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RESEARCH ARTICLE

THE PORE-FORMING TOXINS AND BIOLOGICAL POTENTIAL

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ABSTRACT

Bacteria produce different virulence factors through which they are capable of causing damage to the cells or tissues of the organism they infect. Among the virulence factors we can mention: the production of capsules, flagella, the synthesis of exopolysaccharides, the production of fimbriae, the formation of biofilm, the production of toxins. In this sense, toxins play a very important role in bacterial pathogenesis. For this reason, the toxins of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Escherichia coli*, *Mycobacterium tuberculosis* have been studied. In exotoxins, the largest class is the so-called pore-forming toxins, which function by perforating the membranes of the host cells. However, in recent years the use of pore-forming toxins in cancer therapy has been proposed. This work presents the biological potential of pore-forming toxins.

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INTRODUCTION

Infections caused by bacterial pathogens in human hosts are due to the host-pathogen interaction process. To successfully achieve bacterial pathogens, they use virulence factors with the functions of adhering, invading, antiphagocytizing, secreting proteins. So, they can grow, reproduce, spread and cause damage to the host ranging from mild discomfort to death (Niu *et al.*, 2013). To achieve successful colonization of the host, bacteria define a series of virulence factors, such as bacterial toxins. Classically, bacterial toxins are divided into endotoxins and exotoxins. Endotoxins are bound to the outer membrane of Gram-negative bacteria and provoke an inflammatory response in the host. Exotoxins are protein substances secreted by both Gram-negative and Gram-positive bacteria, which act locally or at a distance from the site of bacterial colonization (Popoff, 2018). The mechanism of action of exotoxins is to damage host membranes, affecting signaling in host cell membranes or entering target cells (Ala'Aldeen and Wooldridge, 2012). In the group of exotoxins, the largest class is the so-called pore-forming toxins (PFT), constituting 25 to 30% of cytotoxic exotoxins. They are the largest group among bacterial virulence factors (González *et al*., 2008). The pore-forming toxins function by perforating the membranes of the host cells, especially the cytoplasmic membrane as well as the membranes of intracellular organelles (Iacovache *et al.*, 2010).

Pore-forming toxins are the most common bacterial cytotoxic proteins and are required for virulence in a large number of important pathogens, including *Streptococcus pneumoniae*, group A and B streptococci, *Staphylococcus aureus*, *Escherichia coli* and *Mycobacterium tuberculosis*. Pore-forming toxins generally disrupt host cell membranes, but they can have additional effects independent of pore formation (Los *et al*., 2013). This work shows the most relevant aspects of biological potential of pore-forming toxins.

THE PORE-FORMING PROTEINS

Plasma membrane acts as a semi-permeability barrier between the cell and the extracellular environment. Plasma membrane integrity is essential for cell survival and sustainability, so disruption of it is considered to be one of the ancient cell-killing mechanisms by which bacteria invade humans. Pore-forming proteins (such as pore-forming toxins) are among such molecules that can alter membrane permeability (Dal Peraro and van der Goot, 2016; Gonzalez *et al*., 2008; Li *et al*., 2021). Killing target cells by pore-forming toxins is a common virulence mechanism in a wide range of pathogenic bacteria. As the largest class of bacterial toxins, pore-forming toxins are mainly produced by pathogenic bacteria. However, pore-forming proteins have been identified in all kingdoms, especially in eukaryotes as part of their immune system (Bischofberger *et al*., 2012; Li *et al*., 2021).

In modern multicellular organisms, interfering with membrane integrity is one the most effective strategies employed in immune defense, and membrane disrupting pore-forming proteins have evolved as key effectors in both innate and adaptive immune responses (Krawczyk *et al*., 2020). Pore-forming proteins can be found in all kingdoms of life. Bacteria use them to facilitate their entry into cells (for example: listeriolysin), to aid in the delivery of effector molecules across membranes (for example: streptolysin O) or as toxic agents (for example: diphtheria or anthrax toxins) (Peraro and van der Goot, 2016). Eukaryotic multicellular organisms (including mammals) use pore-forming proteins as membranolytic pores located on the surface of invasive pathogens or as effectors to selectively kill infected or cancerous host cells (Anderluh *et al.*, 2014; Liu and Lieberman, 2020). While bacteria can specifically attack eukaryotic membranes by recognizing specific host molecules, mammals face the more challenging task of eliminating unwanted cells without accidentally damaging surrounding healthy tissues. Mammalian pore-forming proteins evolved to show limited target membrane specificity in isolation and therefore depend on other proteins of the immune system to safely guide their activity. They are controlled by both the innate and adaptive arms of the immune system (Krawczyk *et al.*, 2020). On the other hand, pore-forming proteins appear to be involved in some disease processes in humans, for example in multiple organ failure which is the major cause of morbidity and mortality in intensive care patients (Kozlov and Grillari, 2022). As is known multiple organ failure is multifactorial, however hypoxia, intense inflammatory response and membrane disruptions (caused by pore-forming toxins that secrete pathogenic bacteria and permeable small molecules and also proteins), are important components as developers of the disease (Deitch, 1992; Iacovache *et al*., 2010; Shatursky *et al.*, 1999; Thiessen *et al.*, 2017). Membrane damage via changes in Ca^{2+} homeostasis and generation of intracellular reactive oxygen and nitrogen species cause widespread oxidation of both the proteins and lipids associated with membrane damage occurring predominantly in intracellular compartments (Cooper and McNeil, 2015; Kozlov and Grillari, 2022).

THE PORE-FORMING TOXINS

Toxins with cytolytic activity are produced by bacteria and various living organisms such as insects, poisonous reptiles and certain marine invertebrates. Of the toxins studied, a large proportion act by altering membranes. They form pores in cell membranes altering permeability and leading to cell death (Alouf, 2001; Parker and Feil, 2005). Pore-forming toxins can be classified in a number of ways. One of them corresponds to the secondary structure of the regions that penetrate the plasma membrane of the host cells, consisting of α-helices or β-barrels, that is depending on whether the structure adopted by their membrane-spanning region is constituted by α-helices or amphipathic β-strands (Dal Peraro and van der Goot, 2016; Gonzalez *et al*., 2008; Gouaux, 1997; Parker and Feil, 2005). Another class of pore-forming toxins are repeats in toxin (RTX), produced by Gram-negative bacteria, but their classification and mechanism of pore formation remain unclear (Dal Peraro and van der Goot, 2016). Many pore-forming toxins represent virulence factors and play multifaceted roles in pathogen infection, by directly or indirectly contributing to pathogen invasion and dissemination. Pore insertion into the eukaryotic plasma membrane causes uncontrolled efflux of nutrients and ions, especially K+, and can also perturb Ca^{2+} signalling (Hu *et al*., 2021; Los *et al*., 2013). The α-pore-forming toxins produce pores using helices. These toxins tend to be highly helical and the largest toxins have pore-forming domains that consist of a three-layer structure of up to ten α-helices sandwiching a hydrophobic helical hairpin in the middle of the structure. This class of toxins includes: exotoxin A from *Pseudomonas aeruginosa*, diphtheria toxin and some insecticidal δ-endotoxins (for example Cry toxin from *Bacillus thuringiensis*) (Allured *et al*., 1986; Choe *et al.*, 1992; Li *et al*., 1991; Parker and Feil, 2005; Parker *et al.*, 1989). Other examples of α-pore-forming toxins are: hemolysin E (HlyE, ClyA or SheA) produced by *E. coli*, *Salmonella* spp., *Shigella* spp., and it binds to the specific cholesterol receptor. In *Yersinia enterocolitica*, the YaxAB system has been reported, which represent a family of binary α-pore-forming toxins with orthologues in human, insect, and plant pathogens (with unknown structures). YaxAB system shown to be cytotoxic and likely involved in pathogenesis (Bräuning *et al.*, 2018; Sathyanarayana *et al*., 2018). Cell lysis mediated by HlyE (or ClyA) involves several steps in which the toxin must recognize and bind to the target cell, then it assembles forming a functional pore, thus the membrane is distorted and a pore is formed by insertion of α-helices in the lipid bilayer. The result is cell lysis similar to hemolysis. It has also been reported that in addition to hemolysis, this α-pore-forming toxin has other effects on target cells, such as producing morphological and cytotoxic changes in human and murine macrophages, and that cytotoxicity depended on dose and time (Mueller *et al*., 2009; Murase, 2022; Oscarsson *et al*., 1999; Sathyanarayana *et al*., 2021; Tzokov *et al*., 2006; Wai *et al*., 2003; Wallace *et al*., 2000). Lai *et al.* (2000) reported that ClyA induced massive apoptosis as was observed from host cell DNA fragmentation. This result is related to the activity reported by other authors by the α-pore-forming toxin from *S. aureus* and hemolysin (HlyA) from *E. coli* (Bantel *et al.*, 2001; Murase, 2022; Russo *et al.,* 2005). Fuentes *et al.*, (2008) reported that ClyA helped *S.* Typhi invade human epithelial cells in vitro and promoted deep organ colonization in mice when heterologously expressed in *S.* Typhimurium.

In recent years, the use of pore-forming toxins in cancer therapy has been proposed. As already mentioned before, pore-forming toxins bind to their target receptors, which can be sugars, proteins, or lipids. After binding, the pore-forming toxins oligomerize to form a pore inserting directly into the plasma membrane, altering the membrane potential (Lesieur *et al*., 1997; Zahaf and Schmidt, 2017). In this context, immunotoxins have been developed. The immunotoxins are chimeric proteins developed for specific targeting of cancer cells. These molecules are composed of two major parts: a receptor-binding moiety, which is in most cases an antibody (from which the name immunotoxin is derived) or a ligand (targeted toxin) directed towards a specific receptor expressed on the cell membrane. The second part of the immunotoxin is usually the catalytic part of a toxin, an enzyme, responsible for the toxin-induced lethality (Zahaf and Schmidt, 2017). Immunotoxins use the specificity of the antibodies produced towards target molecules on the cell membrane. The antibody part of immunotoxin is commonly shortened to its antigen-binding domain in order to decrease immunogenicity. The catalytic part is genetically engineered to improve toxic activity and/or to decrease antigenicity. When bound to the targeted receptor, the immunotoxin is endocytosed. To modify its molecular target, the toxin has to be released into the cytosol. Many toxins have the intrinsic capacity to escape to the cytosol following acidification of the endosomes and pore formation. Others take the route back through the Golgi body and endoplasmic reticulum into the cytosol. Within the cytosol, the catalytic part of the toxin modifies its target, ultimately triggering cell death (Binz and Rummel, 2009; Jank *et al*., 2007; Kreitman, 2006; Lord *et al.*, 1999; Zahaf and Schmidt, 2017). As an example, a pore-forming immunotoxin was generated by fusion of cytolysin equinatoxin II (isolated from the sea anemone *Actinia equina* L) to human transferrin, which is a regulator of cellular growth. Transferrin was an interesting ligand candidate because its receptor was thought to be only present on transformed, activated, and malignant cells (Gatter *et al.*, 1983; Zahaf and Schmidt, 2017). The α-pore-forming toxins from *B. thuringiensis* have been used as insecticides because they kill insects, such as the insecticidal crystal (Cry) proteins, the vegetative insecticidal proteins and the toxin complex (Tc). α-Pore-forming toxins from *B. thuringiensis* break down the midgut epithelia cells of the larval stages of insects, representing a highly effective and interesting ecological strategy for pest control because they are both highly specific against target insects and biodegradable (Crickmore *et al*., 2021; Hinchliffe and Hares, 2010; Pacheco *et al*., 2023; Pardo-López *et al.*, 2013). Another important class of toxins are ß-pore-forming toxins that insert into membranes to form ß-barrel. These toxins tend to be rich in ß-sheets. This class of toxins includes: aerolysin (an α-toxin from *Clostridium septicum*), α-hemolysin from *Staphylococcus aureus*, cytotoxin from *Pseudomonas aeruginosa*, some insecticidal δ-endotoxins, and

cholesterol-dependent cytolysins (Parker, 2003; Parker and Feil, 2005).

On the other hand, it has been reported that the formation of pores consists of the binding of the toxin to a receptor located on the surface of the cell membrane. These receptors can be made up of lipids, glycans or proteins. Oligomerization generally occurs differently for α and ß-pore-forming toxins. For most α-pore-forming toxins, the monomers undergo a "structural change" that exposes their hydrophobic/amphipathic helices to a hydrophilic environment, promoting them to divide in the membrane, so oligomerization and membrane insertion of α-pore-forming toxins are often concomitant (Benke *et al.*, 2015; Parker *et al.*, 1989; Ulhuq and Mariano, 2022; Wilmsen *et al.*, 1992). It has been observed that a consequence of the above is that α-pore-forming toxins are structurally heterogeneous, producing insertions through closed rings, which then perforate the membrane. Such is the case of cytolysin A (ClyA) and fragaceatoxin C (FraC). α-Pore-forming toxins are also known to form partial pores (with an incomplete ring) or toroidal pores (with a lumen formed by protein and lipid segments).

These pores retain their functionality despite their incomplete structure (Antonini *et al.*, 2014; Benke *et al.,* 2015; Churchill-Angus *et al.*, 2021; Dal Peraro and van der Goot, 2016; Sobko*et al*., 2006; Tanaka *et al*., 2015; Wilson *et al.*, 2019). In ß-pore-forming toxins, oligomerization occurs first by orderly adding monomers at the membrane interface, generating the so-called "pre-pore." Then, the pre-pore is inserted into the membrane through some conformational changes. The ß strands of each monomer form a ß-barrel that spans the membrane. Within the ß-barrel structure, a series of hydrogen bonds are established between the amino acid side chains of different monomers, ultimately conferring high structural rigidity and pore stability. Such is the case of some cholesterol-dependent cytolysins, where it was been observed that the process of sequential addition of monomers to the pre-porous structure remains incomplete, leading to the formation of arches as well as complete pores (Ostolaza *et al.*, 2019; Parker and Feil, 2005; Sonnen *et al.*, 2014; Sugawara *et al.*, 2015; Ulhuq and Mariano, 2022; Vögele *et al*., 2019; Yamashita *et al.*, 2014).

CONCLUSION

Pore-forming proteins play an important role in the functioning of eukaryotic and prokaryotic organisms. In particular, pore-forming toxins have potential uses in the possible treatment of some diseases in humans. Therefore, a better understanding of its mechanisms of action is required that can contribute to the development of novel alternatives for the prevention and treatment of bacterial diseases, as well as the treatment of diseases such as cancer.

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