

Keywords

Akt; evolution; HlyA; host response; membrane repair; mesotrypsin; pore-forming toxin; UPEC; urinary tract infection; uropathogenic *Escherichia coli*

An array of taxonomically diverse bacteria inhabit the multifarious environments that make up the vertebrate body. In order to coexist, these single-celled residents employ elaborate mechanisms to adhere to and acquire nutrients from host-associated habitats. However, some bacterial symbionts are distinguished by their ability to gain entry to and survive within restricted tissues where they can cause disease. Therefore, host organisms constantly survey and defend against unwanted barrier breaches and subsequent microbial infiltration of sterile body sites. As a result of enduring selective pressures presented by the host immune system, pathogenic bacteria exhibit a multitude of subversive behaviors. One particular evolutionary innovation that is conserved across multiple bacterial lineages is an ability to impair host cell function by disrupting a ubiquitous and critical structure – the plasma membrane [1]. The phospholipid bilayer of eukaryotic host cells maintains

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homeostasis by facilitating compartmentalization of enzymatic reactions and connectivity of signaling networks that ultimately provide means to organize and execute specialized actions. By compromising membrane integrity, pathogens can evoke a range of effects, from alteration of discrete cellular behaviors to tissue destruction.

RTX proteins are secreted by a phylogenetically varied spectrum of bacteria and have been ascribed a variety of functions ranging from bacterial adherence and biofilm formation to roles as bacteriocins, lipases and proteases [2,3]. Many RTX proteins are pore-forming toxins with the capacity to disrupt host cell membranes. The prototype RTX pore-forming toxin α -hemolysin (HlyA) is secreted by pathogenic variants of *Escherichia coli* and is one of the most extensively studied exotoxins of its kind [4–6]. During what was arguably the heyday of RTX toxin research, the 1980s and 1990s witnessed considerable progress towards understanding the genetic and biophysical principles that govern the function of HlyA and related proteins. In parallel, many observations were made revealing the cytolytic and highly proinflammatory potential of these noxious proteins on host cells [5]. However, it was and still is thought that during the course of infection, HlyA-like toxins act as short-range, labile molecules that only occasionally reach concentrations high enough to induce fulminant lysis of target cells [4]. Therefore, the past decade of research has largely focused on elucidating the precise molecular and biochemical perturbations that occur within host cells and tissues upon exposure to sublytic concentrations of poreforming toxins. The intent of this review is to introduce HlyA as a model RTX toxin and draw attention to the progress made towards resolving determinants of host cell specificity and pore formation, highlight recent studies that have started to elucidate the molecular basis of the influence that HlyA and other pore-forming toxins have on host cell physiology, and provide discussion on what the future of HlyA and RTX toxin research may hold.

α -hemolysin: a prototype RTX toxin

At the turn of the 20th century, Theodor Escherich's *Bacterium coli commune*, also known as *Bacillus coli communis* and later reclassified *E. coli*, was already recognized as a polymorphic microbial species that not only resided among the intestinal microbiota of healthy humans, but also had the potential to infiltrate numerous other tissues, where it caused disease [7–12]. Today, distinct pathogenic subgroups of *E. coli* are now categorized into pathotypes [13,14]. One such pathotype, uropathogenic *E. coli* (UPEC), is responsible for the majority of uncomplicated urinary tract infections (UTIs), which includes both cystitis (bladder infection) and pyelonephritis (kidney infection) [15,16]. During the 1910s and 1920s, it was first noticed that this pathogenic cohort possessed certain characteristics that distinguished it from its nonpathogenic enteric counterparts [17–20]. Approximately half of UPEC isolates were able to lyse erythrocytes and were thus labeled as hemolytic [21]. Because this trait tracked with the incidence of severe UTI and because hemolytic activity was relatively rare among intestinal *E. coli* isolates, the process of hemolysis soon became a focal point for investigation.

In the 1960s, it was conclusively demonstrated that hemolysis was mediated by a labile, secreted molecule subsequently dubbed ' α -hemolysin' (HlyA) [22–24]. During the following decades, the genes responsible for secretion and activation of HlyA were identified, cloned and used to fulfill molecular Koch's postulates in certain experimental systems, demonstrating that HlyA was in fact a *bona fide* virulence factor [25,26]. Below, we provide a basic overview of the biochemical and genetic features of HlyA, highlighting recent progress in addressing two key questions concerning HlyA biology: what dictates target cell specificity and what is the structural nature of the HlyA pore? For further discussion on these topics, the reader is referred to Iacovache *et al.*, Linhartová *et al.*, Welch, Frey and Ludwig and Goebel [1,2,5,27,28].

Biochemical & genetic features of α -hemolysin

HlyA is a 1024-residue, 110-kDa multidomain polypeptide that is released from bacterial cells through a type I secretion mechanism (Figure 1) [2]. The C-terminus contains an uncleaved secretion signal and the hallmark RTX domain, which is made up of several glycine- and aspartate-rich nonapeptide units with the consensus sequence GGXGXDX ϕ X (ϕ represents a bulky hydrophobic residue) [4,5]. The precise function of this domain has not been fully resolved but evidence suggests that, once bound by Ca^{2+} , the region takes on a β -roll structure and actuates a protein-wide conformational change that both stabilizes the toxin and exposes specific peptide surfaces that facilitate interaction with host cell membranes [2,29–31]. In addition, it has recently been demonstrated that the RTX domain may also aid in the initial adsorption phase to the host cell surface [29]. The N-terminus is comprised of ten positively charged amphipathic α -helices [32]. It is thought that this region mediates irreversible anchoring of HlyA to the plasma membrane, contributes structural elements for pore assembly and confers a degree of target cell specificity [33–35]. Bisecting the toxin are two internal lysine residues (K564 and K690) that become post-translationally lipidated (acylated) in the bacterial cytosol prior to secretion. Although not required for secretion, these modifications are essential for conversion of the benign ‘pro-HlyA’ protein into the mature toxic form competent for pore formation [36–39].

Genes encoding HlyA, as well as other RTX toxins, are typically located within an operon with the arrangement *hlyCABD* [27]. HlyC is the acyltransferase responsible for post-translational modification of the two internal lysines of HlyA. The *hlyB* and *hlyD* genes, along with the genetically unlinked *tolC*, encode an ABC transporter, membrane fusion/channel protein and outer membrane protein, respectively, which together form the type I secretion system through which HlyA is translocated. The *hlyCABD* locus is typically localized on the chromosome of pathogenic *E. coli* variants, but can be carried within plasmids by some pathotypes [40–42]. In UPEC, hemolysin genes are primarily harbored within chromosomally bound genomic regions, referred to as pathogenicity islands. These regions are hot spots for integration of foreign genetic elements that have been acquired by a process known as lateral gene transfer [43,44]. This feature suggests that acquisition of the hemolysin operon by pathogenic *E. coli*, such as UPEC, occurred some time in their evolutionary past, perhaps from an extralinear source. Indeed, alignments between phylogenetically distributed RTX toxins indicate that contemporary UPEC hemolysin genes originated from the bacterial lineage *Pasteurellaceae*, which includes several opportunistic animal pathogens [45–48]. Although a number of these evolutionarily related HlyA-like toxins share many biochemical and genetic characteristics, they also exhibit unique properties that include differences in target cell and host range specificities, pore structure and the potential to elicit particular cellular responses [5,28,49,50].

Determinants of target cell specificity & range

As its namesake suggests, HlyA is proficiently cytolytic towards heme-rich erythrocytes. However, it has long been appreciated that HlyA can also effectively lyse other host cell types, including epithelial cells and leukocytes from a variety of human and animal hosts [6,35,51–53]. Interestingly, this lytic promiscuity is not a conserved characteristic of all RTX toxins. For example, Lkt of *Mannheimia haemolytica* primarily targets bovine leukocytes, whereas the Aqx toxin of *Actinobacillus equuli* subsp. *haemolytica* is restricted to causing lysis of equine leukocytes [50]. Given that RTX toxins share many biochemical and genetic features, exactly how target cell specificity is determined is still debated. However, it has been demonstrated that RTX toxins can achieve target-cell recognition, in part, by engaging surface-localized protein receptors.

The cytolytic activity of multiple toxins, including HlyA, is greatly influenced by the presence of the integrin heterodimer CD11a/CD18, known as LFA-1, which is expressed on B and T cells, as well as neutrophils and monocytes [54,55]. Interestingly, RTX toxins expressed by *M. haemolytica*, *Aggregatibacter actinomycetemcomitans* and *Actinobacillus pleuropneumoniae*, which infect cattle, humans and swine, respectively, bind LFA-1 receptors in a host-specific manner [50,56,57]. These observations provide an attractive model whereby target cell range is dictated by the evolved ability of a toxin to bind conserved or unique regions of LFA-1. However, this receptor is not expressed on epithelial cells or erythrocytes, indicating that highly promiscuous RTX toxins have an affinity for multiple receptors and/or are competent for receptor-independent cell association. Indeed, the HlyA of *E. coli* is capable of binding the erythrocyte surface protein, glycophorin, via a conserved region within the C-terminal RTX domain, whereas the promiscuous adenylate cyclase RTX toxin of *Bordetella pertussis*, ACT, appears to lyse erythrocytes in a receptor-independent manner [58–60]. HlyA can also insert and form pores within artificial lipid bilayers and liposomes in the absence of protein receptors, although the kinetics of this phenomenon are greatly influenced by membrane lipid composition [61,62]. Cumulatively, available data suggest that HlyA and other RTX toxins may have evolved receptor-independent and -dependent means of associating with host cells. By having multiple mechanisms of initializing contact, with each varying in effectiveness, it is possible that the polyergic binding capacity of HlyA-like toxins provide an additional layer of functional regulation.

Regardless of the host receptors involved, the broad target cell range of UPEC HlyA and other RTX toxins has largely confused efforts to pinpoint the exact role of these toxins during host–pathogen interactions. It has been loosely suggested that lysis of host cells, particularly iron-rich erythrocytes, releases vital nutrients that are otherwise limiting in pathogenic contexts. However, there exists little to no experimental support for this reasoning, and evidence is building that the property of hemolysis is secondary or even irrelevant to pathogenesis [50]. It is known that the capacity of HlyA to lyse erythrocytes is greatly diminished in the presence of host serum proteins, a property that is corroborated by observations made in our laboratory using bladder epithelial cells [63] [Dhakal BK, Unpublished Data]. By contrast, it was found that HlyA can induce lysis of leukocytes regardless of the presence of serum, indicating that leukocytes are perhaps a more specific and probable *in vivo* target of HlyA [63]. Corroborating the likelihood of such specificity is the well-documented recruitment of phagocytic cells, primarily neutrophils, to infected tissue sites in response to UPEC infection, highlighting the selective need for UPEC to subvert this particular cell type [15]. Additional evidence that leukocytes are primary targets for HlyA comes from a recent study using human UPEC isolates and a surrogate zebrafish host infection model [64]. It was found that the virulence of some UPEC strains is greatly attenuated within zebrafish upon deletion of the *hlyA* gene. However, ablation of neutrophil and macrophage development within zebrafish hosts, using an antisense morpholino technique, rescued the virulence of HlyA-deficient UPEC. Together with findings on the potent targeting of leukocytes by RTX toxins, these observations support a role for HlyA in phagocyte deterrence.

The pore of α -hemolysin

Unlike other pore-forming toxins, such as a toxin of *Staphylococcus aureus*, the putative pores formed by HlyA and related toxins have yet to be observed by electron microscopy, crystal structure determination or other imaging techniques [5,32,65]. Instead, support for the pore-forming capacity of HlyA primarily stems from various indirect osmotic protection and electrophysiological experiments. In the mid-1980s, it was observed that upon HlyA exposure, host cell membranes became permeable to a specific size range of molecules,

inferred from the apparent offset of colloid osmotic pressure by large particles of dextran [5,32,65]. This prompted investigators to conclude that pores formed by this toxin were fixed and approximately 2–3 nm in diameter. However, subsequent studies and observations brought into question the size and dynamic properties of HlyA pores.

HlyA lacks well-defined transmembrane domains and is primarily isolated from host membranes as monomers, raising the question of how a single HlyA polypeptide might form a pore. Soloaga *et al.* found that HlyA may actually contribute to general membrane damage and breakdown by associating solely with the outer leaflet of host cell membranes [32]. Leaflet association may enable HlyA to function in a detergent-like fashion. However, subsequent studies using liposomes and ghost erythrocytes determined that several residues within the amphipathic, α -helix-rich N-terminus of HlyA completely insert into the lipid bilayer, serving as a putative transmembrane domain and possibly contributing to pore formation [33]. In addition, multiple studies have now provided strong evidence that HlyA can oligomerize within the plasma membrane, a process that is understood to be dynamic and dependent on time, toxin concentration and membrane fluidity [38,66]. Notably, it was found that the fatty acylation modifications are specifically required for HlyA oligomerization. It is hypothesized that the lipid decorations adorning the internal lysine residues confer a structural change that exposes disordered regions within the HlyA tertiary structures allowing for protein–protein interactions and pore formation [38,39]. Based on current knowledge, a simplified and speculative model for HlyA pore formation is described below.

The process of pore formation initiates when HlyA monomers, bound by Ca^{2+} , dock to the target cell membrane via electrostatic interactions involving regions contained within both the N- and C-termini; the kinetics of this phase may be enhanced by binding of specific surface receptors [54,67,68]. Once associated, each HlyA unit may then be brought into closer contact through its ability to associate with the outer leaflet, possibly a result of further conformational changes mediated by its proposed molten globule structure [32,39,69]. This insertion phase involves irreversible anchoring of portions of the α -helical domain to the host membrane, potentially exposing disordered regions within the toxin that promote protein–protein interactions [38]. Finally, depending on the localized microdomain and lipid raft content of the host cell membrane, monomers are concentrated and dynamically oligomerize to form pores [38,66,69]. The mechanism of HlyA pore formation is continually being updated and contested. Although it is considered a prototype RTX toxin, it appears HlyA has many unique properties that distinguish it from other canonical pore-forming toxins.

The emerging dichotomy: host cell response versus host cell tampering

So far, we have focused on aspects of HlyA biology that have been a point of focus in RTX toxin research for decades. Currently, there is increasing interest in trying to understand the precise impact of sublytic HlyA concentrations on host cells, as this may be more relevant to the natural progression of disease. It has long been recognized that HlyA-producing bacteria elicit a range of responses from a variety of different host cell types and tissues that are distinct from those triggered by HlyA-deficient bacteria. For example, depending on the target host cell, toxin concentration and duration of exposure, HlyA can promote host cell death via lysis, necrosis or apoptosis [5,70]. Of note, apoptotic-like death resulting from general RTX intoxication occurs within a matter of hours, in contrast to canonical programmed cell death that can take days to complete [5,71–74]. However, HlyA does not necessarily elicit cell death in all cases. Shortly after contact with target host cells, HlyA stimulates cationic fluxes of Ca^{2+} and K^{+} and the release of host metabolites such as ATP [63,65]. Interestingly, leakage of nucleotides such as ATP may stimulate P2X receptors,

which, in turn, may accelerate the cytolytic effects of HlyA-like toxins under some conditions [75–78]. HlyA can also induce proinflammatory responses, including the release of IL-1 β , multiple lipid mediators and the generation of superoxide [79–83]. The plethora of events that ensue upon HlyA intoxication inspired one author to describe the influence of HlyA on host cells as ‘all hell breaking loose’ [5]. Recent studies have started to add some clarity to the *modus operandi* of HlyA by dissecting the specific signaling cascades triggered or altered in response to this toxin.

It is increasingly evident that membrane-damaging proteins, such as HlyA, have dichotomous effects on target host cells (Figure 2). On the one hand, host cells will respond to pore-forming toxins as they would to any membrane-damaging agent, mobilizing systems that repair membrane lesions and recalibrate ion and metabolite gradients. On the other hand, pore-forming toxins may also elicit less generic responses, causing distinct biochemical and molecular alterations that can tweak specific signaling and proteolytic cascades, ultimately benefiting toxin-expressing pathogens. These two types of responses are not expected to always be mutually exclusive, but recent efforts have started to distinguish the general cellular reaction to pore-forming toxins from what appears to be true toxin-mediated manipulation.

General host cell responses to pore-forming toxins

To counter pore-forming toxins, host cells may coordinate several physiological responses to survive the challenge [84]. Below, we describe two mechanisms involving toxin-induced ion fluxes that have recently received attention. Importantly, several of the reported observations that we describe have not been directly demonstrated in the context of HlyA. Since work addressing this particular area of toxin/host biology is somewhat limited and new, the intent of this section is to synthesize a general scheme, using findings involving a variety of RTX and non-RTX pore-forming toxins. The reader is encouraged to consult cited literature for full details.

Similar to mechanical- or detergent-induced membrane damage, insertion of sublytic quantities of pore-forming toxins typically leads to an influx of extracellular Ca²⁺. The Ca²⁺ pulses that are associated with HlyA activity, for example, occur within seconds of toxin exposure, vary in amplitude and frequency in a cell-dependent manner and can alter host cell signaling networks [5,85,86]. It is often supposed that pore-forming toxins modulate Ca²⁺-sensitive physiological functions of host cells and tissues to promote bacterial pathogenesis. However, Ca²⁺ fluxes in response to pore-forming toxins may trigger a cascade of exocytic and endocytic events, initiated as an effort by host cells to repair their membranes. Using the non-RTX pore-forming toxin streptolysin O (SLO), it has been demonstrated that toxin-mediated membrane damage and subsequent rise in intracellular Ca²⁺ stimulate exocytosis of lysosomes, which carry with them to the cell surface healthy membrane and the enzyme acid sphingomyelinase (Figure 2) [87]. Acid sphingomyelinase catalyzes the hydrolysis of sphingomyelin into ceramide, enabling this host enzyme to modify lipids within localized regions of the plasma membrane in a way that favors endocytosis of a damaged, pore-ridden membrane. The ensuing endocytic events following SLO-mediated membrane damage are Ca²⁺ dependent and dynamin independent [88]. The cell recovery mechanism elucidated in the context of SLO intoxication provides a strong platform from which to start understanding how host cells repair lesions produced by other pore-forming toxins. Whether or not RTX toxins such as HlyA, which also induce Ca²⁺ fluxes, stimulate a similar recovery mechanism has yet to be determined. Of note, cellular recovery from α -toxin, a pore-forming toxin made by *S. aureus* that does not induce Ca²⁺ fluxes, requires dynamin-dependent endocytosis and subsequent purging of toxin oligomers through exosomal structures referred to as toxosomes [89,90]. Although we present the hypothesis that Ca²⁺

fluxes may serve as a generic signal to initiate plasma membrane repair, it is clear that not all pore-forming toxins are counteracted in the same way.

Leakage of a second ion, K^+ , is also emerging as a key modulator of host cell survival and recovery following challenge by pore-forming toxins [91–93]. A dramatic efflux of intracellular K^+ occurs as a result of pore formation. Through an unknown mechanism, this event triggers signaling cascades that increase cellular viability in the face of pore-forming toxins (Figure 2) [91–93]. It was found that the efflux of K^+ following pore formation by the non-RTX toxin aerolysin promoted cell survival by stimulating the proteolytic processing and activation of sterol regulatory element binding proteins (SREBPs), transcription factors that are known to upregulate lipogenic genes involved in plasma membrane repair [93]. However, it is not clear if SREBPs provide protection against pore-forming toxins solely via effects on lipogenic genes or if other SREBP-dependent response pathways are involved. Interestingly, K^+ -dependent activation of SREBPs requires assembly of the inflammasome and activation of caspase-1, indicating that the innate immune response to pore-forming toxins is coordinated, to some degree, with regenerative lipid metabolism. The capacity of pore-forming toxins to affect lipid metabolic pathways may tie in with previous reports demonstrating that HlyA can stimulate the release of host immunomodulatory lipid mediators, known as prostaglandins and leukotrienes [79,94,95].

The response of host cells to pore-forming toxins is incredibly complex and far from being fully understood. Numerous other cellular events are likely to contribute to host cell recovery from pore-forming toxin insult. For example, autophagy and the transient arrest of host protein synthesis can provide host cells with increased resistance to pore-forming toxins, possibly by allowing the host cells to focus energy towards membrane recovery and detoxification [92]. Notably, the induction of these protective processes occurs downstream of K^+ efflux from intoxicated cells. Additionally, a recent RNAi screen carried out using the nematode *Caenorhabditis elegans* established that MAPK signaling pathways governed by p38 and JNK orchestrate, the majority of, immunity to pore-forming toxins [96]. Together, this growing body of observations is evidence that many of the signaling events following pore-formation by toxins such as HlyA are part of a coordinated, evolved effort by targeted host cells to survive the attack.

Host cell tampering by HlyA & other pore-forming toxins

Through its destructive, proinflammatory and cytolytic properties, HlyA may indeed act as an instrument by which bacterial pathogens gain the upper hand during infection. However, recent studies have provided molecular evidence that, at sublytic concentrations, this bacterial effector can fine-tune host cell inflammatory and cytoskeletal functions by more discrete mechanisms. Despite earlier observations that HlyA can evoke significant proinflammatory responses, other work suggests that this is not always the case. It is now appreciated that HlyA can actually suppress normal immune signaling during bacterial infection, dependent upon the host cell type analyzed and/or the time points assessed [78,97,98]. It should be noted, however, that conclusions concerning the exact physiological influence that HlyA has on host cells may, in some cases, conflict owing to a tendency for purified toxin preparations to contain the proinflammatory bacterial cell wall component lipopolysaccharide (LPS). Nonetheless, scattered reports hinting at the anti-inflammatory function of HlyA have emerged during recent decades. For example, in 1993, König and König found that a HlyA-negative UPEC variant elicited robust IL-6 and TNF- α production from lymphocyte, monocyte and basophil cell suspensions, whereas cytokine induction was greatly reduced in the presence of HlyA [99]. Furthermore, it was demonstrated that intraperitoneal injection of LPS into mice resulted in dramatic increases in IL-1 α and TNF- α levels within the serum, while injection of a normally avirulent strain of *E. coli* expressing HlyA resulted in marked increases of only IL-1 α [99]. Together, this work provided some of

the first indications that the presence of HlyA during an infection has the potential to alter the immunological landscape.

Recently, the signaling cascades and cellular functions affected by HlyA have become more defined. However, a challenge posed during these investigations is how to identify what distinguishes host response from host cell tampering. Since generic host cell responses to pore-forming toxins appear to occur primarily downstream of Ca^{2+} and K^{+} fluxes, we will assume for the time being that any signaling event or behavior triggered independently of these ions may be considered an instance of true toxin-mediated host cell manipulation for the promotion of bacterial pathogenesis. The two studies detailed below are cases in which the investigators inhibited or artificially stimulated Ca^{2+} and K^{+} fluxes and observed no contribution of these cations towards the pathways in question.

The serine/threonine kinase, Akt, is a central player in host cell survival, immune and metabolic signaling and regulation of the cytoskeleton. In 2008, it was observed that treatment of bladder epithelial cells with HlyA-expressing UPEC or HlyA alone stimulated dephosphorylation and inactivation of Akt (Figure 2) [53]. HlyA-mediated inactivation of Akt is potent, capable of effectively muting strong Akt-activating signals from either EGF or TNF- α . Interestingly, this inactivation of Akt is phenocopied by the pore-forming toxins α -toxin and aerolysin, which are secreted by *S. aureus* and *Aeromonas hydrophila*, respectively [53]. Whether Akt is a primary target by all three pore-forming toxins or simply an inadvertent casualty of their toxic effects has not yet been determined. However, it is notable that membrane damage in general, such as that caused by the host membrane-permeabilizing glycoside saponin, does not stimulate Akt inactivation. The observation that pore-forming toxins from different host backgrounds and evolutionary histories have converged on the same pathway is enticing and suggests that toxin-induced Akt inactivation provides a general benefit to the pathogens.

In contrast to the apparently common ability of pore-forming toxins to dampen Akt signaling, a more recent study demonstrated that HlyA, and not α -toxin or aerolysin, is able to mediate degradation of select host cell proteins involved in immune and cytoskeletal functions (Figure 2) [78]. Specifically, it was found that exposure to HlyA stimulates rapid degradation of the RelA subunit of the central immune regulator NF- κ B, as well as several cytoskeletal regulatory proteins, including paxillin, HDAC-6 and PAK-1. Degradation of these proteins entailed activation of mesotrypsin and possibly other host serine proteases, and was observed in both bladder epithelial cells and macrophages following intoxication with HlyA. HlyA-induced proteolysis of host proteins correlated with an impaired capacity of bladder epithelial cells to produce the proinflammatory cytokine IL-6 in response to LPS. This finding is intriguing given the natural progression of UPEC disease within the urinary tract.

During the course of UTI, UPEC ascend into the bladder, where they can grow and replicate planktonically within the urine or adhere to and invade cells of the bladder epithelium. The presence of UPEC in the urinary tract stimulates a robust influx of phagocytic neutrophils, in addition to the exfoliation of infected bladder epithelial cells [15]. Bladder cell exfoliation is exacerbated in the presence of HlyA-producing UPEC, but the molecular mechanism by which exfoliation is triggered by UPEC and how this phenomenon specifically alters the course of infection is unclear [100]. The unique capacity of HlyA to disrupt immune signaling and cytoskeletal components suggests that this pore-forming toxin may be used by UPEC to facilitate bladder cell exfoliation while also impairing infiltrating phagocytes, potentially altering the outcome of infection.

Future perspective

This review has outlined recent advancements in HlyA research concerning target cell specificity, pore formation and perturbation of normal cellular functions. Despite much progress, investigation of the bioactive properties of HlyA has been, and still is, associated with a certain degree of ambiguity. For example, a host cell receptor that is ubiquitous enough to account for HlyA's promiscuous cell targeting is yet to be identified – does one even exist? We still lack any direct evidence that HlyA forms a pore, at least in the canonical sense. With regards to target cell physiology and pathogenesis, when do the cytolytic and sublytic signal-modulating properties come into play? No doubt, implementation of innovative technologies and experimental design, including the use of new super-resolution and *in vivo* imaging approaches, will yield clarification as we continue to investigate the molecular basis of HlyA and related RTX toxins.

In addition to these fundamental questions, there ultimately remains a gap in our knowledge regarding the precise role of HlyA during the lifecycle and evolution of the pathogenic *E. coli* carrying it. As noted earlier, only 50% of UPEC isolates express HlyA, which is a significantly higher proportion than nonpathogenic *E. coli*, but nonetheless indicates that HlyA is dispensable for some UPEC strains. Genes that confer a decisive fitness advantage are expected to sweep a population and provide a discernable benefit under physiologically relevant conditions. There has yet to be a specific fitness attribute assigned to HlyA within the urinary tract. In addition to our own unpublished observations, it has been reported multiple times that genetically engineered HlyA-deficient UPEC exhibit no reduction in fitness compared with HlyA⁺ parent strains using animal UTI model systems [100–102]. However, it has been demonstrated that HlyA is critical for survival during murine peritoneal infection and within a surrogate zebrafish host [26,64]. Although they serve as proxies for understanding the biology of HlyA, these infection models are unlikely to fully capture the exact selective pressures responsible for the maintenance and evolution of HlyA within UPEC strains. By shifting efforts towards understanding the temporal and spatial effects of HlyA within assorted host environments, novel insight could be gained on unresolved issues concerning the functionality and evolution of this pore-forming toxin.

With the growing number of sequenced bacterial isolates, many comparative approaches can be taken to address the evolutionary features of HlyA and the pathogens that express it. For example, is there a fundamental difference between the gene repertoires or expression profiles of HlyA⁺ and HlyA[−] *E. coli* pathogens? Considering related RTX toxins that exhibit various host and cell type specificities, is there evidence of positive selection within particular toxin domains? To ascertain the natural context under which HlyA is employed, we must also consider the complex and varied life cycle of UPEC. In contrast to diarrheagenic *E. coli*, UPEC and other related extraintestinal pathogenic *E. coli* pathotypes are generally thought to have benign effects within the intestinal tract. Is HlyA production tempered or somehow neutralized in this environment? The microniches and selective pressures that UPEC endures while on route to the urinary tract are still not fully defined; perhaps this is where HlyA is utilized. Additionally, given the strong evidence for HlyA as an effective antiphagocyte toxin, could it also be of benefit in defending *E. coli* against predatory amoebae and nematodes in soil habitats? Some of these points may be moot, but going forward, a synthetic approach that takes advantage of the trove of observations recorded to date while incorporating new perspectives will likely be critical to the next phase of HlyA research.

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Executive summary

α -hemolysin: a prototype RTX toxin

- α -hemolysin (HlyA) is expressed by uropathogenic *Escherichia coli* (UPEC) and is a member of the RTX protein family – a class of toxins that is widely distributed across bacterial phyla. RTX toxins are pore-forming proteins that are secreted by pathogens to disrupt host cell physiology.
- HlyA is a 110-kDa, multidomain protein comprised of: a N-terminal amphipathic region that contributes to membrane anchoring and pore structure; a central region that serves as an activation domain whereby two internal lysine residues become lipidated and subsequently facilitate pore oligomerization; and a C-terminal RTX domain containing several glycine- and aspartate-rich repeats that bind calcium and actuate conformational changes in the toxin necessary for pore formation.

The emerging dichotomy: host cell response versus host cell tampering

- Eukaryotic host cells must contend with constant mechanical, chemical and pathogen-mediated insults to their plasma membrane. Host cells respond to these insults in a generic fashion by upregulating membrane repair systems, but pore-forming toxins, such as HlyA, may also manipulate host cell function by specific manipulation of host signaling and proteolytic cascades.
- Ca^{2+} and K^{+} fluxes, triggered as result of host cell permeabilization by pore-forming toxins, initiate a coordinated response involving exocytic and endocytic events that expedite membrane repair and removal of pores from the cell surface.
- HlyA can repress select host cell signaling cascades involving the prosurvival and immunoregulatory proteins Akt and NF- κ B, as well as a variety of cytoskeletal regulators, including paxillin.

Future perspective

- HlyA has been demonstrated to be an important virulence factor in UPEC, yet only 50% of UPEC isolates express it – how do the virulence mechanisms among HlyA⁻ isolates differ from those of HlyA⁺ isolates?
- The next phase of HlyA research will need to better define the spatial and temporal activities of this toxin during infection.
- Questions concerning the impact of HlyA on the evolution of UPEC have largely gone unaddressed. Investigation into this topic will provide new perspectives and help guide future experimentation.

Figure 1.
 α -hemolysin domain structure and functions.

Figure 2. Influences of pore-forming toxins on host cell biology

Host cell response: Ca²⁺ influx induced by pore-forming toxins stimulates exocytic delivery of new membrane and the enzyme ASM to the cell surface. Accumulation of ceramide as a result of ASM activity favors subsequent endocytosis of the pore-ridden membrane. In some cases, toxin oligomers are discarded by trafficking to exosomal structures called 'toxosomes'. K⁺ efflux triggers autophagy, cellular quiescence, the lipogenic caspase-1/SREBP signaling axis and the MAPK p38. Host cell tampering: flux of unknown metabolites or osmotic stress triggers inactivation of Akt and serine protease-mediated degradation of NF-κB, paxillin, HDAC-6 and PAK-1, resulting in immune suppression and impaired cytoskeletal function. [†]Despite being a MAPK that has been implicated in mediating pore-forming toxin immunity, JNK has not been explicitly demonstrated to respond to K⁺ efflux in the context of pore-forming toxins.

ASM: Acid sphingomyelinase; SREBP: Sterol regulatory element binding protein.