The Aerolysin-Like Toxin Family of Cytolytic, Pore-Forming Toxins

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Abstract: Pore-forming toxins (PFTs) represent the largest known group of bacterial protein toxins to date. Membrane insertion and subsequent pore-formation occurs after initial binding to cell-surface receptor and oligomerization. Aerolysin, a toxin produced by the Gram-negative bacterium *Aeromonas hydrophila* and related species, belongs to the PFT group and shares a common mechanism of action involving β-barrel structures resulting from the assembly of β-hairpins from individual toxin monomers into a heptamer. Aerolysin is also the name given to structurally and mechanistically related toxins called the aerolysin-like toxin family. A universal characteristic of this toxin family involves the diverse life forms that synthesize these proteins throughout Nature. Examples include: 1) epsilon-toxin and *septicum*-alpha-toxin produced by anaerobic, Gram-positive *Clostridium species*; 2) enterolobin by the Brazilian tree *Enterolobium contortisiliquum*; 3) a mushroom toxin *Laetiporus sulphureus* lectin (LSL); 4) mosquitocidal toxins (Mtxs) from the Gram-positive bacteria *Bacillus sphaericus* and parasporine-2 from *Bacillus thuringiensis;* and 6) hydralysins from the tiny aquatic animal *Chlorohydra viridis*. The following review provides an overview of the different members within the aerolysin-like toxin family.

INTRODUCTION

Many pathogens produce pore-forming toxins (PFTs), which represent more than one third of all bacterial toxins. Bacteria such as *Staphylococcus* secrete PFTs to alter host cell membranes and trigger nutrient release to stimulate bacterial growth. In addition, cytotoxicity mediated by PFTs counteracts the host defences based on phagocytosis and other immune responses. Indeed, the killing of cells like leukocytes or macrophages facilitates tissue invasion by PFT-producing bacteria [1]. Disruption of cell barriers by PFT-producing bacteria also facilitates their diffusion throughout the host organism. Production of PFTs is common in Gram-negative and Gram-positive bacteria, as well as in higher plants, fungi, and even animals. Among "higher" organisms, PFTs may serve as a defence system for some plants to avoid attacking herbivores or parasites as well as assist certain animals in hunting or digestion.

Members of the aerolysin-like toxin family are synthesised by a very diverse group of organisms composed of: 1) Gram-negative bacteria, such as *Aeromonas hydrophila* and related *Aeromonas species*; 2) epsilon- and alpha-toxin respectively from the anaerobic, Gram-positive *Clostridium perfringens* and *Clostridium septicum*; 3) enterolobin from *Enterolobium contortisiliquum*, a Brazilian tree; 4) insecticidal toxins, Mtx and parasporin-2, from *Bacillus sphaericus and Bacillus thuringiensis,* respectively;

5) *Laetiporus sulphureus* lectin (LSL) from the mushroom *L. sulphureus*; and 6) hydralysins from aquatic animals in the phylum *Cnidaria*. Despite the fact that these PFT producers originate from diverse kingdoms represented by plants, fungi and bacteria, all of the PFTs share remarkable sequence identity with aerolysin. It is striking that high sequence homology occurs between aerolysin, *septicum*-alpha-toxin (72% similarity [2]), and enterolobin (45% identity and 59% similarity, respectively [3, 4]) (Fig. **1A**). Despite lower sequence homology of aerolysin with epsilon-toxin (identity 13.9 % [5]), LSL lectin (similarity <20% [6]), and parasporin-2 (identity 25.7 % [7]) (Fig. **1B**), these proteins are members of the same toxin family because of structural similarities between their domains. In addition to structure, another common feature of all these toxins is their mode of action. They all act as cytolysins via pore-formation.

PFTs use two general mechanisms to form pores in cell membranes, either through insertion of amphipathic α-helices or ß-hairpins organized into a ß-barrel [1]. Thus, PFTs are respectively divided into two main groups called α-PFT and β-PFT. Members of the aerolysin-like toxin family use amphipathic ß-hairpins and are therefore rich in ß-sheets. Although ß-PFTs share a common mode of pore formation, several types can be distinguished according to their receptor, number of monomers forming oligomers, pore size, and functionality. Like other ß-PFTs, aerolysin-like toxins bind to a cell-surface receptor, oligomerize, and generate ß-hairpins from 6 to 7 individual monomers to form the pore (Table **1**). This is opposed to cholesterol-dependent cytolysins (CDCs) which use 40-50 monomers [8] that form a ß-barrel structure, insert into the lipid bilayer, and subsequently generate a channel. Furthermore, aerolysin-like toxins form, in comparison to CDCs, small transmembrane

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Aerolysin    -------------AEPVYPDQLRLFS--LGQGVCGDKYRPVNREEAQSVKSNIVGMMGQW
Septicum     -----------------------------MSKKSFAKKVICTSMIAIQCAAVVPHV
Enterolobin  TQRDTLTKGMTQGQIVAGPDDTKIDQSIMDQGVCATIALPFDIHVAQSVKN-VMFMFAWT

Aerolysin    QISGLANG--WVIMGPGYNGEIK--------PGTASNTWCYPTNPVTGEIPTLSALDIPD
Septicum     QAYALTN------LEEGGYANHN--------NASSIKIFGYEDN----------------
Enterolobin  QKHGLAVNNLVILGVPGYLNMWKRFGESTDEPSESSKRCCYEKP----TDPVSGEIVTPA

Aerolysin    GDEVDVQWRLVH--DSANFIKPTSYLAHYLGYAWVGGNHSQYVGEDMDVTRDGDG-----
Septicum     ---EDLKAKIQ--DPE-FIRNWANVAHSLGFGWCGGTANPNVGQGFEFKREVGAGGKVS
Enterolobin  FDPADEISETTHQEESADIKPVRLLDARLGYTWVTQNHSWIVAYSDEPIKDGNE-----

Aerolysin    WVIRG---NNDGGCDGYR-CGGDKTAIKVSNFAYNLDPDSFKHGDVTQSDRQLVKTVVGWA
Septicum     YLLSARYNPNDPYASGYR-AKDRLSMKISNVRFVIDNDSIKLGTPKVKKLAPLNSASFDL
Enterolobin  -VVTLRNIGNNNFCDALRKCTDKSACIHQYLQFAWN----SFGDPTVRTRQVVFEVVGWA

Aerolysin    VNDSDTPQSGYDVTLRYDTATNWS-----KTNTYGLSEKVTTKNKFKWPLVG----ETEL
Septicum     INESKT-ESKLSKTFNYTTSKTVS-----KTDNFKFGEKIGVKTSPKVGLEAIADSKVET
Enterolobin  DSDTTN-NNSDTLFYRTTQADNWQVLTESKTNTIGLAEVDWFNFKYET--------KQEV

Aerolysin    SIEIAANQSWASQNGGSTTTSLSQSVRPTVPARSKIPVKIELYKADISYPYEFKADVSYD
Septicum     SFEFNAEQGWSNTNSTTETKQESTTYTATVSPQTKKRLFLDVLGSQIDIPYEGKIYMEYD
Enterolobin  SIEIARQTWTSTLNAGVSTVSR----VPKVP--IKVKINVDTFLKVLFAVYKADLYDIFL

Aerolysin    LTLSGFLRWGGNAWYTHPDNRPNWNHTFVIGPYKDKASSIRYQWDKRYIPGEVKWWDWNW
Septicum     IELMGFLRYTGNAREDHTEDRPTVKLKPGK-NGMSAEEHIKDLYSHKNIN-GYSEWDWKW
Enterolobin  QFASVFKKGGNRQYWLNYTQEPNTKSNYNHKIYNDKIRYLQEDVQVHPNETQSKWWWWSF

Aerolysin    TIQQNGLSTMQNNLARVLRPVRAGITGDFSAESQFAGNIEIGAPVPLAADSKVRRARSVD
Septicum     VDEKFGYLFKNSYDALTSRKLGGIIKGSFTNINGTKIVIREGKEIPLP-DKKRRGKRSVD
Enterolobin  KK----VALTTMTVVALTRFKASGINGQYISARQFSGGETVSPYRLAAPFDSCLWRRSPN

Aerolysin    G-----AGQCLRLEIPLDAQELSGLGFNNVSLSVTPAANQ-----
Septicum     SLDARLQNEGIRIEN-IETQDVPGFRLNSITYNDKKLILINNI--
Enterolobin  P-----LGTDG-LKVALAHHPLAGASNRNKLRVSIPAQCICSITA
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B

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ETX        ------------------------------MKKNLVKSLAIASAVISIYSIVNIVSP-
LSLa       ------------------------------MTDIYTPPEGLYFRLLGFASRQVIFAR-
Aerolysin  AEPVYPDQLRLFSLGQGVCGDKYRPVNREEAQSVKSNIVGMMGQWQISGLANGWVIMGPG

ETX        -----------------TNVIAKEISNTVSNEMSKKASYDNVDTLIEKGRYNTK------
LSLa       -----------------NSPSPDVGLSPVNDQATDQYFSLIYGTGEHAGLYAIK------
Aerolysin  YNGEIKPGTASNTWCYPTNPVTGEIPTLSALDIPDGDEVDVQWRLVHDSANFIKPTSYLA

ETX        ------------YNYLKRMEKYYPNAMAYFDKVTINPQGNDF--------------YINN
LSLa       ------------SKATGKVLFSRRPAEPYVGQIDGDGR--------------------Y
Aerolysin  HYLGYAWVGGNHSQYVGEDMDVTRDGDGWVIRGNNDGGCDGYRCGDKTAIKVSNFAYNLD

ETX        PKVELDGEPSMNYLEDVYVGKALLTNDTQQEQKLKSQSFTCKNTDTVTATTTHTVGTSIQ
LSLa       PDNWFKIEPGKTYLSKYFRLVQPSTGTALVSRTHLQPYFWNHPQTEVFDDQYFTFLFEDM
Aerolysin  PDSFKHGDVTQSDRQLVKTVVGWAVNDSDTPQSGYDVTLRYDTATNWSKTNTYGLSEKVT

ETX        ATAKFTVPFNETGVSLTTSYSFANTNTNTNSKEITHNVPSQDILVPANTTVEVIAYLKKV
LSLa       SIDKIEYDLKD-GRILSSTPNVLATQTLENTSSQTQEMSFN--LSQTLTQTSTFAYTAGF
Aerolysin  TKNKFKWPLVGETELSIEIAANQSWASQNGGSTTTSLSQSVRPTVPARSKIPVKIELYKA

ETX        NVKGNVKLVGQVSG-------------------------------------------SEWG
LSLa       TIAVGTAFKAGVPI-------------------------------------------FAET
Aerolysin  DISYPYEFKADVSYDLTLSGFLRWGGNAWYTHPDNRPNWNHTFVIGPYKDKASSIRYQWD

ETX        EIPSYLAFPRDGYKFSLSDTVNKSDLNEDG----------------TININGKGNYSAVM
LSLa       EFKVDISVDN-QWNWGEENTFSKTYTATFS----------------VRAGPGETVKAVST
Aerolysin  KRYIPGEVKWWDWNWTIQQNGLSTMQNNLARVLRPVRAGITGDFSAESQFAGNIEIGAPV

ETX        GDELIVKVRNLNTNNVQEYVIPVDKKEKSNDSNIVKYRSLYIKAPGIK--
LSLa       VDSGIINVPFTAYLSSKSTGFEVTTEGIWRGVSSWDLRHTLTSVTA----
Aerolysin  PLAADSKVRRARSVDGAGQGLRLEIPLDAQELSGLGFNNVSLSVTPAANQ
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Fig. (1). Sequence alignment of aerolysin-like toxin family members.

A) Alignment of aerolysin with *septicum*-alpha-toxin septicum and enterolobin, sharing both high sequence identity and similarity (*septicum*-alpha-toxin 72% similarity [2]; and enterolobin, 59% similarity / 45% identity). Numbering refers to the enterolobin sequence.

B) Alignment of aerolysin with epsilon-toxin ETX and LSLa. Despite lower sequence homology (epsilon-toxin 13.9% identity, [5] and LSL similarity below <20%, [6]), structural similarities can be observed. Numbering refers to the aerolysin sequence. Strictly conserved regions are shaded in black, and highly conserved regions are light grey.

channels that result from clustering of receptor-bound toxin molecules and oligomerization, followed by insertion into the cell membrane and pore formation. Lipid or protein receptors localized into distinct microdomains facilitate such mobility on cell membranes [1].

Because of the diverse ancestry of PFT-producing organisms, the aerolysin-like toxin family is one of the most phylogenetically-diverse families of PFTs [9]. Resolution of structures that include identification of functional domains within aerolysin, epsilon-toxin, and related toxins [5, 10, 11] allows deeper insight into the structures and mechanisms employed by this toxin family. We now present the most recent data available regarding the aerolysin-like toxin family that includes structures and mechanisms of action.

STRUCTURAL BASIS OF ACTIVITY AND MECHANISM OF ACTION FOR AEROLYSIN, THE PIONEER MEMBER OF THE AEROLYSIN-LIKE TOXIN FAMILY

Aerolysin is the best-studied molecule of the PFT family. It was the first member associated with cytotoxicity; therefore, aerolysin is commonly used to explain the general mode of action for this toxin family. Aerolysin was discovered and partially purified from the ubiquitous, Gram-

Table 1.   Main Characteristics of Aerolysin-like Toxin Family Members

	Aerolysin (*Aeromonas species*)	**Septicum-α-Toxin** (*Clostridium Septicum*)	**Enterolobin** (*Enterolobium Contortisiliquum*)	**Epsilon-Toxin Type B and D** (*Clostridium Perfringens*)	**LSL** (*Laetiporus Sulphureus*)	**MTXs** (Mtx2/Mtx 3) *Bacillus Sphaericus*	**Hydralysins** (*Chlorohydra Viridis*)	**Parasporin-2** **Bacillus Thuringiensis**
Size in kDA: (monomer unprocessed)	52	47.5	55	32.5	35	31.8/35.8	27	37
Propeptide:	yes, N-terminal	yes, C-terminal	unknown	yes, N- and C-terminal	unknown	possibly	unknown	Yes, N- and C-terminal
Pore formation:	yes	yes	possibly	yes	possibly	possibly	yes	possibly
Oligomerization (number of monomers):	7	6,7	unknown	7	4,6	unknown	possibly	unknown
Channel conductance G [pS]:								
0.1 M KCl	72	175	n.m	60	n.m	n.m	n.m	n.m
0.3 M "	220	450	n.m	160	n.m	n.m	n.m	n.m
1 M "	650	1250	n.m	550	n.m	n.m	n.m	n.m
3 M "	1700	3750	n.m	1700	n.m	n.m	n.m	n.m
P_{cation}/P_{anion}:	0.21 (-24 mV) anion selective	0.38 (-14 mV) anion selective	n.m	0.3 (-19 mV) anion selective	n.m	n.m	n.m	n.m
Pore size:	0.7 nm	1.3-1.6 nm	unknown	1 nm	>3.8 nm	unknown	1.2 nm	unknown
Receptor	GPI-anchored proteins	GPI-anchored proteins, including human folate receptor (hFR), Thy-1 (CD90), SAG family	unknown	membrane protein, not GPI-anchored	unknown, contains N-acetyllactosamine glycoconjugates	unknown	unknown	unknown
Target cells	GPI-anchored protein expressing cells	GPI-anchored protein expressing cells	arthropod and mammalian cells	only a few cell lines including kidney cells (MDCK, mpkCCD$_{C14}$, human renal leiomyoblastoma, and G-402 cells) and glutamatergic neurons	unknown	mainly insect cells, larvae of the genus *Culex*, *Aedes*, and *Anopheles*	arthropod and eukaryotic cells	Liver and colon cancer cells and cell lines (HepG2, CACO-2) also human leukemia T-cell lines (Molt-4, Jurkat, Hl-60)

n. m. Means not Measured.

Table 1. Contd….

	Aerolysin (*Aeromonas species*)	Septicum-α-Toxin (*Clostridium Septicum*)	Enterolobin (*Enterolobium Contortisiliquum*)	Epsilon-Toxin Type B and D (*Clostridium Perfringens*)	LSL (*Laetiporus Sulphureus*)	MTXs (Mtx2/Mtx3) *Bacillus Sphaericus*	Hydralysins (*Chlorohydra Viridis*)	Parasporin-2 Bacillus Thuringiensis
Cytotoxicity:	yes	yes	yes	yes	yes	yes	yes	yes
Hemolysis:	yes	unknown	yes	mainly	yes	unknown	yes	no
Apoptosis/Necrosis	apoptosis	necrosis	unknown	apoptosis/necrosis	unknown	unknown	unknown	unknown

negative fresh water bacterium *A. hydrophila* in 1975 by Bernheimer *et al.* [12]. The same toxin was later found to be produced by other species [13] (Table **1**). Members of *Aeromonas* can be pathogenic to a broad spectrum of different hosts including fish, amphibians, reptiles, and mammals. Aerolysin from the different *Aeromonas* species share a high level of sequence identity [14-18]. In humans, aerolysin is mainly associated with gastrointestinal diseases, but the toxin has also been associated with wound infections, septicemia, and meningitis especially among immuno-compromised individuals [13, 19, 20]. Pathogenicity in humans is also a potential problem for the food industry. Among poultry, fish, and shrimp, more than 50% of the samples for commercially-available raw food and between 20-30% of processed, ready-to-eat food are contaminated with haemolytic, multiple-antibiotic resistant *A. hydrophila* [21].

Among the different virulence factors produced by *Aeromonas* species, aerolysin is considered the most important. Immunization against aerolysin leads to protection against the bacterium and toxin. Toxigenic strains of *A. hydrophila* are lethal to mice, whereas toxin-deficient strains are not pathogenic [22, 23]. Intoxication of host cells by aerolysin occurs through a multi-step process involving each of the toxin's functional domains.

BIOCHEMISTRY AND MODE OF ACTION

A) Secretion

Aerolysin is produced as a 52 kDa, biologically-inactive protoxin called proaerolysin (Table **1**). The molecule enters extracellular medium from the bacterium through a type II secretion system (Fig. **2**, step 1) [24, 25]. Translocation across the inner bacterial membrane is mediated by a signal peptide subsequently removed upon transport [24, 25]. At least twelve proteins are involved in the next transport step across the outer bacterial membrane [26, 27]. While proaerolysin is transported across the inner membrane in its unfolded state, the molecule becomes fully folded when transported across the outer membrane [28]. Hardie *et al.* (1995) [29] could show that replacement of Met-41 with Cys results in dimers within the periplasmic space, which are then transported across the outer membrane [29]. Important parts of the secretion system are composed of the inner membrane proteins ExeA and ExeB [30], as well as an outer membrane protein ExeD [31]. Signals for secretion are located within the large lobe (domains 2-4) (see below for structure). The small lobe alone has reduced secretion versus the wild type structure [32]. Proteolytically-activated

monomers oligomerize spontaneously at high concentrations [9, 33] (Fig. **2**, step 2). It was shown for both proaerolysin and the mature form that they remain as dimers in solution. This was demonstrated through ultracentrifugation, chemical crosslinking, and a final electrophoresis step [34]. Fivaz *et al.* [33] claimed that proaerolysin exists only as a dimer at concentrations >100 μg/ml; whereas, it is monomeric at concentrations <100 μg/ml which also favours receptor binding. In contrast, Barry *et al.* [35] claimed that the proaerolysin dimer is also stable at concentrations <100 μg/ml, and in fact down to <5 μg/ml which also binds receptor.

B) 3-D Structure

The crystal structure of proaerolysin dimer has been solved (Fig. **3A**). The monomers are L-shaped molecules with a small N-terminal lobe (domain 1) rich in β-structure with a low number of α-helices [9, 36]. It can not be excluded that monomer structure differs from dimer. Nevertheless, the monomer has a bilobal structure divided into the little lobe, consisting of domain 1 (residues 1 to 82) [37], and a large, elongated lobe split into three additional domains 2 to 4 (residues 83 to 470) [36] with characteristic long β-strands. Domain 1 of aerolysin shares structural homology to S2 and S3 subunits of pertussis toxin, which are involved in receptor binding [38]. Domains 1 (residues 1 to 82) [37] and 2 (residues 83 to 178 and 311 to 398) [37] are involved in receptor binding and dimerization of aerolysin. Important residues for binding are Trp-45, Ile-47, Met-57, Tyr-61, and Lys-66 of domain 1, as well as Tyr-162, Trp-324, and His-332 of domain 2 [38-40]. Disulfide bridges between Cys-19 and Cys-75 in domain 1, and Cys-159 with Cys-164 of domain 2 increase protein stability and protect loop 154-167 from degradation by proteases [41]. Domain 2, together with domain 3 (residues 179 to 194, 224 to 274, 299 to 310, and 399 to 409) [37], is also involved in oligomerization and maintenance of the oligomers [41]. Trp at positions 371 and 373 seems to have a direct effect on oligomerization, since an exchange with Lys accelerates this process [37]. Van der Goot *et al* [34] suggest that these residues contribute to dimer stability or establishing contact between subunits in the oligomers [41]. Domain 3 shows sequence similarities to other pore-forming toxins like *Staphylococcus aureus* alpha-toxin [42] or channel-forming components of binary toxins such as the protective antigen (PA) from *Bacillus anthracis* [37, 43]. The channel-forming region within this domain is defined by disulfide trapping and cysteine scanning mutagenesis coupled to thiol-specific labeling on lipid bilayers. The transmembrane region

consists of ~20 amino acids, forming two amphipathic β-sheets connected by a hydrophobic region of 5 residues that altogether generate a β-hairpin [11]. The hydrophobic turn of the β-hairpin seemingly drives membrane insertion and folds back after membrane crossing in a rivet-like fashion. After heptamerization, these β-hairpins form during membrane insertion and β-barrel formation, comparable to outer membrane porins of Gram-negative bacteria [11, 44]. Domain 4 is located on the major lobe's tip (residues 195 to 223, 275 to 298, and 410 to 470) [37] and contains the C-terminal peptide released upon proteolysis. Proteolytic cleavage occurs between residues 422 to 440 of a long flexible loop by a broad spectrum of proteases (see above). As a consequence of activation, there might be reorganization of the different domains and/or helical pitch of the large loop that ultimately favors oligomer formation. Despite the oligomeric, pore-forming complex being very stable and relatively insensitive to changes in ionic strength and pH [37], crystals of this complex have not been obtained to date. It has only been possible to analyze two-dimensional crystals associated with *Escherichia coli* phospholipids [45] and electron microscopy [37]. The observed oligomers show, like the *S. aureus* alpha-toxin, a mushroom-like structure [37, 45]. According to Rossjohn *et al.* [37], domains 1 and 2 form the mushroom-shaped head while domains 3 and 4 generate the stalk. Parker *et al.* assumed that domain 3 is involved in forming the channel mouth, whereas domain 4 crosses the membrane bilayer to line the pore [10]. This model was proven wrong by Rossjohn *et al.* as they show through site-directed mutagenisis that the trans-membrane loop is located in domain 3 [37]. However, the main assumption is still valid that rapid exposure of hydrophobic residues from the transmembrane loop to the host cell membrane may be triggered through proteolytic activation, thus resulting in conformational changes [10, 37]. Several parameters that include toxin and receptor concentrations are seemingly required for the final event of pore formation [37]. Proteolytic activation induces a distinct change in secondary structure that coincides with reorganization of random structures, thereby increasing the β-sheet content. Release of the propeptide is essential for conformational and structural changes not restricted to the cleavage site, but which propagate throughout the molecule. Subsequent processes like oligomerization and membrane insertion after propeptide release have no influence on the secondary structure, but formation of heptamers changes the tertiary interactions [46]. It could be shown that propeptide release from *Aeromonas sobria* hemolysin converts the prohemo-lysin into an active form with the carboxy terminal region contributing to proteolytic stability. However, despite the fact that the N-terminus of *A. sobria* hemolysin is homologous to aerolysin, the C-terminal region shares no homology [47]. A similar effect of the aerolysin propeptide can therefore not be excluded. Conversion of a hydrophobic channel-forming heptamer into a water-soluble protein complex can easily be achieved by mutating Tyr-221 to Gly. This mutation inhibits hemolytic activity but has no influence upon oligomerization or receptor binding. Overall structure of the mutated complex shows no remarkable difference versus wild type; however, the former molecule does differ in that it is completely hydrophilic [48]. Domains 2-4 are rich in β-sheets and related to domains 1-3 of the single-lobed epsilon-toxin (Fig. **3A, B**), as well as the model of *septicum*-alpha-toxin. Interestingly, the structure of epsilon-toxin is highly related to aerolysin, despite a low (14%) amino acid sequence identity [5, 10]. Some differences in the receptor binding domain 2 suggest that epsilon-toxin binds to a different receptor than aerolysin, which could also be the reason for epsilon-toxin's > 100-fold higher toxicity [5, 49, 50]. Structural comparisons between LSL, epsilon–toxin, parasporin-2 (see Fig. **3A** and **B, C, D**), and aerolysin (see Fig. **3A**) reveal similarities between domains 2 and 3 of LSL and epsilon-toxin with domains 3 and 4 of aerolysin. The *septicum*-alpha-toxin shares a relatively high sequence homology with aerolysin domains 2-4 (72% similarity, 27% identity) [2, 15]. Domain 4 occurs in channel formation and contains the C-terminal peptide released upon proteolytic cleavage. However, one clear difference between the *septicum*-alpha toxin versus epsilon-toxin and aerolysin is an additional domain (domain I) in aerolysin, which seemingly binds to receptor.

In general, all members of the aerolysin-like toxin family are rich inb-strands. This assumption was confirmed by the crystal structure of water-soluble forms of aerolysin, epsilon-toxin, LSL, and most recently parasporin-2 [5, 6, 7, 10] (Fig. **3D**), as well as through CD spectroscopy of epsilon-toxin and LSL [51, 52]. All remaining members of the aerolysin-like toxin family are devoid of a sequence corresponding to domain 1 of aerolysin. Thus, these proteins consist of a single-lobed, elongated structure containing three domains homologous to domains 2-4 of aerolysin [2, 3, 5, 6, 7, 10]. Domains 1 and 3 are generally involved in oligomerization, while receptor binding is restricted to domain 1 [53]. Residues within domain 1 regulate insertion of the transmembrane b-hairpin located within domain 2 [53].

C) Receptor Binding

Aerolysin monomers, but not oligomers, bind to specific cell-surface receptors [33] (Fig. **2**, step 3) identified as glycosyl-phosphatidyl-inositol (GPI)-anchored proteins (Table **1**). The GPI anchor attaches many cell-surface proteins into the cell membrane, especially through lipid rafts. Because of the ubiquitous distribution of GPI-anchored proteins and their multiple functions as enzymes, antigens, adhesion molecules, or receptors for various ligands that include bacterial toxins, aerolysin interacts with a wide variety of cell types [32, 54-56]. Aerolysin recognizes the GPI anchor as well as associated glycosylated proteins. Although aerolysin possesses two different binding regions within the toxin, each recognizing different parts of the receptor, each region is unable to mediate aerolysin binding without the other. Biacore measurements reveal binding of aerolysin to the GPI-anchored proteins Thy-1, variant surface glycoprotein (VSG), and contactin. Relative to whole toxin, binding of the small lobe (domain 1) to Thy-1 is 5000-fold weaker and that of the large lobe (domains 2-4) >50-fold weaker [39]. The posttranslational modification of N-glycan structures (minimal structure containing N-acety-lglucosamine, mannose and asparagine) on glycosylated proteins is recognized by domain 1 of aerolysin (see below), whereas domain 2 binds to the GPI anchor. Many GPI-anchored proteins, containing the proper N-glycan post-translational modification, can be used by aerolysin as a
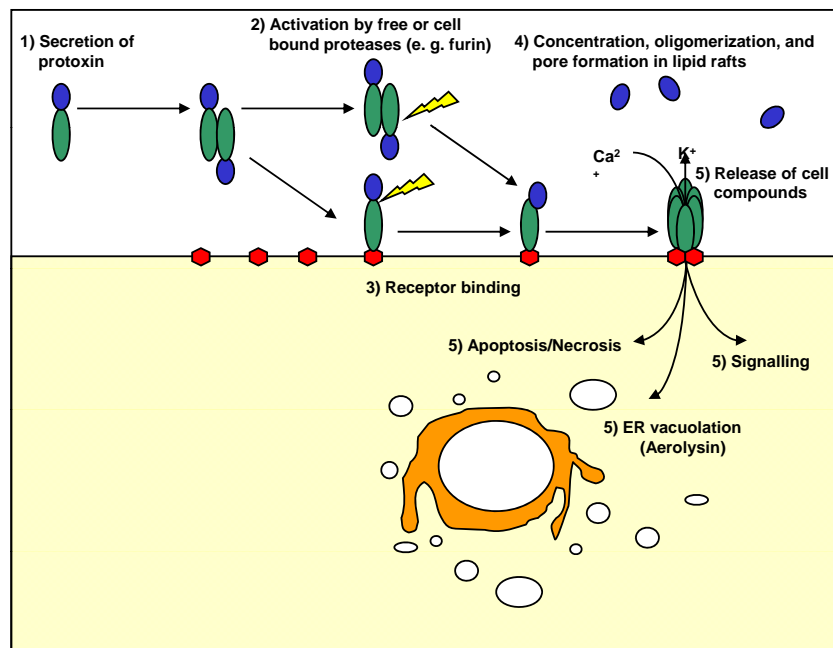
Fig. (2). Schematic model of aerolysin-like toxin uptake. (1) Secretion of toxin (aerolysin) monomer with head-to-tail dimers formed in solution (2/3). Proteolytic cleavage results in activated monomers and propeptide release. Although proteolysis can occur in solution, activation mainly occurs after receptor binding on the cell surface (4). Oligomerization and pore formation occurs in lipid rafts due to concentrating effects (5). Pore formation results in several cellular responses that include vacuolation of the ER by aerolysin.
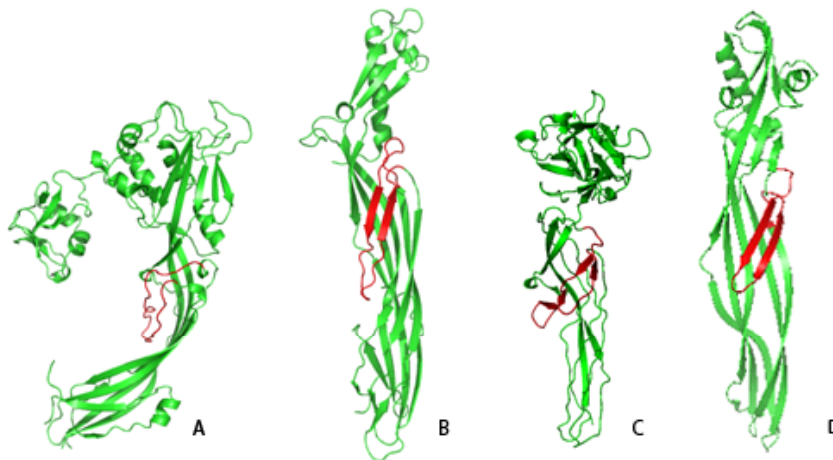
Fig. (3). Crystal structure of aerolysin-like toxin family members.

Crystal structures of (**A**) aerolysin from *A. hydrophila* [10], (**B**) epsilon-toxin from *C. perfringens* [5], (**C**) LSL from *L. sulphureus* [6], and (**D**) Parasporin from *Bacillus thuringiensis* [7]. The pore forming β-hairpins are highlighted in red.

receptor. Receptors include Thy-1 [55], contactin [32], CD14, carboxypeptidase M or NCAM (neural cell-adhesion molecule) [57], and even the variant surface glycoprotein (VSG) of *Trypanosoma brucei* [39]. Aerolysin binding to GPI-anchored proteins can be used to detect and concentrate eukaryotes of the genus *Leishmania* or *Trypanosoma*. Trypanosomes are remarkably insensitive to aerolysin, and the reason might be a dense packing of GPI-anchored proteins [58] that hinder access to the plasma membrane. After a selective lysis of erythrocytes and leukocytes by aerolysin, which does not affect African trypanosomes, the latter can be easily detected [59]. Zheng *et al.* [60] showed that *septicum*-alpha-toxin kills *Leishmania major* parasites, with a 50% lethal concentration of 0.77 nM. However, a few

GPI-anchored proteins like CD59 [61] and gp63 from *Leishmania* prevent aerolysin binding, but the binding ability of gp63 can be restored when expressed in mammalian cell lines [32]. An explanation could be a different posttranslational modification pattern in mammalian cell lines. Surface plasmon resonance experiments by MacKenzie *et al.* [39] identified crucial residues within domains 1 (Trp-34, Tyr-62, Lys-66) and 2 (Lys-171, Trp-324) that affect receptor binding. Indeed, cell lines with a reduced ability to produce the GPI anchor, or those treated with inositol-specific phospholipase C, are less sensitive to aerolysin [55, 62].

D) Proteolytic Activation

Activation of secreted aerolysin precursor consists of proteolytic removal of 41 to 43 C-terminal residues within a flexible loop (domain 4) while the toxin is in solution or bound to receptor (Fig. **2**, step 2). Additionally, no significant difference in binding between proaerolysin and mature aerolysin could be observed [39]. Many eukaryotic proteases found in the digestive tract, such as trypsin (cleavage site on proaerolysin Lys-427), α-chymotrypsin (Arg-429), and furin (Arg-432) in solution or on the cell surface [36, 63], as well as proteases secreted by *Aeromonas* [64], activate the hydrophilic protoxin monomers. Activation is accompanied by a conformational change essential for aerolysin activity, as it enables a water-soluble protein to expose hydrophobic residues necessary for insertion into host cell membranes and forming persistent membrane channels. Such residues can be found normally in the interior of water-soluble proteins; hence, a conformational change is expected to accompany channel formation. The ability to exist as water-soluble proteins and form pores in lipid membranes requires two, alternatively-stable conformations. It seems that peptide removal favours oligomerization by reducing the energy barrier leading to an extremely stable aerolysin heptamer [24]. Differences in conformation throughout the entire molecule have been observed between aerolysin and proaerolysin using fluorescence [65] and infrared spectroscopy [46]. However, these changes promoting oligomerization have not been more closely evaluated [24]. Comparative studies of *A. sobria* hemolysin with aerolysin of *A. hydrophila* reveal that the carboxy terminal region of proaerolysin may also contribute to toxin stability towards proteolytic degradation [47].

E) Oligomerization and Pore-Formation

Binding to GPI-anchored proteins, which are mainly localized in lipid rafts, concentrates toxin monomers into these restricted cell-surface microdomains that favor monomer clustering and subsequent oligomerization (Fig. **2**, step 4) [1]. Proteolytic cleavage occurs after receptor binding and approximately 40 residues are removed from the aerolysin C-terminus. Both events, receptor binding and proteolytic activation, reflect important conditions that trigger oligomerization. Even at low picomolar concentrations, oligomerization takes place mainly on the surface of host cell membranes [66] but aerolysin can also oligomerize in solution to form very stable heptamers (Table **1**). However, oligomerization is much more efficient on cells due to the concentrating effect of receptor binding and accumulation into lipid rafts [66]. During steady state, only about 30% of GPI-anchored proteins are associated with lipid rafts. Thus the majority of GPI-anchored proteins are not localized within lipid rafts but all have the capacity to become transiently associated with these membrane microdomains. Activated monomers assemble into heptamers through domains 3 and 4 interactions, as well as form channels through insertion into lipid bilayers as observed indirectly by electron microscopy since only the mushroom cap and not the transmembrane region are visible [45]. Several mutations affecting oligomerization have been identified. His-132, for example, requires protonation to facilitate oligomerization and mutation of this residue abolishes pore formation [67-69]. Trp-371 and -373 accelerate the heptamer formation process [34]. Unlike other

PFTs like *S. aureus* alpha-toxin [70, 71], aerolysin apparently does not form a pre-pore complex. Heptamerization is accompanied by exposing hydrophobic areas in domain 3 and insertion into membrane [11]. At this point, there are at least two phases of toxin-mediated cytotoxicity: specific binding upon target cells via the GPI-toxin receptor and membrane insertion. Lipid bilayer studies reveal that electrostatic forces play an important role during the reversible adsorption phase of RTX (Repeats in ToXin)-toxins such as *E. coli* alpha-toxin or adenylate cyclase toxin from *Bordetella pertussis* [72-74]. Conformational change drives an unfolding of the antiparallel amphipathic loop from each monomer and formation of an amphipathic transmembrane 14-stranded-β-barrel that inserts into the membrane to form pores [75, 76] (Fig. **2**, step 4). A domain 3 (domain 2 for other toxin family members) loop seems to move away from the core β-sheet and with the loops of other monomers forms a transmembrane channel. Hydrophobic amino acid side chains of each ß-strand interact with the lipid bilayer core, whereas the hydrophilic residues face the channel lumen [50, 62, 76, 77]. Once inserted into the membrane, heptamers cannot be removed without using detergents. A similar effect is also expected for members of the aerolysin-like toxins. An aerolysin heptamer is extremely stable, even to boiling in SDS [78], and has a mushroom-shaped structure like that of *S. aureus* alpha-toxin [42, 79]. However, the pore structure of aerolysin heptamer is not yet well defined. A loop from domain 3 is involved in ß-barrel formation [25]. Heptamers inserted into the membrane form voltage-dependent, anion selective channels with a single channel conductance of about 70 pS in 0.1 M KCl which corresponds to a pore diameter of at least 7 Å [73] (Table **1**). Wilmsen *et al.* estimated a fairly similar diameter of 9.3 Å [45]. Some unclarity still remains for the pore diameter since it is estimated by Parker *et al.* and Tisitrin *et al.* [10, 48] at 17 Å, 30 Å by Howard and Buckley [80], and between 19 and 23 Å by Tschödrich-Rotter *et al.* [81]. These variations might be caused by the use of different techniques. Parker *et al.* used cryo-negative staining electron microscopy [10], Howard and Buckley measured the release of labeled molecular weight markers [80], while Tschödrich-Rotter *et al.* used the scanning microphotolysis method [81]. The channel-forming domain of aerolysin could be identified through sequence alignments with other PFTs like *S. aureus* alpha-toxin [42] or channel forming components of binary toxins like PA from *Bacillus anthracis* [37, 43]. Additionally, the channel-forming domain of aerolysin has been determined through altering channel properties in biophysical experiments by insertion of different mutations (see below; [11]). Therefore, channel formation for not only aerolysin, but also the *S. aureus* alpha-toxin and other β-barrel pore forming toxins, marks a crucial step in biological activity.

F) Consequence of Pore Formation

As a consequence of anion-selective transmembrane channels being formed, a cell can readily lose ions, water, and small molecules (i.e. sugars, amino acids, nucleic acids) that do not include proteins [56, 78]. Besides breakdown of the Na^+/K^+ gradient, an influx of Ca^{+2} can be observed in cells, which seemingly mediates cell signalling [82] (Fig. **2**, step 5). Loss of K^+ facilitates formation and activation of the

inflammasome, cytoplasmic multiprotein complexes harbouring Nod-like receptors such as IPAF and NALP3, as well as caspase-1 [56]. Finally, via caspase-1 the K^+ efflux mobilizes Sterol Regulatory Element Binding Protein (SREBP)-1 and -2. Both proteins are involved in the biogenesis of membranes and their activation enhances cell survival [57]. Caspase-1 is also responsible for the processing and activation of cytokines IL-1β and IL-18 [83], which is also observed during aerolysin intoxication. Furthermore, it has been shown that IL-8 is produced as a consequence of NF-κB pathway activation in macrophages with aerolysin [22]. Intoxicated cells stay alive for several hours, depending upon the toxin concentration [32]. Additional effects, besides pore formation, include increasing cell membrane permeability contingent upon the number of pores and finally cell lysis. Formation of intrinsic ion channels and loss of membrane integrity induce a number of signalling pathways resulting in release of secondary messengers (Fig. **2**, step 5). In granulocytes, secondary messengers activate G proteins, leading to production of inositol(1,4,5)-triphosphate (IP3) and release of Ca^{2+} from the endoplasmic reticulum (ER) through IP3-dependent channels [82]. This effect is directly linked to channel formation since inactive channel mutants (Tyr-221 to Gly, Gly-202 to Cys, and Ile-145 to Cys) do not elicit these effects through receptor binding alone [82]. In T cells, aerolysin channel formation results in DNA fragmentation which is a sign of apoptosis [84] (Fig. **2**, step 5; Table **1**). Aerolysin-induced apoptotic effects have also been shown in murine macrophages [85], and the eliciting factor seems to be Ca^{2+} influx through the aerolysin channels [84] or a secondary effect linked to toxin-induced TNFα [86]. It is thought that apoptosis is caused through a massive entry of Ca^{2+}. Interestingly, induction of apoptosis could not be observed in all cell types [24]. As an additional effect, vacuolation of the ER can be observed in epithelial cells (Fig. **2**, step 5); however, this is seemingly not linked to Ca^{2+} entry and induction of apoptosis, since Ca^{2+} ionophores and overexpression of an anti-apoptotic protein (Bcl-2) does not protect against ER vacuolation [56, 64]. Exposure to EGTA prevents ER vacuolation [57]. Aerolysin does not alter morphology of the Golgi complex and endocytic compartments, or induce degradation of DNA [56, 64]. Vacuolation is inhibited by either ATP depletion or depolymerization of the microtubule network. How this effect is elicited by aerolysin remains unclear, even though there is now evidence that toxin enters the cell [24].

Bacillus thuringiensis Cry5B, a cytolytic pore-forming toxin not related to the aerolysin-like family, induces passive uptake of ions leading to host cell swelling. Such actions ultimately result in up regulation of two MAPK pathways, p38 and c-Jun N-terminal kinase (JNK)-like. Activation of the p38 pathway facilitates divalent cation transport [87]. Furthermore, the p38 pathway is involved in membrane resealing after cell intoxication with *S. aureus* alpha-toxin [88]. MAPK pathways provide a significant defence against the toxin, as inhibition of p38 in aerolysin-treated baby hamster kidney cells results in hypersensitivity. It can be assumed that aerolysin influences both MAPK pathways [87]. Finally, it should be considered that as aerolysin is anion selective, transport of negatively charged ions across the cell membrane could also contribute to the observed effects.

Members of the Aerolysin-Like Toxin Family

1. Alpha-Toxin from Clostridium septicum (Gram-Positive, Prokaryote)

The *septicum*-alpha-toxin is secreted by the highly virulent pathogen *C. septicum*. This anaerobe is a major cause of necrotizing enterocolitis and enterotoxemia in animals. *C. septicum* is also involved in nontraumatic myonecrosis and gas gangrene of the intestinal wall that includes a necrotizing enterocolitis in humans subsequent to colon malignancy [89-91] (Table **1**). The *septicum*-alpha-toxin, a 47.5 kDa protein (Table **1**), is the only known virulence factor of *C. septicum* absolutely required for pathogenesis [92]. Purified toxin is lethal for mice [50], and cytotoxic for CHO cells [93] as well as many other nucleated cell lines [94]. The toxin also causes membrane damage and lysis of tachyzoits from the protozoan parasite, *Toxoplasma gondii* [95]. The *septicum*-alpha-toxin gene has been sequenced from 25 clinical isolates, and although the deduced proteins have very few changes they can still be divided into 7 groups (based upon amino acid sequence variations). However, no differences in molecular weight, toxicity, and antigenic cross-reactions have been observed [96].

Septicum-alpha-toxin is hemolytic, dermonecrotic, and lethal with a reported mouse median lethal dose (LD$_{50}$) of 10 µg/kg [97]. The *septicum*-alpha-toxin is encoded by the *csa* gene [2, 75] and secreted from the bacterium by a signal peptide evident as an inactive protoxin, probably via the type II secretion pathway [97]. Proteolytic activation occurs by removing a C-terminal peptide of 45 residues [93, 98] (Table **1**). Various proteases can activate *septicum*-alpha-toxin, such as trypsin and proteinase K, but furin found on the cell surface seems to be the main activator *in vivo*.

The crystal structure of *septicum*-alpha-toxin has not been solved, but based on that of aerolysin and the high degree of similarity (72% similarity at the amino acid level) between domains 2-4 of both toxins, it is possible to generate a molecular model of the water-soluble form [76]. *Septicum*-alpha-toxin does not form a dimer in solution [98] and possesses a higher amino acid sequence identity (27%) [2] with aerolysin than epsilon-toxin (13.9%) [5] (Fig **1A**). Several residues contributing to oligomerization and receptor binding could be identified through site-directed mutagenesis [53], but one important difference versus aerolysin is formation of a prepore. Furthermore, by using cysteine-scanning mutagenesis with multiple spectrofluorimetric models it is possible to identify the channel-forming domain between residues 203-232. Deletion of up to 90% of the membrane-spanning domain affects pore formation, but has no effect upon other features like activation, cell binding, and oligomerization [76]. A disulfide bond was designed by introducing Cys residues for Ser-220 and Ser-269. Like that for aerolysin [37], reduction of these residues restores alpha-toxin activity and demonstrates necessary flexibility of the β-hairpin.

Precursor for *septicum*-alpha-toxin monomer binds to GPI-anchored membrane proteins on target cells [62] (Fig. **2**,

step 3). Cells unable to synthesize GPI anchors have reduced, or complete, loss of toxin sensitivity [62]. However, receptors for the *septicum*-alpha-toxin and aerolysin are different. Multiple cellular receptors of *septicum*-alpha-toxin include the human folate receptor (hFR), neuronal surface molecule contactin, Thy-1 (CD90) [62], as well as proteins out of the SAG (**S**ensitive to **A**poptosis **G**ene) family which are on the surface of *Toxoplasma gondii* [95] (Table **1**). Whereas carbohydrates linked to the GPI-anchor may be important for binding of aerolysin and *septicum*-alpha-toxin, the N-glycan of anchored proteins is seemingly not important for binding of *septicum*-alpha-toxin [99]. The *septicum*-alpha-toxin lacks domain 1 found in aerolysin, perhaps explaining the different binding behaviors of these toxins to GPI-anchored proteins. Indeed, a hybrid protein consisting of domain 1 from aerolysin fused to *septicum*-alpha-toxin retains binding capability to receptors so far restricted to aerolysin [40].

Receptor bound *septicum*-alpha-protoxin requires activation by furin and possibly other cell-surface proteases (Fig. **2**, steps 2 and 3). Activation of *septicum*-alpha-toxin in solution leads to aggregation of monomers into non-functional oligomers; however, activation of toxin after receptor binding results in functional oligomers. The propeptide stays associated with the activated toxin monomers by non-covalent interactions and acts as an intramolecular chaperon [98], thereby preventing the toxin from undergoing incorrect folding or oligomerization in solution [98]. Dissociation of the propeptide occurs during oligomerization and stabilization of the *septicum*-alpha-toxin monomer is then no longer required. The propeptide is located within domain 3 of *septicum*-alpha-toxin and probably protects an interfacial domain involved in monomer interactions [9]. It has been possible to observe *septicum*-alpha-toxin channels *in vitro* with the black lipid bilayer technique. Channels formed in this assay are slightly anion selective with a single-channel conductance of 140-180 pS in 100 mM KCl [77] (Table **1**). Like that for other PFTs, the binding to cell receptor, oligomerization, as well as subsequent channel formation has different kinetics. Pore formation is completely abrogated at 4°C, whereas binding to cells and oligomerization occurs at 25° and 37°C [98]. Pore diameter formed by *septicum*-alpha-toxin is estimated between 1.3 and 1.6 nm [50, 62, 76, 97] (Table **1**). *Septicum*-alpha-toxin induces a rapid K(+) efflux, deregulation of mitochondrial activity, increased ROS levels, and dramatic ATP depletion followed by cell necrosis, characterized by cell permeabilization to propidium iodide without DNA fragmentation and activation of caspase-3 which is oppositional to the apoptosis-inducing aerolysin [100, 101]. An also reported increase in intracellular Ca^{2+} levels seems to be not the initial effect, since incubation of the cells in K^+-enriched medium delayed cell death. These results suggest that the fast loss of intracellular K^+ represents an early signal of pore-forming toxins-mediated cell necrosis [100, 101]. Ca^{2+} influx seems to be associated with calpain activation and release of cathepsins from lysosomes. Finally, the immunostimulatory histone binding protein HMGB1 was found to be released from the nuclei of alpha-toxin-treated cells [101].

2. Enterolobin from Seeds of Enterolobium contortisiliquum (Eukaryotic Plant - Brazilian Tree)

Enterolobin, a 55 kDa cytolytic, insecticidal protein is produced by seeds of the tropical tree *Enterolobium contortisiliquum* belonging to the family of Mimosaceae [3, 102, 103] (Table **1**). Although this tree grows mainly in coastal areas of Costa Rica and throughout temperate zones of South America, it also resides up to 900 meters above sea level. *E. contortisiliquum* can grow as high as 40m and is well adapted to the dry season. The seeds germinate in high humidity and elevated temperatures, of which the latter includes a heat impulse caused by bush fires. Enterolobin was first purified by Sousa *et al.* in 1989 [102]. Since enterolobin harbours insecticidal activities against larvae of the brucid *Calosobruchus macullatus* [102], and causes inflammation upon injection in rats [104], it may serve as a defence against potential herbivores. Three known outbreaks of intoxication have also been caused by *E. contortisiliquum* pods [105] consumed by cattle, leading to anorexia, depression, photosensitization, and abortion. Generally, these animals fully recovered within 30 to 40 days. Calves fed *E. contortisiliquum* seeds had gastrointestinal irritation and liver degeneration [105]. Furthermore, it was shown that mouse erythrocytes were less susceptible to haemolysis by enterolobin than human and rabbit erythrocytes [102] (Table **1**).

The primary structure of enterolobin appears to be more related to aerolysin than to the *septicum*-alpha- or epsilon-toxins. Enterolobin displays sequence identity (45%) and similarity (59%) with both the small and large lobes of aerolysin (Fig. **1A**). Little is known about the cytolytic mechanism of enterolobin, but it seems to act via oligomerization and pore formation (Fig. **2**). Indeed, enterolobin apparently forms stable dimers in solution at concentrations as low as 10 μg/ml [4, 62, 102, 103]. In addition, similar residues to proaerolysin Trp-371 and Trp-373 responsible for protein dimer maintenance in solution can be found in enterolobin with Trp-386 and Trp-387 [4]. A light-scattering experiment showed that oligomerization of enterolobin was promoted by low pH and high ionic strength. Several key residues involved in oligomerization of aerolysin (His-107, His-132, and Cys-159) are also conserved in enterolobin (His-127, His-154, and Cys-183) [4]. Additionally, Trp-227 involved in secretion of aerolysin [45] is also aligned with Trp-247 in enterolobin. A different receptor-binding domain of enterolobin, when compared to proaerolysin, is to be supposed since residues responsible for receptor binding of proaerolysin are deleted or substituted in enterolobin [103]. These data suggest that enterolobin recognizes a different receptor. The cleavage site for trypsin or α-chymotrypsin in aerolysin corresponds to a non-conserved tripeptide Asp-Ser-Cys (residues 437-439) in enterolobin. Up to now, it is unknown if proteolytic activation of enterolobin further increases haemolytic activity [4].

3. Epsilon-Toxin from Clostridium perfringens (Gram-Positive, Prokaryote)

Epsilon-toxin is produced by the widespread, spore-forming bacterium *C. perfringens*. In addition to soil, *C. perfringens* can also be found as an intestinal resident in a

variety of different animal species, including humans. *C. perfringens* strains are classically placed into five types, depending on the production of four major toxins (Alpha, Beta, Epsilon, Iota). Types B and D produce epsilon-toxin [106] (Table **1**). The epsilon-toxin represents a main pathogenicity factor [107], belonging just beneath the botulinum and tetanus neurotoxins as the most potent clostridial toxins when tested by mouse lethality assay [108]. Epsilon toxin is responsible for fatal enterotoxaemia in sheep, goats, calves, and other domestic animals [42, 109]. Proliferation of clostridia in the intestine produces large amounts of epsilon-toxin. Absorption of toxin occurs via the intestinal mucosal barrier and then spreads into all organs by the blood stream, preferentially accumulating in the brain and kidneys [110]. Within infected animals, the toxin promotes blood pressure elevation and edema throughout the viscera such as intestines and lungs. The toxin induces extensive kidney damage, known as "pulpy kidney disease", and is characterized post-mortem by tubule cell necrosis and medullary interstitial hemorrhage [42, 111-114]. An epsilon-toxin-Green Fluorescence Protein (GFP) fusion injected intravenously into mice binds mainly to the apical membranes of distal-collecting duct cells [115]. Mice injected with epsilon-toxin-GFP exhibit severe kidney damage, including selective degeneration of distal nephrons. Epsilon-toxin also crosses the blood-brain-barrier, causing perivascular edema and neuronal damage that leads to release of glutamate responsible for neurological disorders characterized by convulsions and uncoordinated movements [116]. Because of the extreme potency and possible activity in humans, the National Institutes of Health and the Centers of Disease Control have classified epsilon-toxin as a Catergory B agent [117].

Epsilon-toxin is encoded by a gene located on a large plasmid [118], and the protein secreted as a non-active protoxin like many other clostridial toxins (Fig. **2**, step 1). After secretion, proteolytic activation occurs by serine-type proteases like trypsin, α-chymothrypsin, or the *C. perfringens* lambda protease in the gut of infected animals or *in vitro* (Fig. **2**, step 2). Cleavage of 29 C-terminal residues from epsilon-toxin is essential for the biological activity and formation of large SDS-resistant heptameric complexes in rat synaptosomal membranes and target cell membranes [119, 120] (Table **1**). The removal of 13 N-terminal amino acids from epsilon-toxin is of secondary interest and induces only minor activation of toxin [48] (Table **1**).

The structure of *C. perfringens* epsilon-toxin is highly related to that of aerolysin, despite low sequence identity (13.9 %) (Fig **1B**). Despite high structural similarity, epsilon-toxin shows a remarkable difference in its biological potency being ~ 100 times more lethal in mice versus aerolysin and *septicum*-alpha-toxin [5, 49, 50]. A cluster of aromatic residues within domain 1 of epsilon-toxin might be related to receptor binding [5]. Epsilon-toxin binds to detergent resistant microdomains (DRMs), which leads to the possibility that a putative receptor is located in DRMs (Fig. **2**, step 3 and 4). Phosphatidylinositol-specific phospholipase C (PI-PLC) does not impair binding or formation of epsilon-toxin complexes, suggesting that the receptor for epsilon-toxin is not GPI-anchored [121]. Furthermore, sphingolipid and cholesterol content influences susceptibility of host cells to epsilon-toxin and necessary

heptamerization of the toxin [120, 122]. Addition of extracellular ganglioside G_{M1} causes a dramatic decrease in binding, heptamerization, and cytotoxicity [122]. Toxin binding induces oligomerization and formation of a heptameric complex in rat synaptosomal and Madin-Darby canine kidney (MDCK) cell membranes [119, 120, 123]. However, epsilon-toxin binds to a specific receptor on a restricted number of cell lines that include MDCK, human renal leiomyoblastoma G-402, and mouse renal mpkCCD (Table **1**) [124], which is distinct from the GPI-anchored proteins recognized by aerolysin and *septicum*-alpha-toxin. It has been shown that epsilon-toxin binds to plasma membranes of MDCK cells, but not to any other intracellular organelles [123, 125]. Thus, epsilon-toxin heptamerizes and induces pore formation in target cell membranes, leading to a rapid change in membrane permeability and ultimately cell death [120, 123]. Epsilon-toxin decreases intracellular K^+, and increases Cl^- as well as Na^+, concentrations in MDCK cells[122] (Fig. **2**, step 5). Furthermore, epsilon-toxin influences the cell cycle [126] and induces rapid cell death without altering tight junction permeability, the actin cytoskeleton network, or endocytotic processes [125]. It has also been possible to observe oligomerization and channel formation of epsilon-toxin *in vitro* using the black lipid bilayer technique [127]. Epsilon-toxin forms slightly anion-selective channels in artificial membranes with a single-channel conductance of 60 pS in 100 mM KCl (Table **1**). The channels are permeable to hydrophilic solutes with a molecular mass of at least 1 kDa, which probably represents the basic mechanism of toxin action upon target cells [127].

Epsilon-toxin induces rapid depletion of ATP, stimulates the AMP-activated protein kinase (AMPK), and influences cell signal pathways via mitochondrial membrane permeabilization and mitochondrial-nuclear translocation of apoptosis-inducing factor (AIF) (Fig. **2**, step 5). Different than aerolysin [22, 83], epsilon-toxin induces cell necrosis instead of apoptosis [121] (Table **1**). A characteristic trait of necrosis involves nuclear shrinkage without DNA fragmentation. Treatment of cells with a lipid raft disrupting agent, methyl-β-cyclodextrin (MβCD), diminishes epsilon-toxin oligomerization and distinctly reduces Na^+ and Ca^{2+} influx; however, ATP depletion and cell death is not prevented [121] (Fig. **2**, step 5). The exact mechanism of epsilon-toxin induced cell death remains undetermined. Despite structural similarity with aerolysin, epsilon-toxin recognizes a distinct receptor on DRMs of target cells and is much more cytotoxic by inducing cell necrosis.

4. Toxins from Bacillus: Mtx from Bacillus sphaericus and Parasporin-2 from Bacillus thuringiensis (Gram-Positive, Prokaryotes)

The aerobic, spore-forming *Bacillus sphaericus* produces several insecticidal toxins. More than 380 different strains have been identified which are toxic to the larvae of *Culex*, *Aedes*, and *Anopheles* mosquitoes [128, 129]. The strains can be divided into low-and high-toxin producers [128, 130]. Both low and high toxicity strains produce a 100 kDa toxin, whereas highly-toxic strains produce during sporulation an additional binary toxin consisting of 51.4 and 41.9 kDa proteins [128, 131]. In addition to these toxins, Mtxs (Mtx1, Mtx2, Mtx3) have a different mode of action [132] and are produced during vegetative growth by both high- and low-

toxicity strains [131, 133, 134]. Significant homologies have been found between the 31.8 kDa Mtx2 [134], 35.8 kDa Mtx3 (Table **1**), epsilon-toxin [118], and *Pseudomonas aeruginosa* cytotoxin [134]. Mtx3 shares 38% identity with Mtx2, 30% with epsilon-toxin, and 27% with the *P. aeruginosa* cytotoxin [135]. The N-terminus contains a signal peptide (26 amino acids) [133, 136]. In contrast to wild type Mtx3, recombinant toxin produced in *E. coli* is moderately toxic to *Culex quinquefasciatus* larvae and weakly toxic to *Aedes aegypti* larvae [133] (Table **1**). Nothing is known about the toxic effects on cultured cells. It can be assumed that the receptor(s) for Mtx2 and Mtx3 is highly specific and restricted to gut cells of mosquito larvae. Mtx2 shows 31% identity to the epsilon-toxin and 28% with the *P. aeruginosa* cytotoxin. Both Mtx2 and Mtx3 are widespread within high- and low-toxicity strains [133, 134]. Because of relatively dampened expression for Mtx2, and its degradation by the subtilisin-like serine proteinase sphericase [137, 138], its contribution to the toxicity of spores used for mosquito control is low [139]. However, Mtx2 is toxic to *C. quinquefasciatus* [134] and possesses significant activity against *A. aegypti* larvae [132].

Little is known about the toxic mechanism of the Mtxs and it still remains unknown if Mtx2 and Mtx3 are, like the other members of the aerolysin-like toxin family, secreted as protoxins requiring proteolytic activation [128, 129]. Because of relatively high sequence homologies with the epsilon-toxin and *P. aeruginosa* cytotoxin, it is thought that Mtx may also form pores on suitable target cells [133].

During sporulation, the Gram-positive, spore-forming *Bacillus thuringiensis* produces protein crystals also referred to as parasporal inclusions. These crystal proteins vary in size and composition among bacterial strains, and often exhibit insecticidal activity without any harmful effects on mammals [7]. The structure for one of these parasporal inclusion crystals, parasporin-2, has recently been solved. Four parasporins (parasporin-1 through parasporin-4) have been identified and characterized to date [140-143]. The structure of parasporin-2 is unique for the two currently classified, major crystal protein families: Cry and Cyt proteins [144-150]. Despite differences, parasporins are classified as Cry proteins in the *B. thuringiensis* toxin nomenclature. Interestingly, parasporin-2 cannot be assigned to one of the two groups of crystal proteins produced by *B. thuringiensis*, and displays striking similarities to the aerolysin-like toxin family (Fig. **3**) (Table **1**) [7]. Although parasporin-2 and these other protein toxins share a characteristic strand-dominated structure, there is only marginal sequence similarity (25.7–27.7% identity between parasporin-2 and aerolysin, epsilon-toxin, or LSL). In addition, parasporin-2 shares 41% sequence identity with hydralysin from *Chlorohydra viridissima* [151] (see below). Therefore, it can be assumed that both proteins also share a common structure [7]. Like the epsilon-toxin and LSL, parasporin-2 consists of a single large loop composed of three domains. Domain 2 contains a putative channel-forming β-hairpin. The surface has exposed side chains of serine and threonine residues possibly important during cell binding, oligomerization, and channel formation [7].

Parasporin-2 is produced as an inactive 37-kDa protoxin requiring proteolytic nicking. Proteinase K removes 51 residues from its N-terminus and 36 residues from the C-terminus, resulting in a 30-kDa active toxin that forms pores and increases plasma membrane permeability (Table **1**) [7, 140, 152]. Exposure of cells to the toxin causes further swelling, blebbing, lysis, disassembly of the microtubule network, and fragmentation of mitochondria as well as ER [152]. The receptor for parasporin-2 is not yet known, but the toxin targets lipid rafts, undergoes dimerization, as well as inserts into lipid bilayers and membranes of sensitive cells [153]. Based upon structure homology with the aerolysin-like toxin family, parasporin-2 retains a conserved β-hairpin that forms a pore according to the rivet model, in which a single residue (Phe) between the two amphipathic β-strands contributes to a β-barrel anchor into the lipid bilayer [7].

Aside from the structure, parasporin-2 also has divers activities upon different cell types. While Cry proteins are toxic for specific insect species and Cyt proteins have broad cytolytic/hemolytic activity against invertebrate and vertebrate cells, parasporin-2 is particularly cytocidal to human liver and colon cancer cells. The 50% lethal dose for hepatic and colon cancer cell lines, as well as human leukemia T-cell lines, is around 20 ng/ml [152]. Parasporin-2 is neither insecticidal nor haemolytic [154, 155].

5. Hemolytic Lectins from Laetiporus Sulphureus (LSL) (Eukaryote, Mushroom)

Sugar binding proteins or glycoproteins of non-immune origins which agglutinate cells and precipitate glycoconjugates are called lectins [156]. The lectin produced by the parasitic and saprophytic mushroom *L. sulphurous*, possesses strong hemagglutination and haemolytic activity [52] (Table **1**). This lectin-producing organism is widely distributed and grows namely during summer and fall in large clusters upon the decaying logs and stumps of hardwoods as well as conifers [157]. Closely related *Laetiporus species* (*L. cincinnatus, L. gilbertsonii, L. conifericola*) exist [158] and perhaps share the same haemolytic lectins. The LSL lectin was first isolated by Konska *et al.* [159] and reported as a homotetramer with subunits of ~35 KDa [52] (Table **1**).

Three similar full-length cDNAs, designated as LSLa, LSLb, and LSLc, have been detected through the analysis of a cDNA library derived from *L. sulphureus*. Each cDNA subtype has an open reading frame of 945 bp encoding for 315 amino acids. Sequence homology between the three proteins is 80 to 90% [52]. LSLs share sequence homologies of about 22-25% with the mosquitocidal toxin (MTX2) from *B. sphaericus* and *septicum*-alpha-toxin. The sugar binding N-terminal part has a β-trefoil framework structurally similar to other toxins that interact with galactose-related carbohydrates, such as the hemagglutinin component (HA1) of the progenitor toxin from *C. botulinum* neurotoxin [6, 160]. The C-terminal portion consisting of domains 2 and 3 possesses structural similarity to domains 3 and 4 of aerolysin, as well as domains 2 and 3 of epsilon-toxin [160]. Like these toxins, LSL contains an amphipathic β-hairpin of about 30 amino acids. Furthermore, the crystal structure of LSL reveals a hexamer that contradicts the above-mentioned results indicating that LSL exists as a tetramer [6, 52].

All three lectin subtypes of LSL, as well as their recombinant forms, agglutinate and lyse rabbit, human and

sheep erythrocytes via a colloid-osmotic mechanism [52]. The latter is characterized by compounds, such as sucrose or polyethylene glycol, that delay erythrocyte lysis by temporarily stabilizing osmotic pressure and preventing cell swelling caused by toxin-induced pores [161]. Haemolysis seemingly occurs through pore formation, and results of osmotic protection experiments show that haemolysis involves ion-permeable channels smaller than 3.8 nm diameter [52] (Table **1**). Incubation of LSL with lacto-N-neotetraose and related saccharides inhibits cell binding and formation of trans-membrane pores, indicating that the lectin receptor contains N-acetyllactosamine glycoconjugates. LSL contains in its N-terminal region three tandemly repeated subdomains resembling segments of the ricin B-chain. The N-terminus recognizes carbohydrates, since mutants with a C-terminal deletion retain cell agglutination function but lose haemolytic activity. The C-terminal region is also involved in oligomerization and pore formation [160].

6. Hydralysins from Cnidaria (Eukaryote, Aquatic Animals)

Recently, a new group of toxins designated as the hydralysins has been described in the literature [162, 163]. These toxins are produced by the cnidaria *Chlorohydra viridis* (Table **1**), are distinct from other cnidarian toxins, and show similarities in activity and structure to toxins of the aerolysin-like toxin family. A functional characterization of hydralysins performed by Sher *et al.* [162] reveals that hydralysins are secreted as soluble monomers rich in β-structure [162]. Furthermore, it was shown that hydralysins bind to erythrocyte membranes via an unknown receptor(s), form discrete pores of ~1.2 nm diameter (Table **1**), and are cytolytic upon select cell types. Comparison of aerolysin, epsilon-toxin, alpha-toxin, and LSL sequences with hydralysin reveals conserved sequences, especially around the pore-forming domains. Since hydralysins are secreted onto captured prey by cells of the digestive system, it seems that such proteins are not involved in capturing prey. Instead, the function of hydralysins probably aids digestion of prey tissue. Therefore, hydralysins bind only to membranes of captured animals but evidently not to cells of *Chlorohydra* [163].

MEMBERS OF THE AEROLYSIN FAMILY SHARE A CHANNEL MOTIF WITH THE BINARY TOXINS OF GRAM-POSITIVE BACTERIA

Members of the aerolysin-like toxin family, and PFTs in general, are membrane-damaging toxins. They are generally active on the cell surface, causing pore formation and disruption of plasma membrane that subsequently leads to leakage and death of target cells. Although not described in this review, related β-PFTs include multi-component staphylococcal leukocidins and the oxygen-labile streptolysin O of *Streptococcus pyogenes*. These toxins form pores on cell membranes, but in addition streptolysin O mediates translocation of a NAD glycohydrolase into the cytosol of a target cell [164]. The channel-forming domains of leukocidins and streptolysin O share sequence homology with aerolysin-like toxin family members, especially epsilon-toxin. Similar ß-PFT structures and mechanisms are also used by certain intracellularly-active binary toxins to cross the lipid bilayer.

Binary toxins are structurally organized into two distinct proteins not linked in solution and corresponding to enzymatic (A) plus binding (B) components. The binding component is responsible for receptor recognition and docking/transportation of the enzymatic component across endosomal membranes. Similar to β-PFTs, binding components such as PA from the lethal/edema toxins of *B. anthracis*, Ib of *C. perfringens* iota toxin, and C2-II from C2-toxin of *C. botulinum* are organized into four domains essentially composed of hydrophilic β-strands. The N-terminal domain (domain 1) contains a propeptide and docking site for the enzymatic component, whereas the C-terminal domain (domain 4) is responsible for cell-surface receptor interactions [165, 166]. The smallest domain (domain 3) likely promotes oligomerization, and domain 2 contains long β-strands with an amphipathic flexible loop forming a β-hairpin intimately involved in pore formation [43, 167, 168]. Alignment of the putative channel-forming domain of epsilon-toxin with amphipathic beta hairpins of the binding components from binary iota- and C2-toxins show high sequence identity (Ib: 38%, C2-II: 22%) [169, 170]. In contrast to the aerolysin-like toxins and other cell membrane damaging toxins, the *Clostridium* and *Bacillus* binding components of binary toxins form pores at an acidic pH in endocytic vesicles and selectively transport enzymatic component into the cytosol [1]. Overall though, such data suggest that PFTs of the aerolysin-like and binary toxin families share common mechanisms of pore formation and a protein ancestor. As with many protein toxins, when closely scrutinized by various techniques and by different laboratories around the world, there are remarkable commonalities in structure and modes of actions. Perhaps more remarkable is how such proteins, which are true evolutionary successes, have become conserved amongst very diverse types of organisms and plants.

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ABBREVIATIONS

PFT	=	Pore-forming toxin
PA	=	Protective antigen
LSL	=	*Laetiporus sulphureus* lectin
Mtxs	=	Mosquitocidal toxins
(CD)-	=	Circular Dichroism spectroscopy spectroscopy
RTX-toxins	=	Repeats in **ToXin**-toxins
IP3	=	Inositol(1,4,5)-triphosphate
MAPK	=	Mitogen-activated protein kinase
hFR	=	Human folate receptor
SAG-family	=	Sensitive to apoptosis gene family
GFP	=	Green-Fluorescence Protein
DRMs	=	Detergent resistant microdomains
MDCK cells	=	Madin-Darby canine kidney cells

GPI   =   Glycosyl-phosphatidylinositol

LD_{50} =   Mouse median lethal dose

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