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Clostridial pore-forming toxins: Powerful virulence factors

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Abstract: Pore formation is a common mechanism of action for many bacterial toxins. More than one third of clostridial toxins are pore forming toxins (PFTs) belonging to the β -PFT class. They are secreted as soluble monomers rich in β -strands, which recognize a specific receptor on target cells and assemble in oligomers. Then, they undergo a conformational change leading to the formation of a β -barrel, which inserts into the lipid bilayer forming functional pore. According to their structure, clostridial β -PFTs are divided into several families. Clostridial cholesterol-dependent cytolysins form large pores, which disrupt the plasma membrane integrity. They are potent virulence factors mainly involved in myonecrosis. Clostridial heptameric β -PFTs (aerolysin family and staphylococcal α -hemolysin family) induce small pores which trigger signaling cascades leading to different cell responses according to the cell types and toxins. They are mainly responsible for intestinal diseases, like necrotic enteritis, or systemic diseases/toxic shock from intestinal origin. Clostridial intracellularly active toxins exploit pore formation through the endosomal membrane to translocate the enzymatic component or domain into the cytosol. Single chain protein toxins, like botulinum and tetanus neurotoxins, use hydrophobic α -helices to form pores, whereas clostridial binary toxins encompass binding components, which are structurally and functionally related to β -PFTs, but which have acquired the specific activity to internalize their corresponding enzymatic components. Structural analysis suggests that β -PFTs and binding components share a common evolutionary origin.

Highlights

- Most of clostridial toxins are β -pore-forming toxins
- Clostridial cholesterol-dependent cytolisins are mainly involved in myonecrosis and gangrene
- heptameric β -PFTs are divided into aerolysin and staphylococcal alpha hemolysin families

CLOSTRIDIAL PORE-FORMING TOXINS: POWERFUL VIRULENCE FACTORS

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Key words: *Clostridium*; toxins; pore-forming toxins; cholesterol-dependent cytolysin, aerolysin; Perfringolysin; *Clostridium perfringens* epsilon toxin; *Clostridium perfringens* enterotoxin; *Clostridium perfringens* beta toxin; *Clostridium septicum* alpha toxin; *Staphylococcus aureus* alpha-hemolysin

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Abstract

Pore formation is a common mechanism of action for many bacterial toxins. More than one third of clostridial toxins are pore forming toxins (PFTs) belonging to the β -PFT class. They are secreted as soluble monomers rich in β -strands, which recognize a specific receptor on target cells and assemble in oligomers, Then, they undergo a conformational change leading to the formation of a β -barrel, which inserts into the lipid bilayer forming functional pore. According to their structure, clostridial β -PFTs are divided into several families. Clostridial cholesterol-dependent cytolysins form large pores, which disrupt the plasma membrane integrity. They are potent virulence factors mainly involved in myonecrosis. Clostridial heptameric β -PFTs (aerolysin family and staphylococcal α -hemolysin family) induce small pores which trigger signaling cascades leading to different cell responses according to the cell types and toxins. They are mainly responsible for intestinal diseases, like necrotic enteritis, or systemic diseases/toxic shock from intestinal origin. Clostridial intracellularly active toxins exploit pore formation through the endosomal membrane to translocate the enzymatic component or domain into the cytosol. Single chain protein toxins, like botulinum and tetanus neurotoxins, use hydrophobic α -helices to form pores, whereas clostridial binary toxins encompass binding components, which are structurally and functionally related to β -PFTs, but which have acquired the specific activity to internalize their corresponding enzymatic components. Structural analysis suggests that β -PFTs and binding components share a common evolutionary origin.

1 - Introduction

Life is organized in cells, which are delineated by a membrane. Cell membrane is not just a physical barrier between the intracellular and external compartments, but it is a complex structure, which has a crucial role for cell life notably in regulating the selective exchanges of molecules and in sensing external signals. Thereby, cell membrane integrity is required for survival and membrane represents the first target for pathogen attack. Pore formation through a cell membrane resulting in cellular ion imbalance and eventually to cell death is probably the simplest mechanism to attack a target cell. Many proteins including toxins are able to induce a pore through a membrane. It is possibly the reason why pore-forming toxins (PFTs) are the largest class of bacterial protein toxins. Almost one third of bacterial protein toxins, including clostridial toxins, are PFTs [1-4]. PFTs are also produced by all the classes of organisms including mammals, invertebrates, plants, and mushrooms. Indeed, pore formation

1 is used by host proteins called membrane-attack complex/perforins (MACPF) in physiological
2 processes like immune defenses or development [5, 6]. PFTs and MACPFs share a common
3 feature consisting of secreted soluble proteins, which interact with the hydrophobic membrane
4 bilayer and form a pore. According to their structure, PFTs can be divided into two main
5 classes the α -PFTs and β -PFTs. Clostridial PFTs belong mainly to the β -PFT family and are
6 important virulence factors, whereas α -pore forming activity is conserved in certain single
7 chain protein toxins produced by clostridia and it is involved in the translocation of the
8 enzymatic domain into the cytosol.
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16 **2 - Mechanisms of pore formation**

17 The two main mechanisms of pore formation are related to the PFT structure. α -PFTs
18 are molecules rich in α -helices, the pore-forming domain of which commonly contains a
19 bundle of α -helices with a hydrophobic helical hairpin in the middle, and which is involved in
20 pore formation through the lipid bilayer. In contrast, β -PFTs are mainly constituted of β -
21 sheets and develop a particular structure, which is an amphipatic β -barrel, to form the pore.
22 PFTs can form either small (0.5 – 5 nm) or large (20 – 100 nm) pores and the specificity of
23 the pores is variable according to the PFT.
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32 **2.1 - α -Pore-forming toxins**

33 Colicins produced by *Escherichia coli* are representative of α -PFTs. Colicins are
34 proteins which efficiently kill related *E. coli* strains or closely related bacteria. Some colicins
35 are active through a pore-forming mechanism, others exhibit an enzymatic activity towards
36 RNA, DNA, or peptidoglycan precursors [7, 8]. However, all colicins share a conserved
37 structure consisting of 3 domains rich in α -helices including a N-terminal translocation
38 domain involved in the crossing of the outer-membrane, a central receptor-binding domain,
39 and a C-terminal pore-forming or enzymatic domain. Structure of a representative colicin
40 (colicin E3) is shown in Fig. 1A. Colicins translocate through the inner-membrane by various
41 mechanisms, and when in the periplasmic space, pore-forming colicins, like colicin A, insert
42 into the inner membrane [7, 8]. The colicin A pore-forming domain consists of a bundle of 10
43 α -helices where H8-H9 form a hydrophobic helical hairpin (Fig. 1B). The pore-forming
44 domain associates with lipid bilayer by electrostatic attraction, and upon an unfolding process,
45 H8 and H9 insert into the bilayer in a perpendicular or nearly parallel manner with respect to
46 the plane of the membrane. A local pH decrease (~ 1.5) probably triggers the conformational
47 change allowing the insertion into the membrane and pore formation [9]. This α -helical
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1 structure including a bundle of α -helices with a central hidden hydrophobic helical hairpin is
2 also shared by single chain intracellularly active toxins, which use such a translocation
3 domain to deliver the enzymatic domain into the cytosol. For example, diphtheria toxin
4 contains a translocation domain with 9 α -helices, including helices H8 and H9 forming a
5 helical hairpin which inserts into the endosomal membrane leading to pore-formation (Fig.
6 1C). These helices are probably stabilized by association with a second helical hairpin
7 (helices H5, H6 and H7). Pore formation is required but it is not sufficient for delivery of the
8 catalytic domain across the endosomal membrane. The other helices H1 to H7 are also
9 involved in the translocation mechanism which results of multiple insertion intermediates and
10 which is still a matter of debate [10-12].

11 Another example of α -helical PFT is provided by *E. coli* hemolysin E (HlyE) (also
12 called cytolysin A or silent hemolysin A). HlyE is a 34 kDa long rod shaped molecule formed
13 by a bundle of 4 α -helices with a helical "tail" subdomain and a "head" subdomain containing
14 a short hydrophobic β -hairpin, called β -tongue (Fig. 1D). HlyE is secreted as soluble
15 monomers in a vesicle-mediated pathway. The monomers are activated by cleavage of
16 intramolecular disulfide bonds and then assemble in a dodecameric prepore structure. HlyE
17 undergoes conformational changes including movement and extension of the β -tongue in
18 hydrophobic α -helix. Thereby, each protomer becomes an elongated three-helix bundle. The
19 dodecamer adopts a cone-shaped α -helical barrel inside the pore [13-15]. Thus, HlyE is a
20 model of α -PFT, which oligomerizes and forms a α -barrel which inserts into the target
21 membrane leading to pore formation.

22 2.2 - β -Pore-forming toxins

23 β -PFTs share a common basic mechanism of activity. They are secreted as soluble
24 monomers which diffuse in the extra-bacterial environment and recognize specific receptor(s)
25 on the surface of target cells. Clustering of β -PFT monomers on cell surface promotes their
26 oligomerization and conformational change of one or two amphipatic β -sheet(s) from each
27 monomer which assemble and form a β -barrel, also called the prepore. Insertion of the
28 prepore into the lipid bilayer results in pore formation and subsequent alteration of the
29 membrane permeability (Fig. 2) [16].

30 According to their structure, clostridial β -PFTs able to form a pore in plasma
31 membrane can be classified into three families: cholesterol-dependent cytolysin (CDCs), and
32 the heptameric β -PFTs including the aerolysin family, and the *Staphylococcus aureus* alpha
33 toxin family (Table 1). In addition, certain clostridial β -PFTs form pores in the membrane of
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intracellular compartments, thereby facilitating the translocation of the enzymatic domain into the cytosol.

3 - Cholesterol-dependent cytolytins

The CDC family encompasses toxins, which are produced by numerous Gram positive bacteria such as listeriolysin O of *Listeria monocytogenes*, pneumolysin of *Streptococcus pneumoniae*, and streptolysin O (SLO) from *Streptococcus pyogenes*. Various *Clostridia* produce CDC (*C. botulinum*, *C. chauvoei*, *C. perfringens*, *C. tetani*, ...) (Table 1). The members of this toxin family exhibit 40-80% identity at the primary structure and share common biological properties and structural characteristics [1, 17, 18].

3.1 - Perfringolysin

Perfringolysin O (PFO) or theta toxin is the prototype of the CDC family. PFO is produced by almost but not all *C. perfringens* strains, and the *pfo* gene is located on the chromosomal DNA near the origin of replication [19-21]. PFO is synthesized with a 27 amino acid signal peptide, and the mature protein consists of 472 amino acids (53 kDa) [22].

PFO has an unusual elongated rod shape (Fig. 3). The molecule is rich in β -sheet and it is hydrophilic without significant patches of hydrophobic residues on the surface. Four domains can be distinguished in the PFO molecule. Domain 1 has a seven-stranded antiparallel β -sheet and is connected to domain 4 by the elongated domain 2. Domain 3 consists of β -sheets and α -helices. The C-terminal part (domain 4) folds into a separate and compact β -sandwich domain [23], and contains three loops (L1-L3), which are involved in the binding to cholesterol [24]. Molecular modeling shows that cholesterol binding to this region induces a displacement of a Trp rich loop. It is proposed that the high affinity (Kd 10^{-9} M) of PFO and also other CDCs to the cholesterol receptor is involved in concentrating the toxin in cholesterol molecules organized in arcs on the target membrane, thus promoting oligomerization and membrane insertion [23]. Cholesterol is clustered in membrane microdomains enriched in certain lipids (cholesterol, sphingolipids) or rafts, and PFO is a useful tool to identify the membrane rafts [25].

3.2 - Mode of action

The proposed model of PFO pore formation includes the binding of water soluble PFO monomers to cholesterol of lipid bilayer mediated by the L1-L3 loops from domain 4 (Fig. 3) [24]. The threonine-leucine pair (${}_{490}\text{TL}_{491}$) located at the top of loop L1 plays an essential role in binding to cholesterol [26]. Binding of domain 4 to its membrane receptor is sufficient to

1 trigger an allosteric activation of toxin monomers. Conformational change of domain 4 upon
2 binding to cholesterol induces transition states through the molecule until the distant domains
3 1 and 3, thus permitting the oligomerization and unfolding of transmembrane hairpins leading
4 to formation of the prepore [27, 28]. The mechanism of allosteric PFO activation dependent
5 of binding to cholesterol controls monomer interactions and pore formation when the toxin is
6 in closed contact with the cell membrane and avoids formation of premature and non-
7 productive toxin associations. A conserved undecapeptide motif among CDCs, also known as
8 the Trp-rich loop, located at the tip of the D4 domain, plays a critical role in the allosteric
9 coupling of membrane binding of D4 to structural change of D3 domain [29]. Domain 4 does
10 not insert deeply into the membrane and is not directly involved in creating the pore. PFO
11 monomers bound to cholesterol and orientated perpendicularly to the membrane assemble and
12 oligomerize to form a prepore complex [30]. Oligomers consist of 40 to 50 monomers
13 forming on the membrane surface large arcs and rings. Domains 1, 2 and 4 fit into L-shaped
14 repeating units connected to the corresponding domains of the neighboring partners and
15 forming a cylindrical structure. Oligomer formation results from domain 3-domain-3
16 interaction via hydrogen bonding between β 1-strand of one subunit with β 4-strand of a
17 second subunit. Interaction of domain 4 with cholesterol induces a conformational change of
18 domain 1 causing the moving of β 5-strand which prevents β 1- β 4 interaction in the soluble
19 PFO form and thus permitting the oligomerization process only when PFO interact with cell
20 membrane [18, 28, 31-34]. Thereby, PFO monomers do not oligomerize in solution even at
21 high concentrations. In addition, domains 3 are rotated from domains 2 and form a belt in the
22 outside face of the cylinder. This is accompanied by a flexing of domain 2 leading to a loss of
23 many contacts between domain 3 and domain 2 thus promoting the exposure of hydrophobic
24 residues and the insertion of a transmembrane β -barrel into the lipid bilayer [27, 35, 36]. A
25 bundle of three α -helices of domain 3 unfolds forming two amphipathic β -sheets. Each
26 monomer contributes two amphipathic β -hairpins to the formation of the transmembrane β -
27 barrel (Fig. 3) [37, 38]. Monomers do not insert their transmembrane hairpins individually,
28 but a cooperation between PFO monomers is required to drive the insertion of the prepore
29 complex, which appears to be an all or none process [39]. The prepore complex remains
30 localized above the lipid bilayer. A vertical collapse of the prepore of 40 Å allows the
31 insertion of the β -barrel into the membrane and formation of a large membrane pore 300 Å
32 and 450 Å in diameter (Table 2) [40-42]. The charged face of domain 4 amphipathic β -hairpin
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forms the inner lining of the pore and the other face is protected from the hydrophobic part of the lipid bilayer by cholesterol molecules [23].

Since PFO is more active at low pH (5.5-6) than neutral pH, PFO can act at the surface of cell membranes which are locally acid due to glycosylated proteins and/or in phagosomes [27, 43].

3.3 Role in the pathogenesis

Except intermidilysin (ILY) produced by *Streptococcus intermedius* which binds to CD59 a glycosyl phosphatidylinositol anchored membrane protein, CDCs recognize the cholesterol as cell surface receptor and thus interact with a large number of cell types [18]. CDCs are able to lyse a wide variety of cells *in vitro*. Notably, CDCs recognize red blood cells and are hemolytic, and they were originally called hemolysins. CDCs experimentally form only large pores (up to 40 nm in diameter) without possibility of intermediate pores on plasma membrane leading to osmotic lysis. But, it is not excluded that smaller size pores could result from assembly of fewer monomers [2, 18]. However, the CDC-dependent cell lysis in the initial steps of infection is not fully determined.

The main role of pore formation by CDCs seem to allow the release of nutrients from cells and then to facilitate bacterial growth and dissemination.

Pores induced by PFTs could be used by pathogens to internalize virulence factors into target cells in a similar manner than Gram negative bacteria type III to VI secretion systems. This has been evidenced only for *Streptococcus pyogenes* which uses SLO to transport NAD glycohydrolase, also produced by the pathogen, into cells [44, 45]. The SLO-mediated internalization is specific, since PFO is unable to facilitate the uptake of NAD glycohydrolase [45]. Thereby, translocation of NAD glycohydrolase into keratinocytes leads to apoptosis and cell death, whereas SLO-defective *S. pyogenes* mutants were less cytotoxic (review in [18]).

CCDs can also form pores in membrane of intracellular compartments. The best example is listeriolysin (LLO), which is produced by the intracellular pathogen, *Listeria monocytogenes*. LLO preferentially forms pore at acidic pH (optimal activity at pH5.5) and has an essential role in the *L. monocytogenes* escape from phagosome allowing the bacterial survival in the cytosol. LLO seems to have a more complex activity than just to induce vacuole membrane disruption at the acidic pH of phagosomal vacuole. LLO possibly induces the release of bacterial phospholipases into the cytosol and also acts in concert with host factors such as GILT (γ -interferon-inducible lysosomal thiol reductase) and CFTR (cystic fibrosis transmembrane conductance regulator) [46, 47]. LLO can form large and small size

1 pores, not only in membrane of endosomal compartments but also in plasma membrane.
2 During infection, LLO is produced intracellularly but also extracellularly and thus activates
3 several cell signaling like nuclear factor- κ B (NF- κ B), mitogen-activated protein kinase
4 (MAPK), phosphatidylinositol, and calcium signaling promoting autophagy, inflammasome
5 activation via efflux of K^+ and caspase1 activation, stimulation of the innate immune response
6 notably via the Toll Like Receptor 4 (TLR4), mitochondrial fragmentation, modulation of the
7 SUMOylation pathway, and histone modifications. The pleiotropic effects of LLO contribute
8 to the *L. monocytogenes* infection and escape to the host defense [46, 47].

14 Clostridial CCDs are mainly involved in gangrene lesions by contributing to tissue
15 destruction and preventing bacterial lysis by host immune cells. It is noteworthy that
16 Clostridia responsible for gangrene produce a CCD and also other membrane damaging
17 toxin(s) such as another PFT or a phospholipase and additional hydrolytic enzymes (Table 1).
18 Thereby, clostridial CCDs act synergistically with other membrane damaging toxin(s) to
19 generate the gangrene lesions. Indeed, using *C. perfringens* mutant strains defective either on
20 PFO or alpha-toxin gene, a synergistic effect between PFO and alpha-toxin has been
21 evidenced in experimental *C. perfringens* gangrene [48-50].

29 Among clostridial CCDs, PFO is the most well characterized regarding its mode of
30 activity. PFO by forming large pores on plasma membrane induces a cell lysis by a colloid
31 osmotic mechanism [51]. Albeit PFO can induce or interfere with cell signalings like the
32 SUMOylation pathway [52], its main activity resides in alteration of the membrane integrity.
33 A hallmark of clostridial gangrene lesions caused by *C. perfringens* and other clostridia, is the
34 total absence of inflammatory cells at the site of infection. At high concentration, PFO is
35 cytotoxic for polymorphonuclear lymphocytes (PMNL) and macrophages. At lower
36 concentrations, PFO impairs respiratory burst, superoxide anion production, and phagocytosis
37 of complement opsonized particles in PMNL [53, 54]. In addition, *C. perfringens* can survive
38 in macrophages, and PFO has a major role in the escape of the bacteria from phagosome by
39 lysis of the endosome membrane and in macrophage cytotoxicity [55, 56].

49 At the periphery of the necrotic lesions, PFO at sublethal concentrations reduces the
50 migration of PMNL/macrophages and induces their adherence to endothelial cells (Fig. 4).
51 PFO upregulates the expression/activation of adherence molecules such as neutrophil
52 CD11b/CD18, endothelial adherence molecules, platelet activating factor (PAF) and
53 subsequent phospholipase A2 synthesis [53, 57, 58]. Moreover, PFO prevents actin filament
54 polymerization in leucocyte and migration of neutrophils in response to chemoattractant [53,
55 54]. Accumulation of PMNL and macrophages in the vessels around the site of infection and
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1 inhibition of their migration mostly contribute to the lack of inflammatory response. In
2 addition, PFO synergistically with *C. perfringens* alpha toxin triggers platelet/platelet and
3 platelet/leucocyte aggregation through activation of the platelet fibrinogen receptor gpIIb/IIIa
4 [59, 60]. PFO also stimulates the expression of intercellular adherence molecule 1 (ICAM-1)
5 in endothelial cells, but to a much lesser extent than *C. perfringens* alpha toxin [57]. These
6 events result in the formation of intravascular platelet/leucocyte/fibrin aggregates leading to
7 vessel obstruction, hypoxia, and tissue destruction. Indeed the blood flow is reduced in the
8 microvasculature of the infected tissues [61, 62]. Adherence of the aggregates to the vascular
9 endothelial cells leads to vascular injury and subsequently contributes to the impairment of
10 leucocyte migration by diapedesis and tissue hypoxia [63-65].

11 The late stage of clostridial gangrene is characterized by cardiovascular collapse,
12 tachycardia, low blood pressure, and multiorgan failure (Fig. 4). Toxins, like PFO and alpha
13 toxin, are released in the blood circulation and act at distance of the site of infection on the
14 cardiovascular system. Notably, PFO reduces the systemic vascular resistance and increases
15 the cardiac output, decreases heart rate without drop in mean arterial pressure [66, 67]. PFO
16 contributes also indirectly to the toxic shock (hypotension, hypoxia, reduced cardiac output)
17 by promoting the release of inflammatory interleukins (TNF, IL1, IL6, PAF, and
18 prostaglandin I₂) and by acting synergistically with *C. perfringens* alpha toxin [65, 68].

19 The role in pathogenesis of botulinolysin and tetanolysin which are not associated
20 with other cytotoxins in *C. botulinum* and *C. tetani*, respectively, remains to be determined.
21 These CDCs could facilitate the local tissue colonization and resistance to macrophages of *C.*
22 *botulinum* and *C. tetani* during the early steps of wound botulism and tetanus, respectively.
23 Indeed, tetanolysin is able to form pores and to induce membrane damages in macrophages
24 [69]. In addition, botulinolysin is active on vascular endothelium leading to vasoconstriction,
25 hypotension and heart dysfunction in experimental rats. This seems to be a common activity
26 of CDCs, since SLO has also been found to be cardiotoxic [70, 71]. However, the effect of
27 botulinolysin on the cardio-respiratory system in the natural disease is not known.

28 **4 - Clostridial heptameric β -Pore-forming toxins**

29 An important group of clostridial β -PFTs is that of the heptameric β -PFTs. In contrast
30 to CDCs, they associate in smaller oligomers, heptamers or to a lower extent hexamers or
31 octamers, leading to the formation of small pores into membrane (Table 2) [72]. Whereas all
32 CDCs recognize a unique cell surface receptor, which is the cholesterol, except
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intermedilysin, heptameric β -PFTs bind to distinct receptor(s). Thereby, they are active on different subsets of cell types and they are responsible for specific diseases. Clostridial heptameric β -PFTs are mainly involved in intestinal diseases rather than in myonecrosis like clostridial CDCs (Table 1). Most of them are produced by *C. perfringens*.

Based on their structure, clostridial heptameric β -PFTs are divided into two families: the aerolysin family and the *Staphylococcus aureus* alpha hemolysin family.

4.1 - Aerolysin family

C. perfringens epsilon toxin (ETX), *C. perfringens* enterotoxin (CPE), and *C. septicum* alpha-toxin (ATX) are structurally related to aerolysin produced by Gram-negative bacteria of *Aeromonas sp.*, although ETX and CPE show no significant homology with aerolysin at the amino acid level (Fig. 5) [73-77]. ATX shares a low level (27%) of amino acid sequence identity with aerolysin [78]. The β -PFT aerolysin family also contains toxins from diverse origin, bacteria, animal, plant, like mosquitocidal toxins (Mtxs) from the Gram-positive bacteria *Bacillus sphaericus*, hydralysins from the animal *Chlorohydra viridis*, enterolobin from the Brazilian tree *Enterolobium contortisiliquum*, *Laetiporus sulphurous* lectin (LSL) from the mushroom *Laetiporus sulphurous*, and lysenin from the earthworm *Eisenia fetida* (review in [79-81]). Aerolysin has been extensively analyzed and is the prototype of this toxin family [82].

Aerolysin and clostridial β -PFTs of the aerolysin family are secreted through a N-terminal signal peptide as prototoxin monomers, except CPE which contains no signal peptide and accumulate in sporulating bacterial cells [83]. Aerolysin is converted into mature toxin by proteolytically removing of a C-terminal peptide (38 to 43 amino acids) by bacterial or host eukaryotic proteases [82]. Similarly, clostridial β -PFTs of the aerolysin family are released as inactive monomers. ETX is activated by cutting of 11 to 13 N-terminal and 29 C-terminal residues [84], and ATX processing results from the cleavage of 45 C-terminal amino acids [85]. ETX is mainly activated in solution by *C. perfringens* λ -protease or proteases of the host digestive tract, and ATX is mainly cleaved by furin, a cell surface associated protease [85]. CPE has also been found to be activated by trypsin or chymotrypsin which removes 24 or 36 N-terminal amino acids, respectively Kokai`-Kun, 1997 #560]. As shown in ATX, the propeptide acts as an intramolecular chaperone, which stabilizes monomers in solution, prevents unproductive aggregates, and drives correct oligomerization when the toxin is bound to membrane [86].

4.1.1 – Structure of aerolysin family β -PFTs

1 β -PFTs from the aerolysin family exhibit a more elongated shape than PFO (Fig. 2).
2 They consist of 3 to 4 domains and associate mainly in heptamers. The domain interacting
3 with the receptor is the N-terminal domain 1, except in CPE, in which, like in PFO, it is the
4 C-terminal domain. β -PFTs of aerolysin family recognize GPI-anchored proteins or
5 membrane proteins as receptors instead of lipids which are receptors of CDCs and some β -
6 PFTs of the α -hemolysin family (see below). A hallmark of heptameric PFTs is that each
7 monomer deploys only one β -hairpin forming the transmembrane β -barrel [80, 82, 87].
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10 Aerolysin is a L-shaped molecule rich in β -structure with a small N-terminal lobe
11 (domain 1) and a big elongated lobe spilled in three more domains (2 to 4) with the
12 characteristic feature of the presence of long β -strands (Fig. 5). Domains 1 and 2 are involved
13 in the recognition of GPI-anchored proteins through a double binding mechanism leading to
14 high affinity interaction of aerolysin with its receptor. Domain 1 binds to N-linked sugar of
15 the protein part of GPI-anchored proteins and domain 2 to the glycan core. Domain 2 with
16 domain 3 are involved in the oligomerization process. Domain 4 is located on the tip of the
17 major lobe and contains the C-terminal peptide which is released upon proteolytic cleavage.
18 Removing of the propeptide probably induces a conformational change and reorganization of
19 the domains which facilitate the formation of oligomers [2, 80, 82].
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22 The structures of ETX and CPE have been solved and show a similar fold than that of
23 aerolysin despite no significant amino acid sequence homology (Fig. 5). The main difference
24 is the absence of aerolysin domain 1. ETX and CPE retain an elongated shape with three
25 domains [73, 75, 88]. ATX probably shares a similar structure than aerolysin based on related
26 (27%) amino acid sequence identity [76-78]. Like ETX and CPE, ATX lacks the aerolysin
27 domain 1. Similarly to aerolysin, domains 1 of ETX and ATX are involved in the interaction
28 with the receptor [77, 89], whereas the CPE binding domain to receptor is located on the C-
29 terminal domain 3 [73, 75]. ATX recognizes GPI-anchored proteins as receptors, which are
30 different from those interacting with aerolysin, except Thy-1 and contactin, two common
31 receptors for both toxins [90]. CPE binds to claudins, which are membrane proteins involved
32 in intercellular junctions [91, 92], and the membrane protein HAVCR1 (hepatitis A virus
33 cellular receptor 1) has been proposed as the receptor for ETX [93]. Domains 2 and 3 of ETX
34 and ATX are involved in oligomerization and maintenance of the oligomers.
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37 The domain 3 of aerolysin and domains 2 of ETX, ATX, and CPE, contain the
38 membrane spanning β -hairpin. In aerolysin, the transmembrane region consists of 20 amino
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1 acids forming two amphipatic β -sheets connected by a hydrophobic 5 amino acid long stretch
2 which folds in an amphipatic β -hairpin upon oligomerization and membrane insertion. The
3 hydrophobic turn of the β -hairpin is thought to drive membrane insertion and folds back after
4 membrane crossing in a rivet-like fashion, thereby anchoring the β -barrel in the membrane
5 [94]. A similar structure is conserved with some differences in the clostridial β -PFTs of the
6 aerolysin family. In soluble CPE monomer, the transmembrane region folds in a helix and β -
7 strand [73, 75]. Hydrophobic residues also lie in the interconnection between the two
8 amphipatic β -strands in ETX, ATX and CPE.
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17 **4.1.2 - Mode of action**

18 The first step of β -PFTs of the aerolysin family as for the other PFTs is the binding to
19 cell surface receptor. This step has been further analyzed with ETX and ATX labeled with
20 photostable nanoparticle (Europium). Toxin monomers bound to their receptor are mobile on
21 the cell surface, but in confined areas corresponding to lipids rafts [95]. Indeed, ETX and
22 ATX receptors are localized in lipid rafts [90, 96, 97]. The confinement seems to be mainly
23 due to the composition and spatial organization of the lipids around the proteins and
24 subsequent molecular interactions (local electrostatic interactions, hydrophobic
25 interactions, lipid-protein specific and/or non-specific interactions) in the lipid rafts.
26 Thereby, membrane depletion in cholesterol or sphingolipids results in the release of
27 confinement, and ETX and ATX bound to their receptors move in a wider area. The actin
28 and microtubule cytoskeleton is not directly involved in the ETX and ATX mobility [98,
29 99]. However, albeit the toxin receptors are not directly linked to actin filaments, other
30 lipid raft proteins are connected to the actin cytoskeleton which mediates the
31 displacement of the whole lipid rafts in the membrane [100]. Mobility of toxin
32 monomers bound to their receptors in confined areas leads to a concentration of toxin
33 molecules and facilitates their interactions and subsequent oligomerisation.
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49 Pore formation has been solved at the structural level with aerolysin. Aerolysin
50 heptamer adopts a mushroom shape similar to that of α -hemolysin (see below).
51 However, in contrast to α -hemolysin, aerolysin heptamer associates with the membrane
52 in an inverse orientation, the mushroom cap facing the membrane and the stalk in the
53 extracellular milieu, since domains 1 and 2 which binds to the receptor are located in
54 the cap. Then, the heptamer undergoes a vertical collapse. Domain 3 and 4 rotate and
55 completely flatten, and the β -hairpin from domain 3 moves through a cavity between
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1 two monomers. The β -hairpins of the seven monomers refold in a β -barrel which lies in
2 the opposite orientation to that of the prepore mushroom stalk and which inserts into
3 the membrane (Fig. 5A) [101]. In contrast, CDCs and α -hemolysin show no drastic
4 conformational change during the prepore to pore conversion.
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7 ETX and ATX are cytotoxic for sensitive cells and induce a rapid and drastic decrease
8 in cell monolayer integrity. Both toxins seem to share a similar mechanism of cytotoxicity,
9 which has been investigated in more details with ETX [97, 102-104]. The cytotoxicity is
10 associated with a rapid loss of intracellular K^+ , and an increase in Cl^- and Na^+ , whereas the
11 increase in Ca^{++} occurs later. In addition, the loss of viability also correlates with the entry of
12 propidium iodide, indicating that the epsilon-toxin forms pores in cell membrane. ETX causes
13 a rapid cell death by necrosis characterized by a marked reduction in nucleus size without
14 DNA fragmentation. Toxin-dependent cell signaling leading to cell necrosis is not yet fully
15 understood and includes ATP depletion, AMP-activated protein kinase stimulation,
16 mitochondrial membrane permeabilization, and mitochondrial-nuclear translocation of
17 apoptosis-inducing factor, which is a potent caspase-independent cell death factor. The early
18 and rapid loss of intracellular K^+ induced by ETX and ATX, seems to be the early event
19 leading to cell necrosis [105] (review in [106-108]. Change in cell membrane permeability
20 with K^+ , Ca^{++} , and ATP as the main signaling molecules is a common feature of PFTs [109].
21 Cellular responses might differ between the distinct PFTs according to their pore selectivity
22 and to their specific receptor. Indeed, in addition to target certain epithelial and endothelial
23 cells, ETX has a specific activity on the nervous system. ETX is able to cross the blood brain
24 barrier and to interact with specific neuronal cells leading to an increased release of
25 glutamate, an excitatory neurotransmitter. But the mechanism of the stimulation of glutamate
26 release is not yet fully understood. Instead of a direct ETX effect on glutamatergic neuronal
27 cells through pore formation, rise in intracellular Ca^{++} , and subsequent signaling leading to
28 stimulation of vesicular exocytosis, ETX could induce a neuron depolarization following pore
29 formation. ETX also targets oligodendrocytes which are involved in the myelination process,
30 and thus could have a demyelinating effect [106, 108, 110]
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51 CPE binds to certain claudin isoforms which are tight junction proteins and have an
52 essential role in the integrity of epithelial barrier such as the intestinal barrier. When bound to
53 cell membrane, CPE forms small complexes (90-100 kDa) and then large complexes (160-
54 200 kDa) by association with a membrane protein, possibly occludin, which is also a tight
55 junction component. CPE induces cell death by a mechanism not yet well understood. At high
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concentration, CPE seems to trigger cell necrosis, and at low concentration cell apoptosis subsequently to Ca^{++} entry into cells [83, 92, 111].

4.1.3 - Role in the pathogenesis

Clostridial β -PFTs of the aerolysin family are responsible for mid to severe intestinal diseases or diseases from intestinal origin. The most potent toxin of this family is ETX, the lethality of which in experimental animals ranges just below the botulinum neurotoxins. The ETX lethal dose in mice upon intraperitoneal injection is 70 ng/kg. ETX is the major virulence factor of *C. perfringens* types B and D, and is responsible for enterotoxemia in sheep, goat and more rarely in cattle. Enterotoxemia is a rapidly fatal disease which causes important economical losses through the world.. Overgrowth of *C. perfringens* type D in the intestine of susceptible animals, generally as a consequence of overeating of food containing a large proportion of starch or sugars, produce large amounts of ETX. The toxin is absorbed through the intestinal mucosa and spreads in the different organs by the blood circulation causing blood pressure elevation, vascular permeability increase, lung edema and kidney alteration (pulpy kidney disease in lambs characterized by a post-mortem kidney softening). The terminal phase of enterotoxemia is characterized by neurological disorders (opisthotonus, convulsions, agonal struggling). ETX increases the permeability of the brain vasculature leading to perivascular edema and stimulates the release of glutamate (review in [106, 107]. ETX seems not to be involved in natural disease in humans since only a few cases have been described (review in [106]. However, ETX has been recently reported to be a potential virulence factor causing demyelination such as in multiple sclerosis [112].

ATX is involved in gas gangrene and also in non-traumatic myonecrosis of the intestinal mucosa, which occurs in patients with intestinal malignancy, neutropenia, leukemia or diarrhea. This infection is accompanied by a profound shock and it is fulminant and often fatal. The precise mode of action of ATX in these pathologies is no well understood. ATX might target vascular endothelial cells, which could result in the extravasation of fluid from the circulatory system and subsequent shock [113]. In animals, *C. septicum* is responsible for gangrene, enterotoxemia, and necrotizing abomasitis (braxy or bradsot) [76, 114].

CPE is one of the most common causative agent of food poisoning in humans. Most of the *C. perfringens* ingested with food die upon exposure to the stomach acidity. But, when ingested in high number, some bacteria can pass into the small intestine. Food involved in *C. perfringens* food poisoning contains at least 10^5 enterotoxigenic *C. perfringens*/g. Then, *C.*

perfringens multiply rapidly in the intestine and sporulate. Sporulation is a prerequisite step for CPE production [4]. CPE accumulates in the intestine resulting in alteration of the integrity of the intestinal epithelial barrier, desquamation of the intestinal epithelium, and diarrhea. High CPE doses cause enterocyte necrosis, inflammation, diarrhea, and abdominal pain.

Enterotoxigenic *C. perfringens* are also involved in hospital- and community-acquired antibiotic associated diarrhea, in chronic non-food borne diarrhea, and have been suspected in infant death syndrome in humans [115-119] [120, 121], as well as in diarrhea in foals and piglets [122].

4.2 - α -hemolysin family

C. perfringens delta toxin and NetB toxin constitute a β -PFT family structurally related to staphylococcal β -PFTs, the prototype of which is the staphylococcal α -hemolysin (or α -toxin) (Fig. 6) [123-126]. Albeit containing three domains, the β -PFTs of the α -hemolysin family show a more globular structure than the β -PFTs of the aerolysin family with a pore forming domain packed against domain I (Fig. 6). Contrarily to β -PFTs of the aerolysin family, β -PFTs of the α -hemolysin family are not activated by trypsin or other proteases. In contrast, they are sensitive to proteolytic degradation, notably beta and beta2 toxins.

4.2.1 – Structure of α -hemolysin family β -PFTs

Staphylococcal α -hemolysin is a 33 kDa protein secreted via a 26 amino-acid signal peptide. The protein is water-soluble and does not undergo further proteolytic cleavage. α -Hemolysin is organized in three structural domains: a N-terminal β -sandwich domain formed of two six stranded anti-parallel β -sheets, a C-terminal rim domain that is rich in β -strands, and a central domain called a stem (Fig. 6). A hallmark of α -hemolysin and related β -PFTs is that the central stem domain of monomers contains three short β -strands packed against the β -sandwich domain [127-130]. *C. perfringens* delta toxin (32.6 kDa) and NetB (33 kDa) secreted monomers share similar size and structure compared to α -hemolysin. They are also organized in three domains, β -sandwich, rim and stem domains. In addition, the heptameric assembly of NetB retains a similar conformation than that of α -hemolysin prepore [123]. However, the rim domain of delta toxin and NetB exhibit significant sequence and conformation differences with that of α -hemolysin [123-125]. Since the α -hemolysin rim domain is involved in the binding to cell surface receptor(s), these rim differences support that delta toxin and NetB recognize distinct receptors. Indeed, α -hemolysin interacts with a

1 protein receptor (see below) and delta toxin uses the ganglioside GM₂ as receptor [126, 131].
2 NetB receptor is still unknown but could be membrane cholesterol [123].

3 This family also includes *C. perfringens* beta toxin, which shares significant amino
4 acid sequence homology with delta toxin and staphylococcal β -PFTs and likely related
5 structure [132]. Beta2-toxin, which has been identified from a *C. perfringens* strain isolated
6 from a piglet that died of necrotic enteritis, shows no significant amino acid sequence
7 homology with beta toxin or other β -PFTs [133]. In contrast to beta toxin, beta2 toxin
8 exhibits sequence and expression variations. Two main alleles, termed consensus and atypical
9 *cpb2*, have been described. Consensus beta2 is mainly found in porcine *C. perfringens*
10 isolates, whereas atypical beta2 is more prevalent in non-porcine isolates [134]. Since beta
11 and beta2 toxins have in common a similar size, highly sensitivity to trypsin degradation,
12 activity limited to some cell types, and potency to induce experimental necrotic enteritis,
13 beta2 toxin is likely structurally related to β -PFTs of the staphylococcal α -hemolysin.
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25 **4.2.2 – Mode of action**

26 The mode of pore formation through a lipid bilayer has been investigated at the
27 structural level with staphylococcal α -hemolysin. Based on their structural relatedness with α -
28 haemolysin, clostridial β -PFTs likely retain the same mode of activity.
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31 The first step of intoxication consists of the binding of α -hemolysin monomer to
32 specific receptor(s) on the cell surface. Phospholipids and cholesterol were initially identified
33 as high affinity receptors for α -hemolysin but it was evidenced that the membrane protein,
34 ADAM10 (A disintegrin and metalloprotease 10), is the specific receptor [135, 136]. When
35 bound to its cell surface receptor via the rim domain, α -hemolysin units oligomerize into a
36 heptameric (or hexameric) pre-pore. Upon heptamerization (or hexamerization), the stem β -
37 strands unfolds and moves from the β -sandwich to form a β -hairpin. The β -hairpin of the
38 stem domains associate into a 14-strand antiparallel β -barrel that inserts into the plasma
39 membrane and forms the transmembrane pore (Fig. 6). The pore has a mushroom shape with
40 an inner diameter ranging from 22 to 30 Å. Overall, α -hemolysin and related toxins share a
41 similar mechanism of pore formation than CDCs, except that they form small pores resulting
42 from oligomerization of 6-7 units instead of 30-50 in CDCs, and that each monomer
43 contribute for one hairpin to form the β -barrel instead of two in CDCs [128, 129, 137].
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57 The primary activity of β -PFTs forming small pores on cell membrane is a disruption
58 of the membrane permeability to small molecules leading notably to K⁺, ATP efflux and
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influx of Ca^{++} , and subsequent deregulation of mitochondrial activity, activation of caspase 1,
release of proinflammatory proteins. At high concentrations, β -PFTs generally kill cells by
necrosis resulting in particular from mitochondria dysfunction, and at lower concentrations
they induce cell death via programmed necrosis or apoptosis [138]. At sublethal doses they can
induce multiple effects on cells including membrane repair, changes in metabolism, activation
of signaling pathways like activation of the p38 MAPK pathway, activation of caspases
leading to inflammasome activation and released of inflammatory molecules [128, 129]. In
addition, α -hemolysin activates the ADAM10 receptor with subsequent cleavage of E-
cadherin and decreased endothelial barrier integrity, which facilitates pathogen dissemination
in the host [139].

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The mode of action of the clostridial β -PFTs from the α -hemolysin family is likely
related to that of α -hemolysin, notably regarding the effects due to the formation of small
pores. Thereby, Beta-toxin associates with human umbilical vein endothelial cell membranes
in multimeric complexes [140], and forms cation selective channels in artificial phospholipids
bilayers [141]. Beta-toxin pore formation has also been evidenced in phosphatidyl choline-
cholesterol liposomes [142]. Beta-toxin induces swelling and lysis of the lymphocytic HL60
cell line, which are preceded by toxin oligomer formation (hexamer or heptamer) in
membrane lipid rafts, K^+ efflux, and Ca^{++} , Na^+ , and Cl^- influxes [143, 144]. The beta-
dependent K^+ loss in HL60 was found to trigger the activation of the p38 and JNK MAPK
pathways which could have a protective effect of host cell [145]. When injected
intradermally, Beta-toxin induces oedema and dermonecrosis, which seem to be mediated by
stimulation of sensory nerves containing tachykinins such as substance P and release of tumor
necrosis factor alpha ($\text{TNF}\alpha$) [143, 144].

4.2.3 – Role in the pathogenesis

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The clostridial heptameric β -PFTs of the α -hemolysin family are mainly involved in
intestinal diseases like necrotic enteritis (Table 1), while staphylococcal α -hemolysin causes
necrosis and abscesses in various organs. The role of beta toxin in the pathogenesis has been
established several decades ago, whereas NetB is a recently identified virulence factor in
necrotic enteritis in birds. In contrast, the involvement of delta toxin in natural disease is still
poorly understood. Delta toxin might have a synergistic effect with beta toxin, since both
toxins are produced together by certain *C. perfringens* type C strains [126].

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Beta-toxin is responsible for necrotic enteritis in young animals and in humans (Pigbel and Darmbrand), and in sheep enterotoxemia. Enteritis due to Beta-toxin are characterized by necrosis and inflammation of the intestinal mucosa with bleeding to the lumen [146]. Beta-toxin is very labile and sensitive to protease degradation. For this reason, the Beta-induced pathology is only observed in particular circumstances such as in newborns in which the protease activity of the digestive tract is low. The risk factors involved in human disease are low-protein diet inducing low trypsin activity in the intestine and consumption of sweet potatoes, which contain a trypsin inhibitor. The low-protease activity permits a high level of active toxin into the intestinal lumen.

The exact mode of action of beta toxin in the genesis of enteric necrotic lesions is not yet fully understood. Which are the intestinal target cells of beta toxin? In naturally occurring cases of necrotic enteritis in piglets and in on human patient, beta toxin has been found to bind to vascular endothelial cells in the intestinal mucosa. It is speculated that beta toxin binding to endothelial cells is an early event which subsequently induces vascular necrosis and then alteration of the intestinal mucosa [147-149]. In primary human and porcine endothelial cells, beta toxin causes the loss of the actin cytoskeleton and cell death, thus promoting alteration of the integrity of endothelial cell monolayer *in vitro* [150, 151]. Beta toxin forms pores in endothelial cell membranes leading to release of K⁺ and ATP, increase in cytosolic Ca⁺⁺, and then cell death by programmed necrosis subsequently to activation of a non yet defined cell signaling pathway [152]. It remains to elucidate how beta toxin reaches the vascular endothelial cells in the intestinal mucosa from the intestinal lumen, whether the vascular endothelial cells are the only intestinal target cells, and which are the cell surface receptor(s) and the exact mode of cytotoxicity.

According to epidemiological data, consensus beta2-toxin strains are mainly involved in piglet necrotic enteritis and in horse typhlocolitis, whereas atypical beta2 strains have a broader animal species distribution and its pathogenicity remains to be defined [153-158]. Interestingly, the *cpb2* gene of *C. perfringens* strains isolated from horses differs from that of strains from pigs by an adenine deletion downstream of the start codon resulting in a premature stop codon after only nine amino acid codons. Therefore, the equine strains do not produce beta2 (92% identity with consensus beta2) under standard culture conditions [159, 160]. However, antibiotics of the aminoglycoside family such as gentamycin and streptomycin are able to induce expression of *cpb2* through a frameshift process.

Beta2-toxin has been immunohistochemically localized in the intestinal wall of diseased horses [161] indicating that this toxin is directly implicated in the genesis of lesions.

1 The involvement of beta2-toxin in intestinal diseases in other animal species and humans is
 2 still discussed [158, 162-165]. Beta2 toxin might be an additional virulence factor in *C.*
 3 *perfringens* associated diarrhea in human [163, 164].
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5 Necrotic enteritis in chickens is an important economical disease in poultry industry.
 6 This disease was associated to *C. perfringens*, but the toxin and virulence factors responsible
 7 for the lesions remain controversial until the discovery of the new toxin NetB. Evidence that
 8 NetB is the main virulence factor involved in necrotic enteritis is based on: (i) a *C.*
 9 *perfringens netB* null mutant failed to cause experimental intestinal necrotic lesions, but was
 10 as virulent as the wild type strain when complemented with the *netB* gene [166], (ii) NetB is
 11 cytotoxic for a chicken epithelial cell line *in vitro* [166], (iii) most of strains isolated from
 12 necrotic enteritis outbreaks contain *netB* gene and non-necrotic enteritis derived isolates lack
 13 this gene [166-171], and (iv) only the *netB* positive isolate from a naturally occurring
 14 outbreak was able to reproduce experimental lesions [172].
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25 **5 – Clostridial intracellularly active toxins and pore formation**

26 Pore formation is not directly involved in the mode of action of intracellularly active
 27 toxins, but it is an essential step in the mechanism of translocation of the enzymatic
 28 component or enzymatic domain from the endosome to the cytosol, where they recognize and
 29 modify a specific intracellular target. Two main groups of intracellularly active toxins can be
 30 distinguished on the basis of their structure: the single chain protein toxins and the binary or
 31 multiple component toxins. Toxins of the two groups use distinct mode of pore formation: the
 32 single chain protein toxins form α -pores, whereas binary toxins exploit β -pores.
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42 **5.1 - Pore-forming domains of clostridial intracellularly active toxins**

43 Clostridial neurotoxins and large clostridial glucosylating (LCGT) toxins are single
 44 chain protein toxins, which enter target cells through a receptor-mediated endocytosis and
 45 deliver through the endosomal membrane an enzymatic domain into the cytosol.
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48 Clostridial neurotoxins, including botulinum neurotoxins (BoNTs) and tetanus
 49 neurotoxin (TeNT), retain a conserved overall structure consisting of three main domains: a
 50 C-terminal receptor binding domain (half C-terminal heavy (H) chain), a central translocation
 51 domain (half N-terminal H chain), and a N-terminal enzymatic domain (light (L) chain). The
 52 translocation domain (TD) contains two unusually long twisted α -helices, which are
 53 reminiscent of α -helical hairpin of some colicins or viral fusion proteins (Fig. 7A) (review in
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1 [173]. At the acidic pH of endosome (pH 5.3), TD inserts into the endosomal membrane and
2 forms small conductance channels (20-40 pS and estimated inner diameter of 15 Å). The
3 mode of passage of the L chain through the endosomal membrane remains unclear. The
4 unfold L chain at acidic pH seems not to be able to pass through these small channels, unless
5 several TDs cooperate to form larger channels. The fact that during the L chain translocation,
6 the Na⁺ conductance progressively increases, supports a passage through the TD channels.
7 Another possibility is a chaperone activity between TD and L chain including a partial
8 structure rearrangement (molten globule state) facilitating the exposition of hydrophobic
9 helices and their subsequent insertion into the membrane. However, no conformational
10 change of TD has been detected [173-176].

11 LCGTs contain a central hydrophobic domain (amino acids 956-1128 in *C. difficile*
12 toxin B), which is involved in pore formation at acidic pH. However, it is not yet established
13 that the translocation of the catalytic N-terminal domain (amino acids 1-543) exploits the
14 LCGT channels [177-179]. LCGTs might use a similar mechanism of translocation than that
15 of clostridial neurotoxins.

26 5.2 - Binding components of clostridial binary toxins

27 Binary toxins consist of two independent proteins including an enzymatic component
28 and a binding component (BC), which are encoded by distinct, yet adjacent, genes. BCs of
29 clostridial binary toxins (*C. perfringens* iota toxin, *C. botulinum* C2 toxin, *C. difficile*
30 transferase (CDT), and *Clostridium spiroforme* toxin) share a similar structural organization
31 to that of *Bacillus anthrax* toxins (protective antigen, PA) and *Bacillus* vegetative insecticidal
32 protein 1 (VIP1) [4, 180, 181]. The structures of clostridial and *Bacillus* BCs are reminiscent
33 of that of CDCs (Fig. 7B). Indeed, PA which is the prototype of BCs, consists in 4 domains
34 rich in β-strands highly related to those of PFO (Fig. 7B and 7C). The N-terminal domain
35 1 contains the binding sites for the enzymatic components, and the C-terminal domain 4
36 is involved in the recognition of the cell surface receptor. Domain 2 contains a long β-hairpin,
37 which assembles with adjacent β-hairpins in the oligomeric structure to form the β-barrel. No
38 specific function has been attributed to domain 3, which is the smallest one [182, 183].
39 However, BCs share functional similarities with β-PFTs of the aerolysin family. Thereby,
40 BCs are proteolytically activated by removing a N-terminal propeptide. But, the BC cleaved
41 propeptide is much more longer (20 kDa) than those of aerolysin family β-PFTs. In addition,
42 in contrast to CDCs and like aerolysin family β-PFTs, BCs use only one amphipathic β-hairpin
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1 from each monomer to built the β -barrel and form heptamers instead of large oligomers.
2 Furthermore, BC amphipatic β -hairpin share significant amino acid sequence with the
3 corresponding sequences of aerolysin family β -PFTs. For example the β -hairpin of Ib (BC of
4 iota toxin) shows 45% identity with that of ETX.
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7 8 9 **5.3 – Role in the pathogenesis**

10 Pore formation of the intracellularly active toxins is not directly involved in the
11 mechanism of pathogenesis. But, it is a prerequisite step allowing the internalization of the
12 enzymatic component or domain. BCs form no functional or only weakly active pores in the
13 plasma membrane. BCs drive the translocation of the corresponding enzymatic component or
14 domain at the acidic endosomal pH through the endosome membrane.
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20 Clostridial neurotoxins are responsible for severe neurological diseases: botulism and
21 tetanus [173, 184, 185].
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23 LCGTs produced by *C. difficile* (Toxin A and Toxin B) are the main etiological
24 virulence factors of pseudomembranous colitis and about 30% of the postantibiotic
25 diarrhea, which are the most frequent nosocomial intestinal diseases [186]. LCGTs of *C.*
26 *sordellii* and *C. novyi* are involved in gangrene, and *C. sordellii* is also an agent of
27 hemorrhagic enteritis and enterotoxemia in cattle [187-194].
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33 Clostridial binary toxins are involved in necrotizing enteritis and diarrhea in
34 animals and occasionally in humans. Iota toxin from *C. perfringens* E causes
35 enterotoxemia in calves and other young animals. *C. spiroforme* is responsible for
36 enteritis and death in rabbits and rarely in humans. *C. botulinum* C2 toxin induces
37 intestinal hemorrhagic lesions in avian [4, 181]. CDT from epidemic *C. difficile* strains is
38 considered as an additional virulence factor in pseudomembranous colitis and recurrent
39 post-antibiotic diarrhea [195].
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48 **6 - Evolution of pore-forming domains**

49 The structure relatedness between β -PFTs and BCs strongly suggests that all β -PFTs
50 and BCs have evolved from a common ancestor, possibly a transmembrane protein. BCs have
51 retained a core structure of β -PFTs, and they have acquired the ability to recognize and
52 translocate specific enzymatic components, whereas β -PFTs form pores in plasma membrane
53 of eukaryotic cells leading to drastic cellular effects (Fig. 8). It is intriguing that homologues
54 of aerolysin and CDC families are wide spread in all the kingdoms of life. As mentioned
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1 above, structural homologous proteins of aerolysin (hydralysins, enterolobin, LSL, ...) are
2 produced by plants, fungi and animals [80]. In addition, more than 300 proteins from diverse
3 groups of organisms share aerolysin domain similarity based on local sequence alignment and
4 phylogenetic analysis. It is hypothesized that these proteins derive from a common ancestor
5 probably in early bacterial lineages, which has been transmitted between organisms of
6 different phylum by horizontal gene transfer. This analysis suggests that at least six
7 independent transfer events have occurred between distantly related organisms including
8 between bacteria and eukaryotic cells [196]. The structural homology between MACPF and
9 CDC proteins restricted to the pore-forming domain, whereas the other domains are distantly
10 related [31], rather suggests a convergent evolution of eukaryote and prokaryote proteins of
11 these families to interact with the lipid bilayer.
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22 **7 – Concluding remarks**

23 Pore formation is a common mechanism of action for many bacterial toxins including
24 clostridial toxins. Disruption of the membrane integrity of cell host is a direct and an efficient
25 way for a pathogen to have access to indispensable nutrients and seems to be the ancestor
26 mode of action of most of bacterial toxins. Structural analysis put in light that all clostridial
27 PFTs derive probably from a common ancestor and retain a similar global mode of insertion
28 into lipid membrane including the formation of an amphipatic β -barrel, which inserts into the
29 lipid bilayer leading to a functional pore. During the evolution, certain PFTs have acquired
30 additional or more specialized function possibly in order to address specific requirements of
31 pathogens. Thereby, instead to interact with an ubiquitous receptor on cell surface and to form
32 large pores which abruptly impair the membrane integrity, heptameric PFTs target receptors
33 specific of some cell types and induce small pores with subsequent intracellular signaling
34 leading to specific response like attack of the nervous system, the vascular endothelial barrier,
35 or the intestinal epithelial barrier. A more specialized pore forming activity concerns the
36 intracellular active toxins. Binding components of binary toxins have evolved from β -PFTs to
37 selectively mediate the translocation of the enzymatic components through the endosomal
38 membrane at the acidic pH of endosome. In contrast, intracellularly active single chain
39 protein toxins retain a different mode of insertion into the lipid bilayer based on α -helices.
40 The selective pressure, which has controlled the evolution of β - and α -pore forming
41 structures, remains mysterious. We can hypothesize that β -pore formation, which results from
42 a more complex structure and mechanism than those of α -pore forming proteins, confer
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selective advantages in stability, efficiency in pore forming activity and beneficial cellular effects for the bacterial pathogens, notably in terms of bacterial growth and dissemination in the host.

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FIGURE LEGENDS

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4 **Figure 1.** Examples of bacterial α -pore-forming toxins (PFT). A) Colicins retain a conserved
5 structural organization consisting of a translocation domain, receptor-binding domain, and
6 catalytic or pore-forming domain, rich in α -helices. Colicin E3 (pdb 1JCH) is shown as an
7 example. B) The C-terminal pore-forming domain of colicin A (pdb 1 COL) consists of a
8 bundle of 10 α -helices. The hydrophobic H8 and H9 α -helices are in magenta. C) Diphtheria
9 toxin (pdb 1DDT) consists of a N-terminal catalytic domain (blue), translocation domain
10 (red), and receptor-binding domain (green). Hydrophobic H8 and H9 α -helices are in
11 magenta. D) *Escherichia coli* hemolysin E, monomer and dodecamer assembled in a (pdb
12 1QOY). Figures were produced with the program MacPyMOL.

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22 **Figure 2.** General model of β -pore-forming toxin (β -PFT) mechanism of action. The secreted
23 soluble monomers recognize specific cell surface receptor(s), assemble, oligomerize, unfold
24 amphipatic β -hairpin(s), which form a prepore and then insert into the membrane.

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30 **Figure 3.** Structure of Perfringolysin (PFO) and pore formation. A) Structural PFO
31 organization in 4 domains (pdb 1PFO). B) View of domain D3 with the transmembrane
32 hairpins (TMH) in the helical conformation and the β 5 strand bended on β 4 strand. C)
33 Schematic representation of two assembled PFO monomers; Binding of domain D4 to
34 cholesterol triggers a conformational change relayed by the undecapeptide segment to domain
35 D3 and leading to displacement of β 5 strand from β 4 strand thus allowing assembly of two
36 monomers and unfolding of α -helices of domain D3 in two amphipatic β -hairpins. D)
37 Schematic representation of a PFO pore inserted into the lipid bilayer. Subsequently to a
38 vertical collapse of oligomerized PFO molecules, the prepore inserts into the lipid bilayer.
39 Figures were produced with the program MacPyMOL.

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50 **Figure 4.** Main steps in the pathogenesis of clostridial gangrenes.

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54 **Figure 5.** Structure of aerolysin and related clostridial β -PFTs: *C. perfringens* epsilon toxin
55 and *C. perfringens* enterotoxin. (A) aerolysin monomer (Pdb, 1PRE) and schematic
56 representation of the pore formation according to [101]. Binding receptor sites are localized
57 in domains 1 and 2. Upon binding to their receptor, monomers heptamerize and form a
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prepore showing an inverted mushroom shape, of which domains 1 and 2 constitute the cap. Then, the stalk, which is constituted of domains 3 and 4, rotates and completely collapses, and the β -barrel extends in the opposite orientation to that of the stalk in the prepore conformation. Two monomers (red and blue) are shown in the heptameric structure. Structures of (B) *C. perfringens* epsilon toxin monomer (Pdb, 1UYJ), and (C) *C. perfringens* enterotoxin (CPE) Pdb, 2XH6, 2QUO). The receptor binding domains are in green, the domains containing the pre-stem loop (red) are in yellow, and the domains, which contain the propeptide and which are involved in the control of oligomerization, are in blue. Figures were produced with the program MacPyMOL.

Figure 6. Structure of *Staphylococcus aureus* alpha hemolysin (Pdb 7AHL, 4IDJ), and related clostridial β -PFTs: *C. perfringens* delta toxin (Pdb, 2YGT), and NetB (410N, 4H56). The receptor binding domain (rim) is in green, the domain (stem) which unfolds in amphipatic β -hairpin in the open conformation and forms the β -barrel is in red, and the domain forming the cap of the mushroom shaped oligomer is in blue. Figures were produced with the program MacPyMOL.

Figure 7. Pore forming domains of clostridial intracellularly active toxins. (A) Botulinum neurotoxin type A (BoNT/A) (Pdb, 3BTA) is a single chain protein toxin which contains two long α -helices in its translocation domain located in the N-terminal half of the heavy chain (H_N) and which mediates the translocation into the cytosol of the catalytic light chain (L). (B) Binding components of the clostridial binary toxins such as C2-II (Pdb, 2J42), the binding component of *C. botulinum* C2 toxin, are structurally related to the protective antigen (PA) (Pdb, 3QBB) of *Bacillus anthracis* toxins, and show a common structural organization with that of the pore-forming toxin, PFO. However, domain 2 of PA or C2-II contains only one β -hairpin forming the β -barrel, instead of two trans membrane hairpins in domain 3 of PFO. (C) PA prepore bound to receptor. PA heptamer with each monomer bound to the domain VWA of the receptor CMG2 (Pdb, 1TZN). One monomer is coloured in 4 domains and one receptor molecule is in brown. In the prepore state the β -barrel is not yet formed. Figures were produced with the program MacPyMOL.

Figure 8. Hypothetical evolutionary lineages of bacterial pore-forming toxins (PFTs) toxin genes. PFTs likely derive from a common ancestor, probably a transmembrane

1 protein ancestor. Except clostridial single chain protein toxins, which use α -helices to
2 form a pore, clostridial PFTs belong to the β -PFT family. The cholesterol dependent
3 cytolysins (CDC) form large pores, whereas the heptameric β -PFTs (staphylococcal α -
4 hemolysin family and aerolysin family) induce small pores. Interestingly, binary toxins
5 produced by certain *Clostridium* and *Bacillus*, seem to have emerged from a convergent
6 or cross evolution between β -PFTs and toxins having an enzymatic activity. Binding
7 components, which are structurally related to β -PFTs of the CDC family and retain
8 similarity with aerolysin toxins, have evolved to specifically internalize an enzymatic
9 protein into cell through a pore-forming mechanism. The single chain protein
10 intracellularly active toxins probably derive from an enzyme ancestor and have acquired
11 by gene duplication/modification or by fusion with other gene precursors new
12 functional domains mediating the transport into the cytosol of target cells. The single
13 chain protein intracellularly active toxins use α -helices for the translocation of the
14 enzymatic domain across the endosomal membrane.
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Clostridium species	Toxin	Receptor	Associated disease	Target species	References
Cholesterol-dependent cytolysin family					
<i>C. perfringens</i>	Perfringolysin (or theta toxin)	Cholesterol	Myonecrosis, gangrene Associated toxin: <i>C. perfringens</i> alpha toxin, gelatinase, neuraminidases	Human, animals	[65]
<i>C. histolyticum</i>	Histolyticolysin	Cholesterol	Myonecrosis, gangrene Associated toxins: collagenases	Human, animals	[18]
<i>C. novyi</i>	Novyilysin	Cholesterol	Myonecrosis, gangrene Associated toxins: <i>C. novyi</i> alpha toxin (TcnA), phospholipase	Human, animals	[18, 197]
<i>C. septicum</i>	Septicolysin	Cholesterol	Myonecrosis, gangrene Associated toxin: <i>C. septicum</i> alpha toxin	Human, animals	[64, 197]
<i>C. sordellii</i>	Sordellilysin	Cholesterol	Myonecrosis, gangrene Associated toxin: <i>C. sordellii</i> lethal toxin (TcsL), <i>C. sordellii</i> hemorrhagic toxin (TcsH), phospholipase	Human, animals	[64, 197]
<i>C. chauvoei</i>	Chauveolysin	Cholesterol	Myonecrosis, black leg Associated toxins: <i>C. chauvoei</i> cytolysin A (CctA), neuraminidase	Mainly cattle	[198, 199]
<i>C. bifermentans</i>	Bifermentolysin	Cholesterol	Myonecrosis, gangrene in association with other	Human,	[18, 197]

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			agents of gangrene Associated toxin: phospholipase	animals	
<i>C. botulinum</i>	Botulinolysin	Cholesterol	Botulism Associated toxin: botulinum neurotoxin	Human, animals	[70, 71]
<i>C. tetani</i>	Tetanolysin	Cholesterol	Tetanus Associated toxin: tetanus neurotoxin	Human, animals	[69]
Aerolysin family					
<i>C. perfringens</i>	Epsilon toxin	MLA HAVCR1?	Enterotoxemia	Sheep, goat, cattle	[106, 200]
<i>C. perfringens</i>	Enterotoxin	Claudin	Food borne poisoning, sporadic diarrhea Diarrhea	Human Pig	[115, 122, 201, 202]
<i>C. septicum</i>	Alpha-toxin	GPI-anchored protein	gas gangrene gangrene of intestinal wall enterotoxemia (braxy/bradsot)	Human, animals Human Sheep	[203, 204] [205, 206]
<i>Staphylococcus aureus</i> alpha-toxin family					
<i>C. perfringens</i>	Delta toxin	GM2	?	Human ? animals ?	[126, 207]
<i>C. perfringens</i>	NetB	?	Necrotizing enteritis	Chickens	[166, 168- 171]
<i>C. perfringens</i>	Beta toxin	?	Necrotizing enteritis (Pig Bel)	Human	[208]

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			Necrotizing enteritis Enterotoxemia (struck)	Piglet, calve Sheep	[209]
<i>C. perfringens</i>	Beta2 toxin	?	Necrotizing enteritis Typhlocolitis	Piglet Horse	[153, 154, 156, 157].
<i>C. chauvoei</i>	CctA	?	Gangrene, black leg	cattle	[198]

Table 1. Clostridial pore-forming toxins, receptor, and associated pathology.

Toxin	Pore size (nm)	Number of monomers forming the pore	Channel conductance (pS) In 1M KCl	Pore selectivity	Toxicity Mouse lethal dose ⁵ µg/kg by intraperitoneal route	References
Perfringolysin	25 - 45	40-50	4000-6000 ^{&}		13-16 ²	[30, 210]
Aerolysin	0.7 - 1	7	650	anionic	10	[211]
Epsilon toxin	1	7	550	anionic	0.07	[212]
<i>C. septicum</i> alpha toxin	1.3 – 1.6	7	1250	anionic	0.04 – 0.06 ²	[103]
CPE	0.6 - 0.7	7?	565 ³	cationic	80	[213]
<i>S. aureus</i> α-hemolysin	0.8 - 1	7 (6 to 8)	820	anionic	0.04 – 0.06 ²	[211]
NetB	?	7	325 ³	cationic		[124]
Delta toxin	4	7?	130	anionic	5 ²	[126]
Beta toxin	1.2	7?	550 60 - 110 ⁴	cationic	<0.4	[126, 141]
Protective antigen	1.2	7 - 8	165	cationic		[214, 215]
Iota toxin binding component (ib)	≈ 1	7	85	cationic		[216]
C2 toxin binding component (C2-II)	1 - 2	7	150	cationic		[216, 217]

¹ in 0.2 M NaCl

² intravenous administration

³ in 0.1 M KCl

⁴ in 0.1 M NaCl

⁵ according to [218]

Table 2. Main properties of channel activity of clostridial and related pore-forming toxins.

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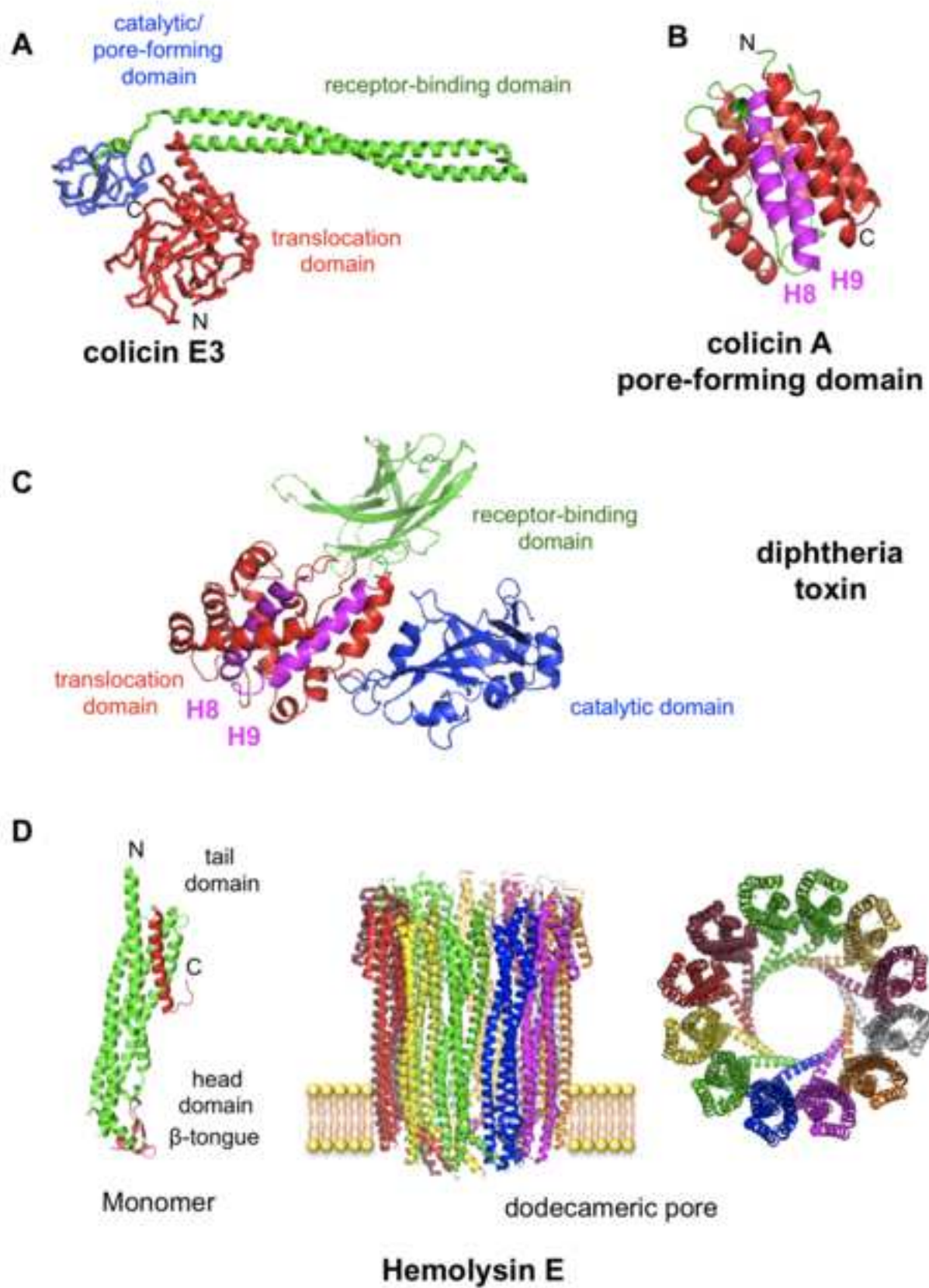


Figure 1

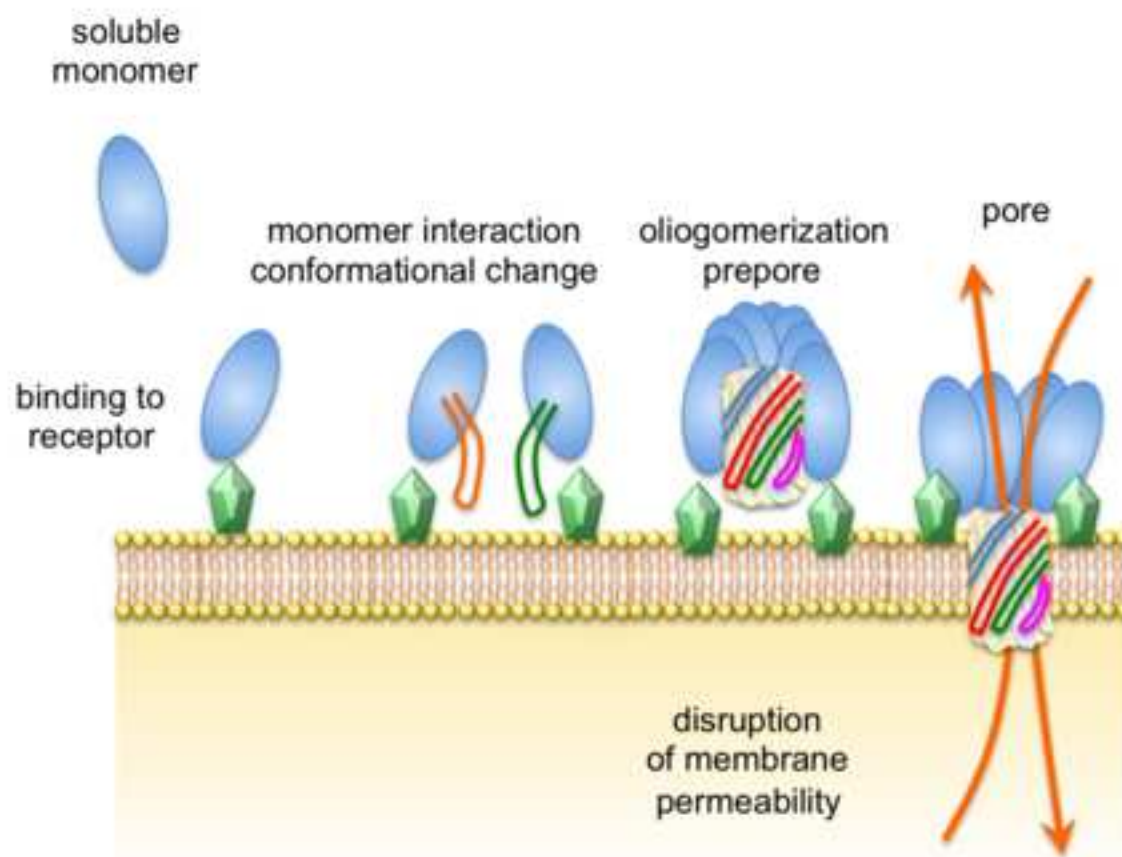


Figure 2

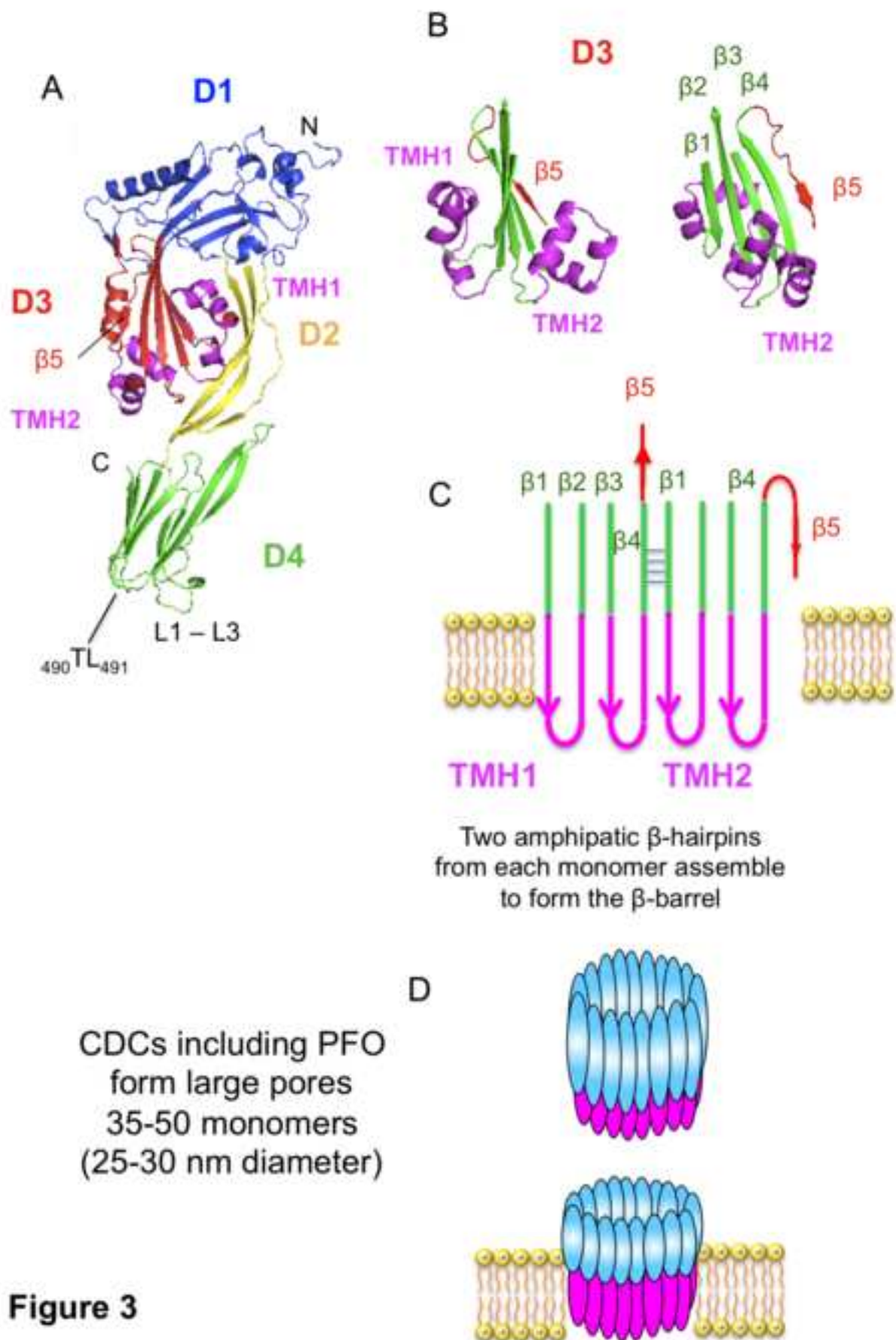


Figure 3

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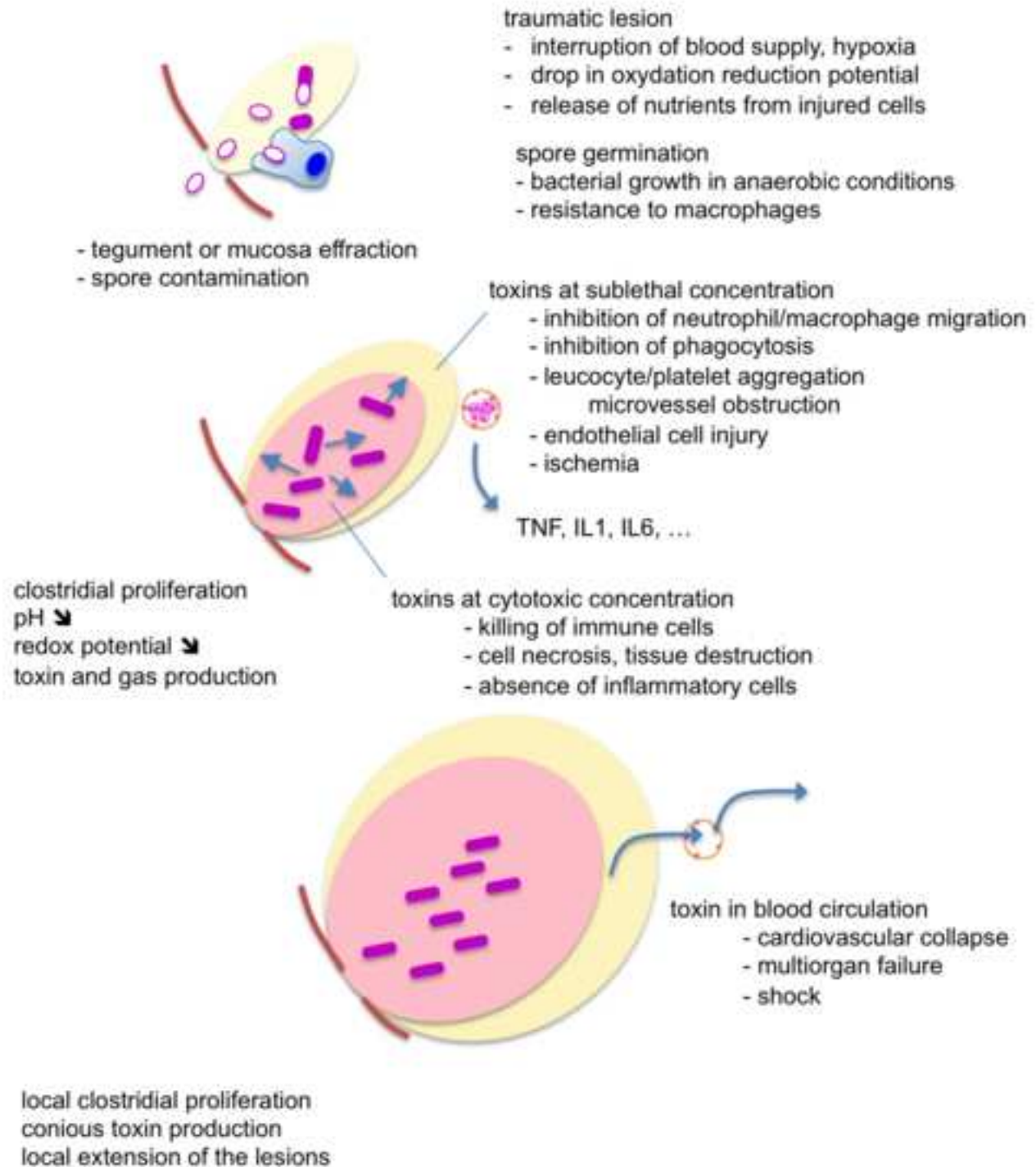
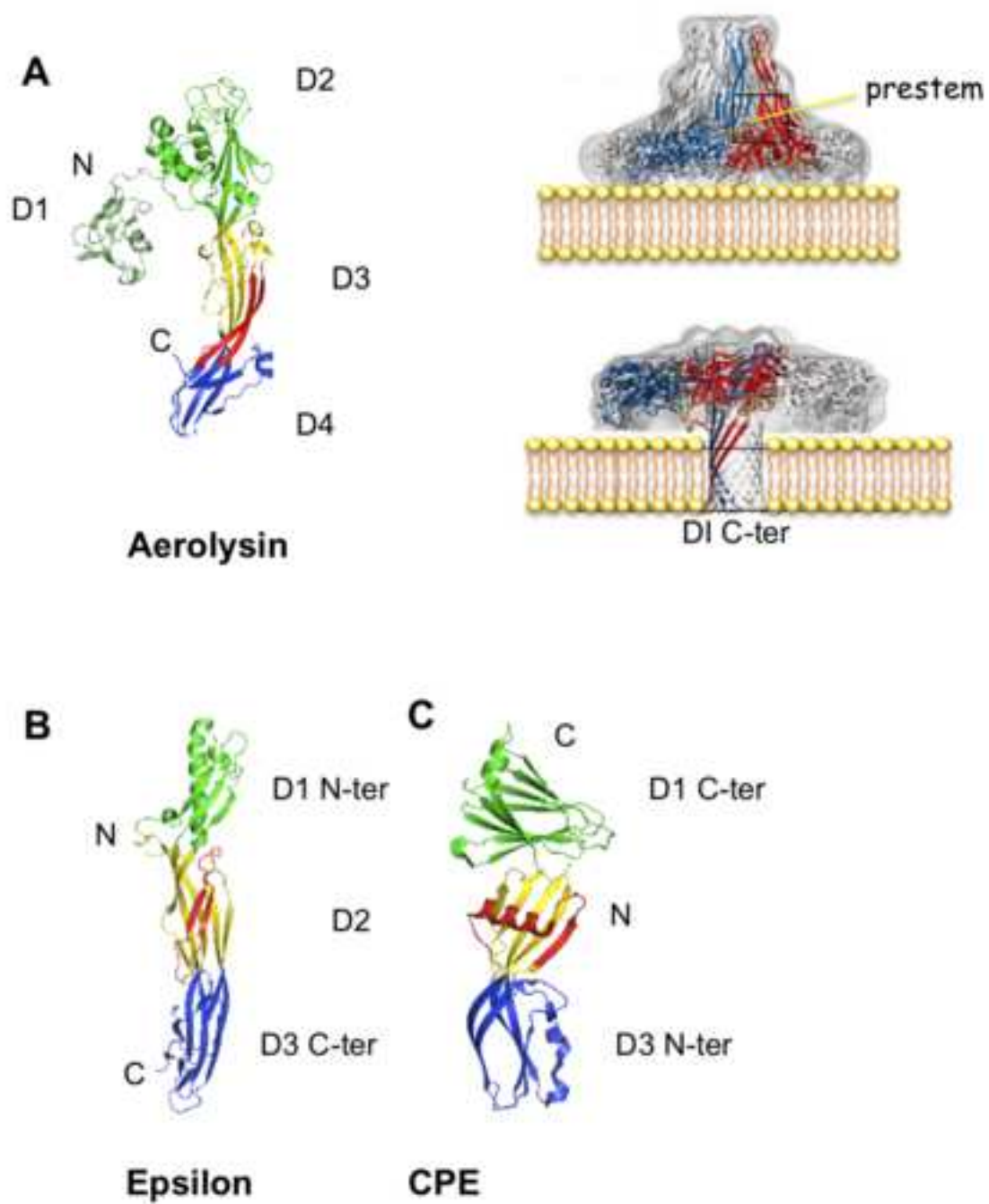


Figure 4

**Figure 5**

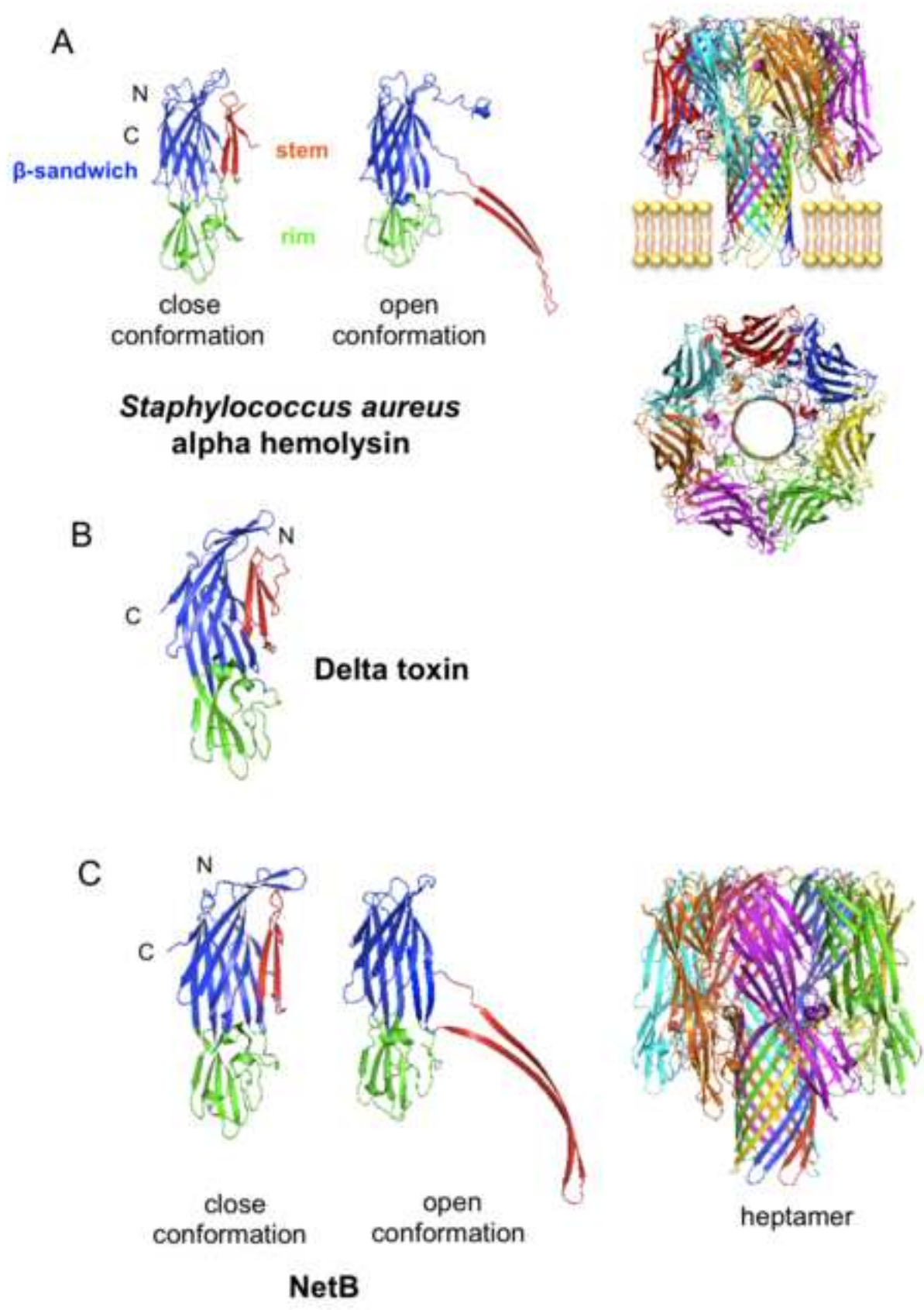


Figure 6

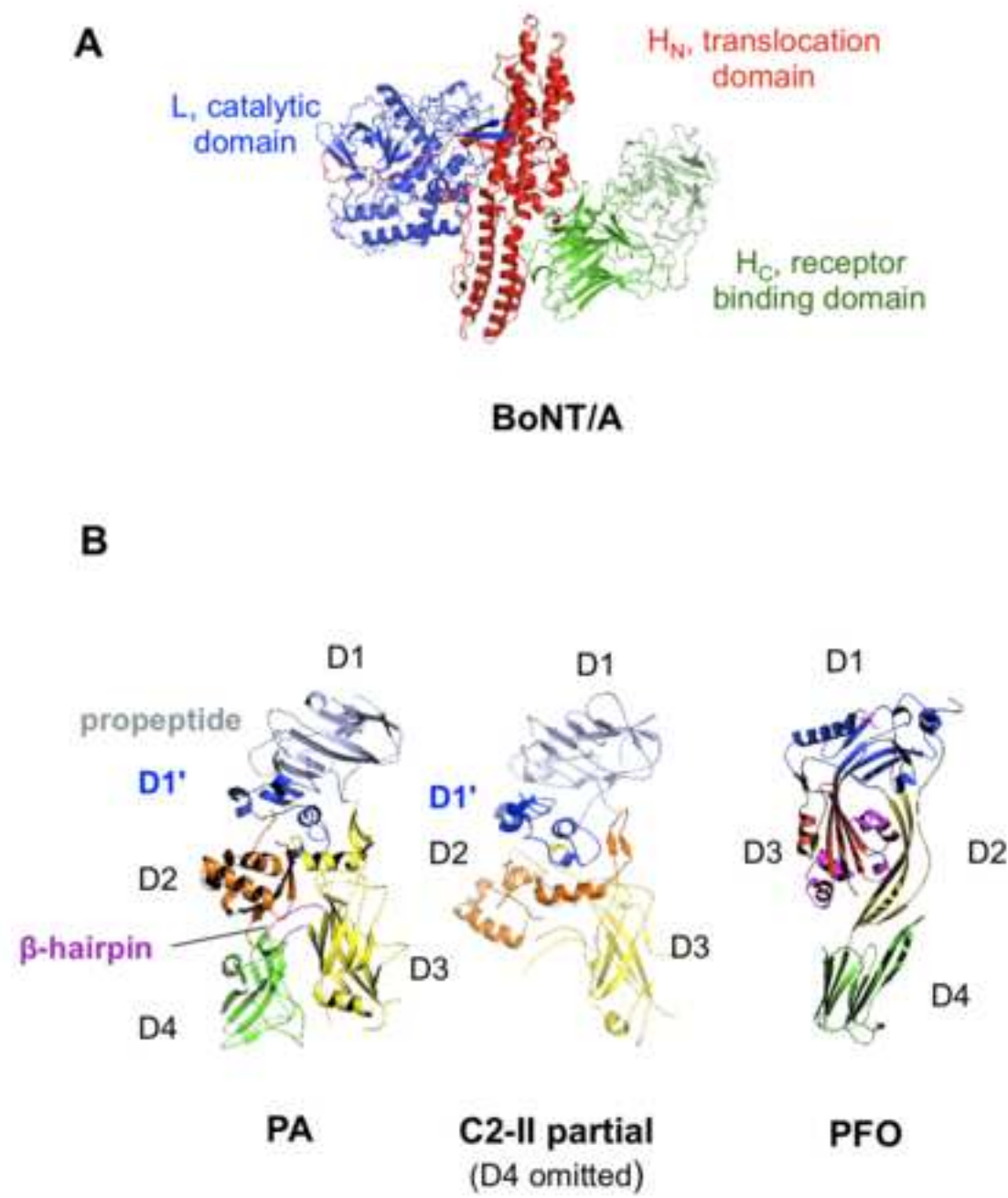
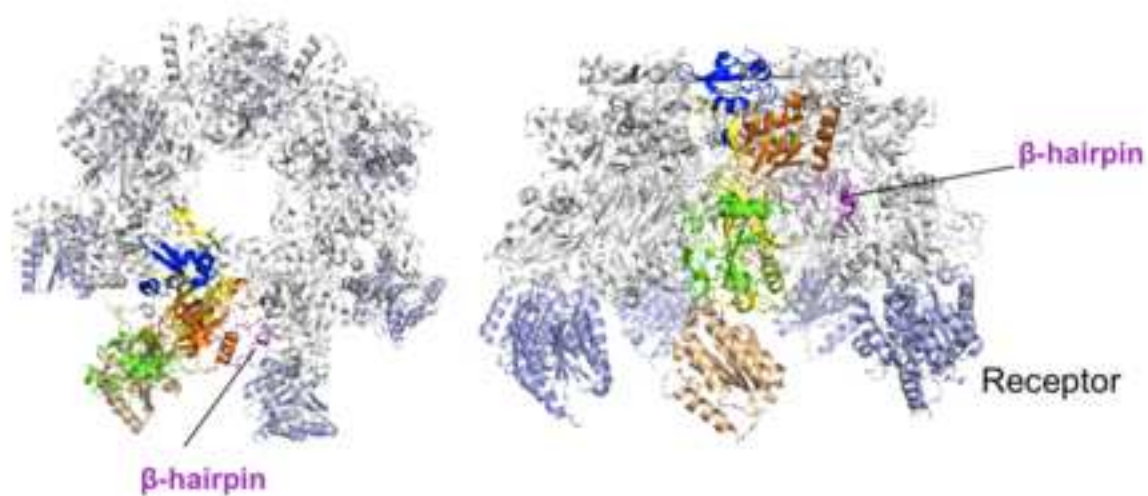


Figure 7

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PA heptamer

Figure 7 (followed)

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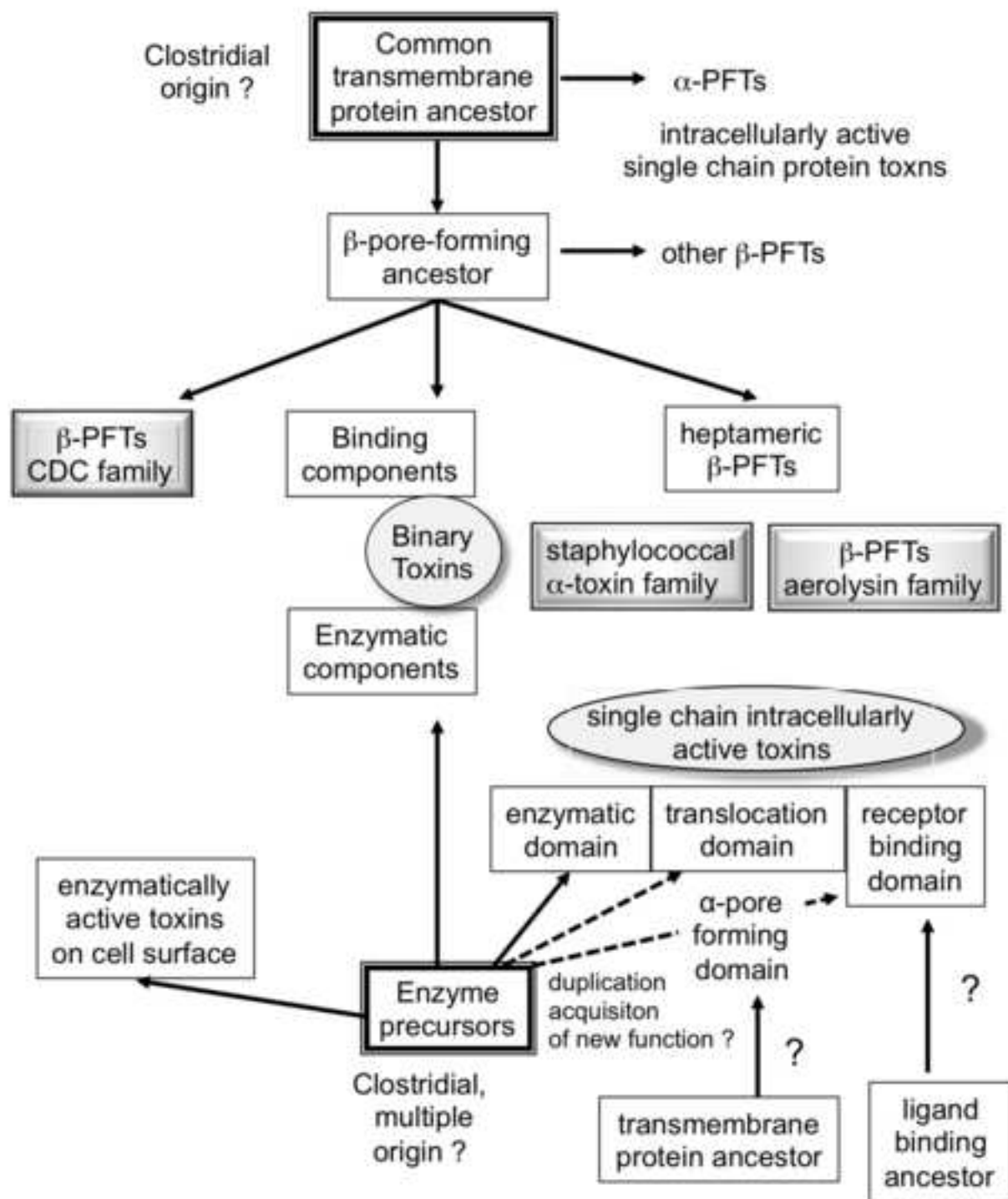


Figure 8