CHAPTER 1.28

Bacterial Toxins

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Introduction

Toxins were the first bacterial virulence factors to be identified and were also the first link between bacteria and cell biology. Cellular microbiology was, in fact, naturally born a long time ago with the study of toxins, and only recently, thanks to the sophisticated new technologies, has it expanded to include the study of many other aspects of the interactions between bacteria and host cells. This chapter covers mostly the molecules that have been classically known as toxins; however, the last section also mentions some recently identified molecules that cause cell intoxication and have many but not all of the properties of classical toxins. Tables 1 and 2 show the known properties of all bacterial toxins described in this chapter, while Figure 1 shows the subunit composition and the spatial organization of toxins whose structures have been solved either by X-ray crystallography or by quick-freeze deep-etch electron microscopy.

Abbreviations: SEA–SEI, staphylococcal enterotoxin A through I; TSST-1, toxic shock syndrome toxin 1; SPEA, B and C, streptococcal pyrogenic enterotoxins A, B and C; ETA and B, exfoliative toxins A and B; MHC, major histocompatibility complex; Vβ or Vγ, T-cell-receptor variable domains; LukF, leucocidin F; PA, protective antigen; RTX, repeats-in-toxin; CryIA, CytB, G_i, G_s, G_o, G_t, G_{olf}, GTP-binding proteins; MAPKK1 and 2, mitogen-activated protein kinases 1 and 2; EF2, elongation factor 2; Rho, Rac and Cdc42, GTP-binding proteins that control assembly of actin stress fibers; IL2, 4 and 5, interleukins 2, 4 and 5; TeNT, tetanus neurotoxin; VAMP, vesicle-associated membrane protein; BoNT, botulism neurotoxin; SNAP, synaptosome-associated protein; YOP, *Yersinia* outer-membrane proteins; AvrRxv, plant-pathogen virulence protein; Ipa, invasion-plasmid antigen; ICE, interleukin-converting enzyme; Sop, *Salmonella* outer-membrane protein; Tir, translational initiation region; CagA, cytotoxin-associated gene A; YpkA, *Yersinia* protein kinase A.

For abbreviations, refer to the footnote in Table 1.

Toxins have a target in most compartments of eukaryotic cells. For simplicity, the toxins are divided into three main categories (Fig. 2): 1) those that exert their powerful toxicity by acting on the surface of eukaryotic cells simply by touching important receptors, by cleaving surface-exposed molecules, or by punching holes in the cell membrane, thus breaking the cell permeability barrier (panel 1); 2) those that have an intracellular target and hence need to cross the cell membrane (these toxins need at least two active domains, one to cross the eukaryotic cell membrane and the other to modify the toxin target) (panel 2); and 3) those that have an intracellular target and are directly delivered by the bacteria into eukaryotic cells (panel 3).

Toxins Acting on the Cell Surface

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

Toxins Acting on the Immune System (Superantigens)

Superantigens (Fig. 2, panel 1) are bacterial and viral proteins that share the ability to activate a large fraction of T-lymphocytes. They are bivalent molecules that have been shown to simultaneously bind two distinct molecules, the major histocompatibility complex (MHC) and the T-cell receptor variable domains (Vβ or Vγ; Kotzin et al., 1993; Fig. 3). Binding of these molecules to MHC class II requires no prior processing and occurs outside the antigen-binding groove. This results in the activation of between 2–15% of all T cells, ultimately leading to T-cell proliferation, the production of a variety of cytokines, and expression of cytotoxic activity.

Bacterial superantigens, also known as pyrogenic toxins, comprise a class of secreted proteins mostly produced by *Staphylococcus aureus* and *Streptococcus pyogenes* (Bohach et al., 1990; Alouf and Muller-Alouf, 2003). So far, they include the group of staphylococcal enterotoxins (SEA, SEB, SECn, SED, SEE, SEG, SEH and

Table 1. Classes of toxins described in the text, their features and activity.

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
Toxins acting on the cell surface	Immune system (Superantigens)	SEA-SEI, TSST-1, SPEA, SPEC, SPEL, SPEM, SSA, and SMEZ	Staphylococcus aureus and Streptococcus pyogenes	Binding to MHC class II molecules and to Vβ or Vγ of T cell receptor	T cell activation and cytokines secretion	SEB SEC2, SEC3, SED, SEH TSST1, SPEA SPEC
		MAM	Mycoplasma arthritidis	Binding to MHC class II molecules and to Vβ or Vγ of T cell receptor	Chronic inflammation	+
		YPMa	Yersinia pseudotuberculosis	Binding to MHC class II molecules and to Vβ or Vγ of T cell receptor	Chronic inflammation	−
		SPEB	S. pyogenes	Cysteine protease	Alteration in immunoglobulin-binding properties	+
		ETA, ETB, and ETD	S. aureus	Trypsin-like serine proteases	T-cell proliferation, intraepidermal layer separation	ETA, ETB
	Surface molecules	BFT enterotoxin	Bacteroides fragilis	Metalloprotease, cleavage of E-cadherin	Alteration of epithelial permeability	−
		AhyB	Aeromonas hydrophyla	Elastase, metalloprotease	Hydrolization of casein and elastine	−
		Aminopeptidase	Pseudomonas aeruginosa	Elastase, metalloprotease	Corneal infection, inflammation and ulceration	−
		ColH	Clostridium histolyticum	Collagenase, metalloprotease	Collagenolytic activity	−
		Nhe	Bacillus cereus	Metalloprotease and collagenase	Collagenolytic activity	−
	Cell membrane			Cell membrane permeabilization	Cell death	
	Large pore-forming toxins	PFO	C. perfringens	Thiol-activated cytolysin, cholesterol binding	Gas gangrene	+
		SLO	S. pyogenes	Thiol-activated cytolysin, cholesterol binding	Transfer of other toxins, cell death	−
		LLO	Listeria monocytogenes	Induction of lymphocyte apoptosis	Membrane damage	−
		Pneumolysin	S. pneumoniae	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	−
	Small pore-forming toxins	Alveolysin	B. alveis	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	−

Category	Toxin	Organism	Mechanism	Effect	
	ALO	B. anthracis	Induction of lymphocyte apoptosis	Complement activation, cytokine production, apoptosis	–
	α-Toxin	S. aureus	Binding of erythrocytes	Release of cytokines, cell lysis, apoptosis	+
	PVL leukocidin (LukS-LukF)	S. aureus	Cell membrane permeabilization	Necrotic enteritis, rapid shock-like syndrome	LukF
	γ-Hemolysins (HlgA-HlgB and HlgC-HlgB)	S. aureus	Cell membrane permeabilization	Necrotic enteritis, rapid shock-like syndrome	HlgB
	β-Toxin	C. perfringens	Cell membrane permeabilization	Necrotic enteritis, neurologic effects	–
RTX toxins	Hemolysin II	B. cereus	Cell membrane permeabilization	Hemolytic activity	–
	CytK	B. cereus	Cell membrane permeabilization	Necrotic enteritis	–
	HlyA	E. coli	Calcium-dependent formation of transmembrane pores	Cell permeabilization and lysis	–
Membrane-perturbing toxins	ApxI, ApxII, and ApxIII	A. pleuropneumoniae	Calcium-dependent formation of transmembrane pores	Lysis of erythrocytes and other nucleated cells	–
	LtxA	A. actinomycetem-comitans	Calcium-dependent formation of transmembrane pores	Apoptosis	–
	LktA	P. haemolytica	Calcium-dependent formation of transmembrane pores	Activity specific versus ruminant leukocytes	–
Other pore-forming toxins	δ-Hemolysin	S. aureus	Perturbation of the lipid bilayer	Cell permeabilization and lysis	–
	Aerolysin	A. hydrophila	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+
	AT	C. septicum	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+ (model)
Insecticidal toxins	PA	B. anthracis	Perturbation of the lipid bilayer	Cell permeabilization and lysis	+
	HlyE	E. coli	Perturbation of the lipid bilayer	Osmotic lysis of cells lining the midgut	+
	CryIA, CryIIA, CryIIIA, etc	Bacillus thuringiensis	Destruction of the transmembrane potential	Osmotic lysis of cells lining the midgut	CryIA, CryIIIA

(Continued)

Table 1. *Continued*

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
		CytA, CytB	*B. thuringiensis*	Destruction of the transmembrane potential	Osmotic lysis of cells lining the midgut	CytB
		BT toxin	*B. thuringiensis*	Destruction of the transmembrane potential	Cytocidal activity on human cells	–
Toxins acting on intracellular targets	Protein synthesis	DT	*Corynebacterium diphtheriae*	ADP-ribosylation of EF-2	Cell death	+
		PAETA	*P. aeruginosa*	ADP-ribosylation of EF-2	Cell death	+
		SHT	*S. dysenteriae*	N-glycosidase activity on 28S RNA	Cell death, apoptosis	+
	Signal transduction	PT	*Bordetella pertussis*	ADP-ribosylation of G_i	cAMP increase	+
		CT	*Vibrio cholerae*	ADP-ribosylation of G_s	cAMP increase	+
		LT	*E. coli*	ADP-ribosylation of G_s	cAMP increase	+
		α-Toxin (PLC)	*C. perfringens*	Zinc-phospholipase C, hydrolase	Gas gangrene	+
		Toxins A and B (TcdA and TcdB)	*C. difficile*	Monoglucosylation of Rho, Rac, Cdc42	Breakdown of cellular actin stress fibers	–
		Adenylate cyclase (CyaA)	*B. pertussis*	Binding to calmodulin ATP→cAMP conversion	cAMP increase	–
		Anthrax edema factor (EF)	*B. anthracis*	Binding to calmodulin ATP→cAMP conversion	cAMP increase	+
		Anthrax lethal factor (LF)	*B. anthracis*	Cleavage of MAPKK1 and MAPKK2	Cell death, apoptosis	+
		Cytotoxin necrotizing factors 1 and 2 (CNF1, 2)	*E. coli*	Deamidation of Rho, Rac and Cdc42	Ruffling, stress fiber formation.	CNF1 (catalytic domain)
		DNT	*Bordetella species*	Transglutaminase, deamidation or polyamination of Rho GTPase	Ruffling, stress fiber formation	–
		CDT	*Several species*	DNA damage, formation of actin stress fibers via activation of RhoA	Cell-cycle arrest, cytotoxicity, apoptosis	–
Cytoskeleton structure		Toxin C2 and related proteins	*C. botulinum*	ADP-ribosylation of monomeric G actin	Failure in actin polymerization	–

		Toxin	Organism	Mechanism	Effect	
		Lymphostatin	E. coli	Block of interleukin production	Chronic diarrhea	–
		Iota toxin and related proteins	C. perfringens	Block of interleukin production	Chronic diarrhea	+ (C2I)
Intracellular trafficking		TeNT	C. tetanii	Cleavage of VAMP/synaptobrevin	Spastic paralysis	+ (Hc domain)
		BoNT-B, D, G and F neurotoxins	C. botulinum	Cleavage of VAMP/synaptobrevin	Flaccid paralysis	BoNT-B
		BoNT-A, E neurotoxins	C. botulinum	Cleavage of SNAP-25	Flaccid paralysis	BoNT-A
		BoNT-C neurotoxin	C. botulinum	Cleavage of syntaxin, SNAP-25	Flaccid paralysis	–
Toxins injected into eukaryotic cells	Mediators of apoptosis	Vacuolating cytotoxin VacA	H. pylori	Alteration in the endocytic pathway	Vacuole formation, apoptosis	–
		NAD glycohydrolase	S. pyogenes	Keratinocyte apoptosis	Enhancement of GAS proliferation	–
		IpaB	Shigella	Binding to ICE	Apoptosis	–
		SipB	Salmonella	Cysteine proteases	Apoptosis	–
		YopP/YopJ	Yersinia species	Cysteine protease, blocks MAPK and NFkappaB pathways	Apoptosis	–
	Inositol phosphate metabolism	SopB	Salmonella species	Inositol phosphate phosphatase, cytoskeleton rearrangements	Increased chloride secretion (diarrhea)	–
		IpgD	S. flexneri	Inositol phosphate phosphatase, cytoskeleton rearrangements	Increased chloride secretion (diarrhea)	–
	Cytoskeleton	ExoS	P. aeruginosa	ADP-ribosylation of Ras, Rho GTPase	Collapse of cytoskeleton	+ (GAP domain)
		C3 exotoxin	C. botulinum	ADP-ribosylation of Rho	Breakdown of cellular actin stress fibers	+
		EDIN-A, B and C	S. aureus	ADP-ribosylation of Rho	Modification of actin cytoskeleton	EDIN-B
		SopE	S. typhimurium	Rac and Cdc42 activation	Membrane ruffling, cytoskeletal reorganization, proinflammatory cytokines production	+
		SipA	S. typhimurium	Rac and Cdc42 activation	Membrane ruffling, cytoskeletal reorganization, proinflammatory cytokines production	+

(Continued)

Table 1. *Continued*

Class of toxin	Target	Toxin	Organism	Activity	Consequence	X-ray
		IpaA	*Shigella species*	Vinculin binding	Depolymerization of actin filaments	−
		YopE	*Yersinia species*	GAP activity towards RhoA, Rac1 or Cdc42	Cytotoxicity, actin depolymerization	+
		YopT	*Yersinia species*	Cysteine protease, cleaves RhoA, Rac, and Cdc42 releasing them from the membrane	Disruption of actin cytoskeleton	−
		VirA	*Shigella flexneri*	Inhibition of tubulin polymerization	Microtubule destabilization and membrane ruffling	−
	Signal transduction	YpkA	*Yersinia species*	Protein serine/threonine kinase	Inhibition of phagocytosis	−
		YopH	*Yersinia species*	Tyrosine phosphatase	Inhibition of phagocytosis	+
		Tir	*E. coli EPEC*	Receptor for intimin	Actin nucleation and pedestalformation	−
		CagA	*H. pylori*	Tyrosine phosphorylated	Cortactin dephosphorylation	−
		YopM	*Yersinia species*	Interaction with PRK2 and RSK1 kinases	Cytotoxicity	+
		SptP	*S. typhimurium*	Inhibition of the MAP kinase pathway	Enhancement of Salmonella capacity to induce TNF-alpha secretion	+
		ExoU	*P. aeruginosa*	Lysophospholipase A activity	Lung injury	−
Toxins with unknown mechanism of action		Zot	*V. cholerae*	?	Modification of intestinal tight junction permeability	−
		Hemolysin BL (HBL)	*B. cereus*	Hemolytic, dermonecrotic and vascular permeability activities	Food poisoning, fluid accumulation and diarrhea	−
		BSH	*L. monocytogenes*	?	Increased bacterial survival and intestinal colonization	−

Abbreviations: SEA-SEI, staphylococcal enterotoxins; TSST, toxic shock syndrome toxin; SPE, streptococcal exotoxin; SSA, streptococcal superantigen; SMEZ, streptococcal mitogenic exotoxin z; MAM, *Mycoplasma* arthritidis mitogen; YPMa, *Y. pseudotuberculosis*-derived mitogen; ETA and ETB, exfoliative toxins; ColH, collagenase; Nhe, nonhemolytic entertoxin; PFO, perfringolysin O; SLO, streptolysin O; LLO, listeriolysin O; ALO, anthrolisin O; AT, α-toxin; PA, protective antigen; DT, diphtheria toxin; PAETA, *Pseudomonas aeruginosa* exotoxin A; SHT, Shiga toxin; PT, pertussis toxin; CT, cholera toxin; LT, heat-labile enterotoxin; DNT, dermonecrotic toxin; CDT, cytolethal distending toxin; TeNT, tetanus neurotoxin; RTX, repeats in the structural toxin; Hly, hemolysin; Cry, crystal; BoNT, botulinum neurotoxin; Ipa, invasion plasmid antigen; Sip, *Salmonella* invasion protein; EDIN, epidermal cell differentiation inhibitor; Sop, *Salmonella* outer protein; Ipg, invasion plasmid gene; Yop, *Yersinia* outer protein; GAP, GTPase-activating protein; GAS, group A *Streptococcus*; Vir, virulence protein; YpkA, *Yersinia* protein kinase A; Tir, translocated intimin receptor; EPEC, enteropathogenic *E. coli*; CagA, cytotoxin-associated gene A; SptP, *Salmonella* protein tyrosine phosphatase; VAMP, vesicle-associated membrane protein; ICE, interleukin-1β-converting enzyme; SNAP, synaptosome-associated protein; MAPKK, mitogen-activated protein kinase kinase; Zot, zonula occludens toxin; and BSH, bile salt hydrolase.

Table 2. Toxins classified according to their enzymatic activities.

Toxin	Substrate	Effect
Glucosyl-transferases		
Clostridium difficile toxins A and B	Rho/Ras GTPases	Breakdown of cytoskeletal structure
Deamidases		
E. coli CNF1	Rho, Rac and CdC42	Stress fiber formation
Bordetella DNT	Rho	Stress fiber formation
ADP-ribosyltransferases		
DT	Elongation factor EF-2	Cell death
PAETA	Elongation factor EF-2	Cell death
PT	G_i, G_o and transducin	cAMP increase
CT	G_s, G_t and G_{olf}	cAMP increase
E. coli LT	G_s, G_t and G_{olf}	cAMP increase
Clostridium botulinum C2	Actin	Failure in actin polymerization
P. aeruginosa ExoS	Ras	Collapse of cytoskeleton
Clostridium botulinum C3	Rho	Breakdown of cellular actin stress fibers
N-Glycosidases		
Shiga toxin	Ribosomal RNA	Stop of protein synthesis
Metalloproteases		
Bacillus anthracis LF	Macrophages	Disruption of normal homoeostatic functions
Clostridium tetanii TeNT	VAMP/synaptobrevin	Spastic paralysis
C. botulinum BoNTs	VAMP/synaptobrevin, SNAP-25	Flaccid paralysis

Abbreviations: CNF1, cytotoxin necrotizing factor 1; DNT, dermonecrotic factor; DT, diphtheria toxin; PAETA, *Pseudomonas aeruginosa* exotoxin A; PT, pertussis toxin; CT, cholera toxin; LT, heat-labile enterotoxin; ExoS, exoenzyme S; LF, lethal factor; TeNT, tetanus neurotoxin; BoNT, botulinum neurotoxin; VAMP, vesicle associated membrane protein; and SNAP-25, synaptosome-associated protein of 25kDa.
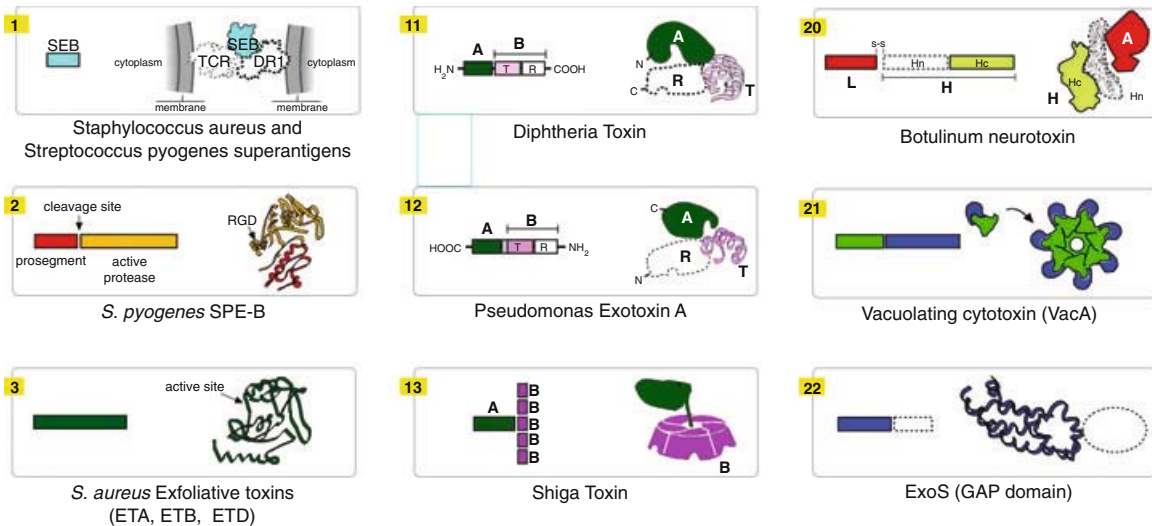

Fig. 1. Structural features of bacterial toxins. (Left) Scheme of the primary structure of each toxin. For the A/B toxins, the domain composition is also shown. The A (or S1 in PT) represents the catalytic domain, whereas the B represents the receptor-binding domain. The A subunit is divided into the enzymatically active A1 domain and the A2 linker domain in Shiga toxin, CT, *Escherichia coli* LTI and LTII, and PT. The B domain has either five subunits, which are identical in Shiga toxin, CT, and *E. coli* LTI and LTII and different in size and sequences in PT, or two subunits (the translocation [T] and the receptor-binding [R] subunits) in DT, Pseudomonas exotoxin A, botulinum toxin, and tetanus toxin. (Right) Schematic representation of the three-dimensional (3D) organization of each toxin. For *Staphylococcus* enterotoxin B, the protein is shown in the ternary complex with the human class II histocompatibility complex molecule (DR1) and the T-cell antigen receptor (TCR). For *Salmonella* SptP, the structure is shown in the transition state complex with the small GTP binding protein Rac1. Similarly, toxin SopE is represented in complex with its substrate Cdc42. In the case of *E. coli* CNF1 and *Pseudomonas* ExoS, only one domain has been crystallized. In the case of SipA, a 3D reconstruction of SipA bound to F-actin filaments is also reported. For all toxins, the schematic representation is based on the X-ray structure, except that for VacA, whose structure has been solved by quick-freeze, deep-etch electron microscopy.
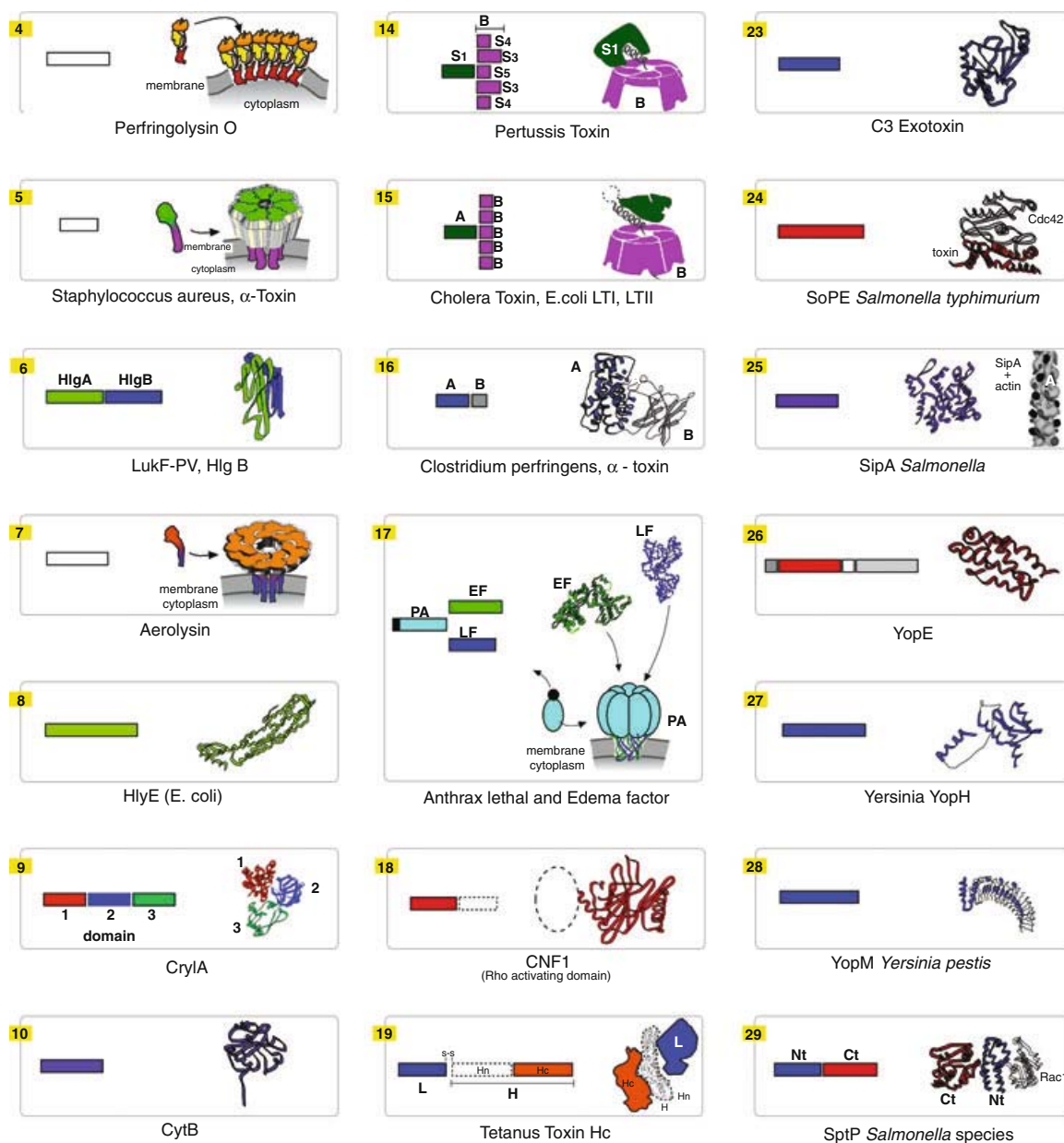
(*Continued*)

4 membrane cytoplasm
Perfringolysin O

14 B, S4, S1, S3, S5, S3, S4, S1, B
Pertussis Toxin

23
C3 Exotoxin

5 membrane cytoplasm
Staphylococcus aureus, α-Toxin

15 A, B, B, B, B, B
Cholera Toxin, E.coli LTI, LTII

24 Cdc42 toxin
SoPE Salmonella typhimurium

6 HlgA HlgB
LukF-PV, Hlg B

16 A B, A, B
Clostridium perfringens, α - toxin

25 SipA + actin A
SipA Salmonella

7 membrane cytoplasm
Aerolysin

17 LF, EF, PA, EF, LF, PA, membrane cytoplasm
Anthrax lethal and Edema factor

26
YopE

8
HlyE (E. coli)

27
Yersinia YopH

9 1 2 3 domain, 1, 2, 3
CryIA

18
CNF1
(Rho activating domain)

28
YopM Yersinia pestis

10
CytB

19 s-s, Hn, Hc, L, H, L, Hc, Hn, H
Tetanus Toxin Hc

29 Nt Ct, Rac1, Ct, Nt
SptP Salmonella species

Fig. 1. *Continued.*

SEI), exfoliative toxins (ETA and ETB), the toxic shock syndrome toxin-1 (TSST-1; Dinges et al., 2000), the streptococcal pyrogenic enterotoxins (SPEA and SPEC; Papageorgiou et al., 1999) and streptococcal superantigen SSA (Sundberg and Jardetzky, 1999).

These toxins play an important role in diseases such as the staphylococcal toxic shock syndrome induced by TSST-1 (Schlievert et al., 1981), vomiting and diarrhea caused by staphylococcal enterotoxins, and the exanthemas caused by the pyrogenic streptococcal exotoxins. Furthermore, these toxins have been linked to the pathogenesis of several acute or chronic human disease states such as the Kawasaki syndrome (Leung et al., 1993), which is the leading cause of acquired heart disease among children in the United States, and to the pathogenesis of other life-threatening events such as food poisoning (Blackman and Woodland, 1995).

In addition to their functional similarities, the staphylococcal enterotoxins share a number of genetic and biochemical characteristics, as well as similar primary (Schlievert et al., 1995) and 3D structures (Swaminathan et al., 1992; Prasad et al., 1993; Papageorgiou et al., 1995; Schad et al., 1995). The genes for these toxins are generally carried on plasmids, bacteriophage chromosomes, or other heterologous genetic elements (Lindsay et al., 1998; Zhang et al., 1998), and all
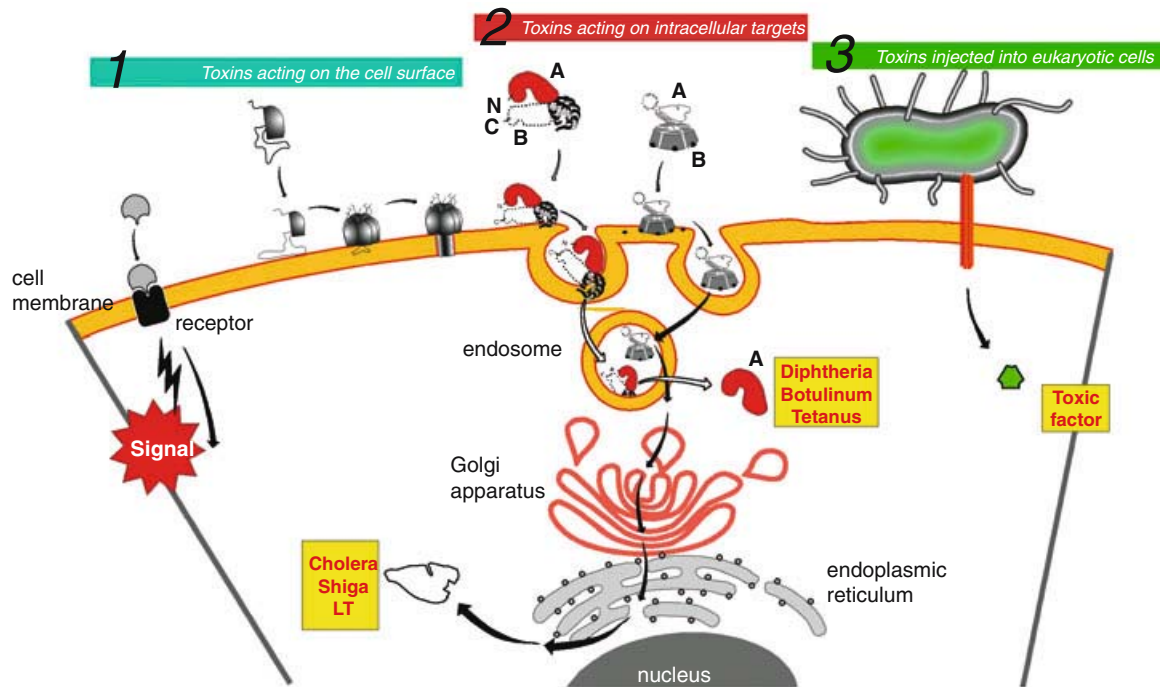
Fig. 2. Schematic representation of the three groups of bacterial toxins. Group 1 toxins act either by binding receptors on the cell membrane and sending a signal to the cell or by forming pores in the cell membrane, perturbing the cell permeability barrier. Group 2 toxins are A/B toxins, composed of a binding domain (B subunit) and an enzymatically active effector domain (A subunit). Following receptor binding, the toxins are internalized and located in endosomes, from which the A subunit can be transferred directly to the cytoplasm by using a pH-dependent conformational change or can be transported to the Golgi and the endoplasmic reticulum (ER), from which the A subunit is finally transferred to the cytoplasm. Group 3 toxins are injected directly from the bacterium into the cell by a specialized secretion apparatus (type III or type IV secretion system).
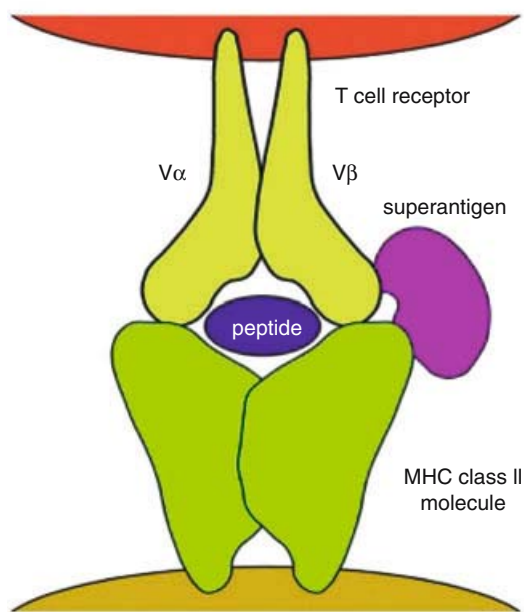
Fig. 3. Schematic representation of the interaction of a superantigen with a major histocompatibility complex (MHC) class II molecule and T-cell receptor.

of them are translated into a precursor protein containing an amino terminal signal sequence that is cleaved during export from the cell. The mature products are small nonglycosylated polypeptide molecules with molecular weights ranging from 20 kDa to 30 kDa and are moderately stable to chemical inactivation, proteolysis and denaturation by boiling.

Staphylococcal and streptococcal superantigens share 20–80% sequence similarity (Fig. 4); in particular, staphylococcal SEA is more related to SEE and SED, whereas SEB has greater homology with SEC, TSST-1, and streptococcal superantigens SPEA and SSA. The overall homology found in the staphylococcal enterotoxins has been suggested to stem from duplication of a gene encoding a common "ancestral" toxin (Iandolo, 1989).

Computer analysis of the *S. pyogenes* genome has revealed the presence of novel superantigen genes, and among them the one coding for the mitogenic exotoxin Z (SMEZ). This toxin is particularly similar to the SPE-C group of superantigens and, although present in all group A streptococci (GAS) strains, it shows extensive
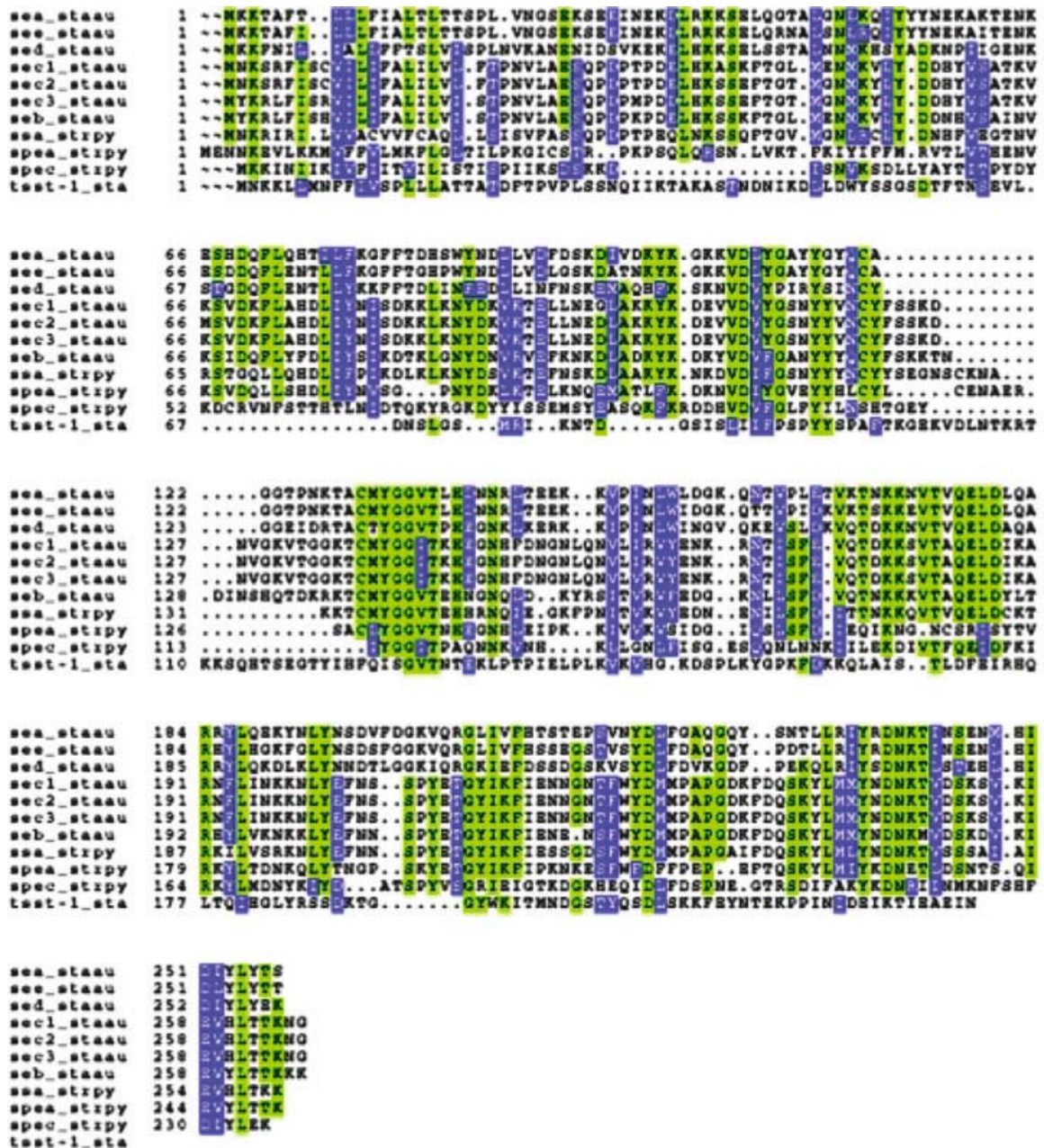
Fig. 4. Multiple sequence alignment of staphylococcal and streptococcal superantigens. Green indicates identity, whereas blue stands for amino acid similarity.

allelic variation. Further genetic characterization has shown that SMEZ is the most potent bacterial superantigen so far discovered and that it strongly contributes to the immunological effects of GAS both in vitro and in vivo by eliciting a robust cytokine production (Unnikrishnan et al., 2002).

Three novel streptococcal genes (*spe-g*, *spe-h* and *spe-j*) have been identified from the *Streptococcus pyogenes* M1 genomic sequence, while a fourth novel gene (*smez-2*) was isolated from the strain 2035. Of these, SMEZ-2, SPE-G and SPE-J are most closely related to streptococcal

pyrogenic exotoxin SPEC, whereas SPE-H is more similar to the staphylococcal toxins than to any other streptococcal toxin (Proft et al., 1999).

Finally, other pyrogenic toxin superantigens recently discovered by genome mining include proteins SPEL and SPEM produced by several isolates of *S. pyogenes* of the M18 serotype. The corresponding genes are contiguous and coded within a bacteriophage. Both toxins were shown to be lethal in different animal models and to directly participate in the host-pathogen inter-

Fig. 5. Comparison of the X-ray structures of SEA (*left*), SEB (*right*) and TSST-1 (*below*). The colors follow the secondary structure succession where the N-terminus is blue, the C-terminus is red, and the long central helix is pale yellow. The zinc atom and the coordination site are colored pink and the cysteines involved in the disulfide bond are dark-red.
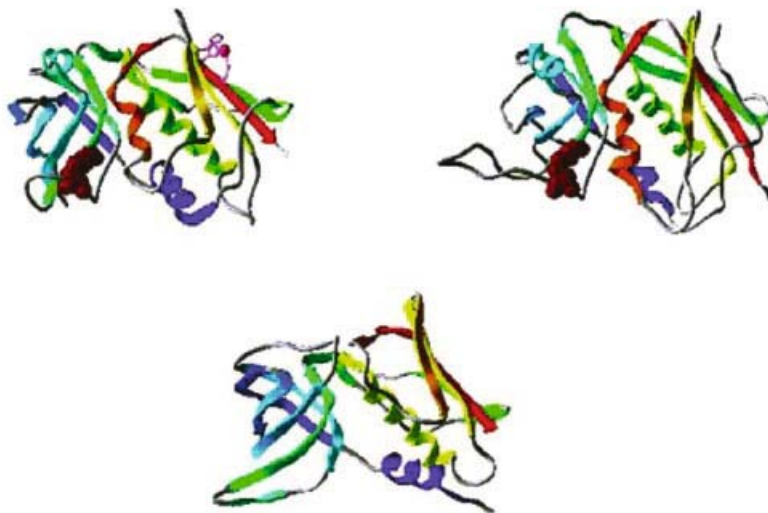
action in some acute rheumatic fever (ARF) patients (Proft et al., 2003).

Crystallographic structures are currently available for most of the described staphylococcal and streptococcal superantigens, such as SEA (Schad et al., 1995), SEB (Swaminathan et al., 1992), SEC2 (Papageorgiou et al., 1995), SEC3 (Fields et al., 1996), SED (Sundstrom et al., 1996), TSST-1 (Prasad et al., 1993; Prasad et al., 1997), SPEA (Papageorgiou et al., 1999), SPEB (Kagawa et al., 2000), SPEC (Roussel et al., 1997) and SSA (Sundberg et al., 1999). However, primary sequence homology among superantigens does not assure homology in their secondary and tertiary structures, and vice versa; in fact SEA, SEB, SEC and TSST-1, despite their low level of sequence similarity, all fold into very similar 3D structures. Below are the X-ray structures of SEA, SEB and TSST-1 that share a very similar fold despite low levels of sequence similarity that range from less than 20% identity in the case of SEA and TSST-1, to 33% in the case of SEA and SEB.

All of these toxins have a characteristic two-domain fold composed of a β-barrel at the *N*-terminus and a β-grasp at the C-terminus connected by a long α-helix that diagonally spans the center of the molecule (Fig. 5). Moreover, all of these toxins are characterized by a central disulfide bond (with the exception of TSST-1, which has no cysteines) and by a Zn^{+2} coordination site which is believed to be involved in MHC class II binding (Abrahmsen et al., 1995).

The presence of two zinc-binding sites in SpeC indicates different modes in the assembly of the MHC-superantigen-T-cell receptor (TcR) trimolecular complex.

The crystal structures of SEB and TSST-1 in complex with an MHC class II molecule, and those of SEC2/SEC3 in complex with a TcR Vβ chain have been solved (Li et al., 1998; Fields et al., 1996). As an example, the complex between SEB and the Vβ domain of a TcR is reported (Fig. 6).

Superantigen molecules have also been identified in other pathogens, where they represent important virulence determinants.

MaM is a T-cell mitogen produced by *Mycoplasma arthritidis*, which contributes to the acute and chronic inflammatory disease mediated by this organism (Cole and Atkin, 1991). The recently determined X-ray structure of MaM in complex with HLA-DR1 has revealed that this protein has a fold and a mode of binding, which are entirely different from those of the known pyrogenic superantigens (Zhao et al., 2004; Fig. 7).

Another superantigenic toxin is the YPMa produced by a subset of *Yersinia pseudotuberculosis* strains. This 14.5-kDa protein was originally purified from bacterial lysates and found to exert a mitogenic activity on human peripheral blood
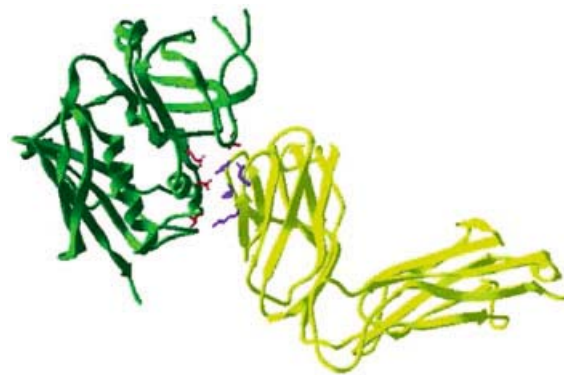
Fig. 6. Crystal structure of the complex between SEB (green) and TcR (yellow). The residues involved in hydrogen bonds between the two molecules have side-chains colored in red and blue, respectively.
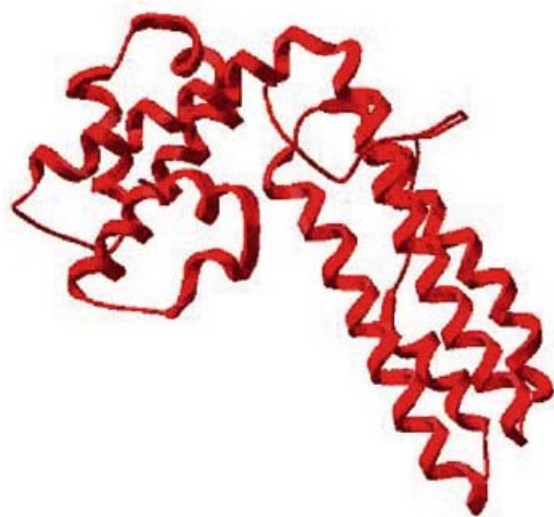
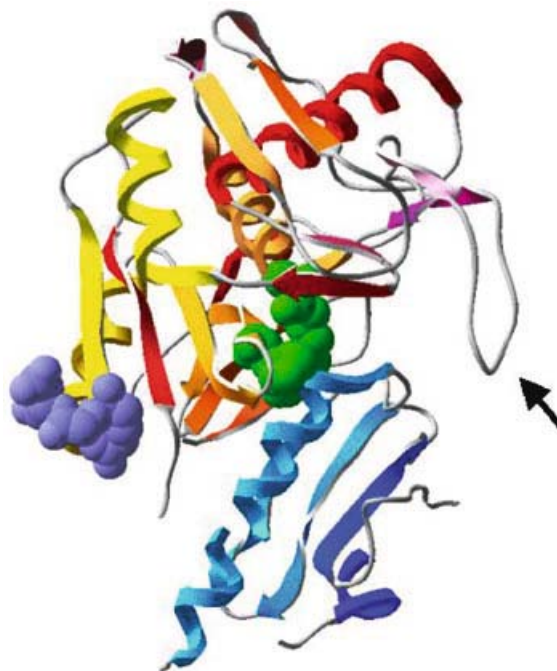Fig. 7. Three-dimensional structure of MaM superantigen.

Fig. 8. The three-dimensional structure of the precursor form of streptococcal cysteine protease SpeB. The prosegment (blue) and active protease (yellow-orange) are indicated with different color scales. The solvent-exposed Arg-Gly-Asp (RGD) motif is violet, and the active site (Cys-47-His-195-Trp-212) is buried by the prosegment and is colored in green. The highly conserved finger loop is also indicated (arrow).

mononuclear cells. Although the precise role of this protein is currently unknown, the data show that YPMa contributes to the virulence of *Y. pseudotuberculosis* in systemic infection in mice (Carnoy et al., 2000).

Other toxins that have long been known as superantigens are the streptococcal pyrogenic exotoxin B (SPEB), a virulence factor with cysteine protease activity produced by all isolates of group A streptococci, and the exfoliative toxins A and B produced by *S. aureus* (Fig. 2, panels 2 and 3).

Although these proteins strongly contribute to the virulence of the corresponding microorganism, their role as mitogenic factors has been disproved when it was shown that all the nonrecombinant forms were in fact contaminated with trace amounts of the SMEZ superantigen (Unnikrishnan et al., 2002).

SPEB appears to contribute to *S. pyogenes* pathogenesis in several ways, including proteolytic cleavage of human fibronectin and vitronectin, two abundant extracellular matrix proteins involved in maintaining host tissue integrity. SPEB causes a cytopathic effect on human endothelial cells and represents a critical virulence factor in human infection and in mouse models of invasive disease. Despite low levels of sequence similarity, this toxin can be considered as a structural homologue of the papain superfamily that also includes the mammalian cathepsins B, K and L (Kagawa et al., 2000). Like other proteases, the enzyme SpeB is produced as an inactive precursor (zymogen) of 40 kDa which, following autolytic cleavage of the N-terminal 118 residues, is converted to the mature, active 27.6-kDa protease. The catalytic site lacks the Asn residue generally present in the catalytic Cys-His-Asn triad, which is in this case substi-

tuted by a Trp. The structure also reveals the presence of a surface-exposed integrin-binding Arg-Gly-Asp (RGD) motif that is a feature unique to SpeB among cysteine proteases and is linked to the pathogenesis of the most invasive strains of *S. pyogenes* (Stockbauer et al., 1999). Sequence analysis performed on more than 200 streptococcal isolates has revealed an overall limited structural variation in SPEB, with the entire active site being completely conserved. Interestingly, the prominent finger loop that extends from the N-terminal domain (Fig. 8) is also invariant, suggesting that antibodies directed against this region could be effective therapeutic agents.

The exfoliative toxins ETA and ETB of *Staphylococcus aureus* are produced during the exponential phase of growth and excreted from colonizing staphylococci before being absorbed into the systemic circulation. They have been recognized as the causative agents in staphylococcal scalded skin syndrome, an illness characterized by specific intraepidermal separation of the layers of skin between the stratum spinosum and the stratum granulosum (Ladhani et al., 1999). The two ETs are about 40% identical, with no apparent sequence homology to other bacterial
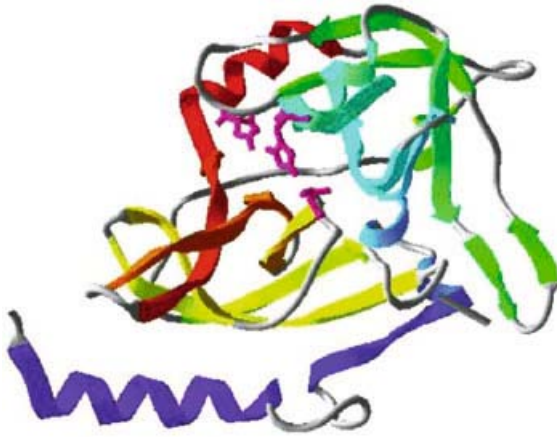
Fig. 9. Crystal structure of exfoliative toxin A (ETA) of *Staphylococcus aureus*. The three residues of the catalytic triad responsible for the serine protease activity are colored in magenta.

toxins. Both superantigens have been proved to act as serine proteases, and this enzymatic activity could be one of the mechanisms hypothesized as the cause of epidermal separation. In fact, at least in the case of ETA (Fig. 8), substitution of the active site serine residue with cysteine abolishes its ability to produce the characteristic separation of epidermal layers but not its ability to induce T-cell proliferation (Redpath et al., 1991). The two ETs are about 40% identical, with no apparent sequence homology to other bacterial toxins. The overall structures of ETA and ETB are similar to that of the chymotrypsin-like serine protease family of enzymes, with the catalytic triad being composed of His-57, Asp-102 and Ser-195 (Vath et al., 1997, 1999).

Recently, a novel member of the exfoliative group of toxins has been discovered in *S. aureus*. This protein, termed "ETD," is encoded within a pathogenicity island, which also contains the genes for a serine protease and the edin-B gene. When injected in neonatal mice as recombinant protein, ETD has been shown to induce exfoliation of the skin with loss of cell-to-cell adhesion in the upper part of the epidermis (Yamaguchi et al., 2002).

Toxins Acting on Surface Molecules

Bacteroides fragilis enterotoxin (BFT) is a protein of 186 residues that is secreted into the culture medium. The toxin has a zinc-binding consensus motif (HEXXH), characteristic of metalloproteases and other toxins such as tetanus and botulinum toxins. In vitro, the purified enterotoxin undergoes autodigestion and can cleave a number of substrates including gelatin, actin, tropomyosin and fibrinogen. When added to cells in tissue culture, the toxin cleaves the 33-kDa extracellular portion of E-cadherin, a 120-kDa transmembrane glycoprotein (responsible for calcium-dependent cell-cell adhesion in epithelial cells) that also serves as a receptor for *Listeria monocytogenes*. In vitro, BFT does not cleave E-cadherin, suggesting that the membrane-embedded form of E-cadherin is necessary for cleavage.

BFT causes diarrhea and fluid accumulation in ligated ileal loops. In vitro, it is nonlethal but causes morphological changes such as cell rounding and dissolution of tight clusters of cells. The morphological changes are associated with F-actin redistribution. In polarized cells, BFT is more active from the basolateral side than from the apical side, decreases the monolayer resistance, and causes dissolution of some tight junctions and rounding of some of the epithelial cells, which can separate from the epithelium. In monolayers of enterocytes, BFT increases the internalization of many enteric bacteria such as *Salmonella*, *Proteus*, *E. coli* and *Enterococcus* but decreases the internalization of *L. monocytogenes* (Sears, 2001).

BFT belongs to a large family of bacterial metalloproteases that usually cleave proteins of the extracellular matrix. *Pseudomonas aeruginosa* and *Aeromonas hydrophila* elastases (aminopeptidase and AhyB) and *Clostridium histolyticum* collagenase (ColH) are the best-known examples (Yoshihara et al., 1994; Cascon et al., 2000; Cahan et al., 2001).

Lately, a novel member of this family of protein toxins has been identified in *Bacillus cereus*. The protein, termed "Nhe" (nonhemolytic enterotoxin), is a 105-kDa metalloprotease, which shares homologies to the above-mentioned elastases and collagenases. Biochemical characterization has shown that Nhe possesses both gelatinolytic and collagenolytic activities (Lund and Granum, 1999).

Toxins Acting on the Cell Membrane

Protein toxins forming pores in biological membranes occur frequently in Gram-positive and Gram-negative bacteria (Braun and Focareta, 1991). Pore-forming toxins, also known as "lytic factors," work by punching holes in the plasma membrane of eukaryotic cells, thus breaking the permeability barrier that keeps macromolecules and small solutes selectively within the cells (Sugawara et al., 1997; Gilbert, 2002; Fig. 2, panel 1). Because erythrocytes have often been used to test the activity of these toxins, some of them are also called "hemolysins"; however, whereas erythrocytes appear to be very good targets in vitro, they are never the main physiological targets of this class of proteins in vivo (Tomita et al., 1997).

The pathogenicity of the toxin-producing organisms in eukaryotes is clearly related to the toxins they produce. Furthermore, pore-forming toxins represent the most potent and versatile tool with which invading microbes damage the host cell (Bhakdi et al., 1994). Cell permeabilization exerted by the toxic activity of these proteins generally results in release of cytokines, activation of intracellular proteases, induction of apoptosis, and finally, death of the eukaryotic cell (Alouf and Geoffrey, 1991).

To generate channels and holes in the cell membrane, this class of toxins must be able to fold in a characteristic amphipathic structure typical of porins (Weiss et al., 1991; Cowan et al., 1992), with one side facing the internal hydrophilic cavity, and the other side interacting with the lipid chains or the nonpolar segments of integral membrane proteins.

Most of the toxins are produced or stored in a protoxin inactive form. The activation step varies from the cleavage of an N_{term} acidic peptide as in the case of melittin, to a C_{term} proteolytic cleavage as in aerolysin (van der Goot et al., 1992); in the particular case of the Gram-negative hemolysins (cytolysins), these toxins are usually synthesized as precursor proteins, then covalently modified to an acylated, active form and finally secreted via specific export systems, which differ for various types of hemolysins (Issartel et al., 1991; Stanley et al., 1994). All such steps increase the affinity for the membrane, which appears to be essential for activity.

A large proportion of these proteins are produced by Gram-positive bacteria and can be divided into large pore-forming and small pore-forming toxins on the basis of the dimension of the holes produced on the plasma membrane and also of the kind of interaction that they establish with the eukaryotic receptor. In addition, the pore-forming, repeats-in-toxin (RTX) family of toxins includes a large group of Ca^{+2}-dependent hemolysins (secreted by both Gram-positive and Gram-negative bacteria), which are characterized by a conserved glycine- and aspartate-rich motif of nine amino acids (Welch, 1991; Coote, 1992). Given their predominant role on cellular membranes, we have included in this section also the so-called "membrane perturbing toxins" and the insecticidal toxins produced by *Bacillus thuringensis*.

Large Pore-Forming Toxins

This class of cytolysins (Fig. 2, panel 2) comprises more than 20 family members, which are generally secreted by taxonomically diverse species of Gram-positive bacteria and which have the common property of binding selectively to cholesterol on the eukaryotic cell membrane (Alouf

and Geoffrey, 1991). Each toxin consists of a single 50- to 80-kDa polypeptide chain, and they are characterized by a pretty remarkable sequence similarity, also suggesting possible similar 3D structures. These proteins are produced by *Streptococcus pyogenes*, *S. pneumoniae*, *Bacillus*, a variety of *Clostridia*, including *Clostridium tetanii* and *C. perfringens*, and *Listeria*.
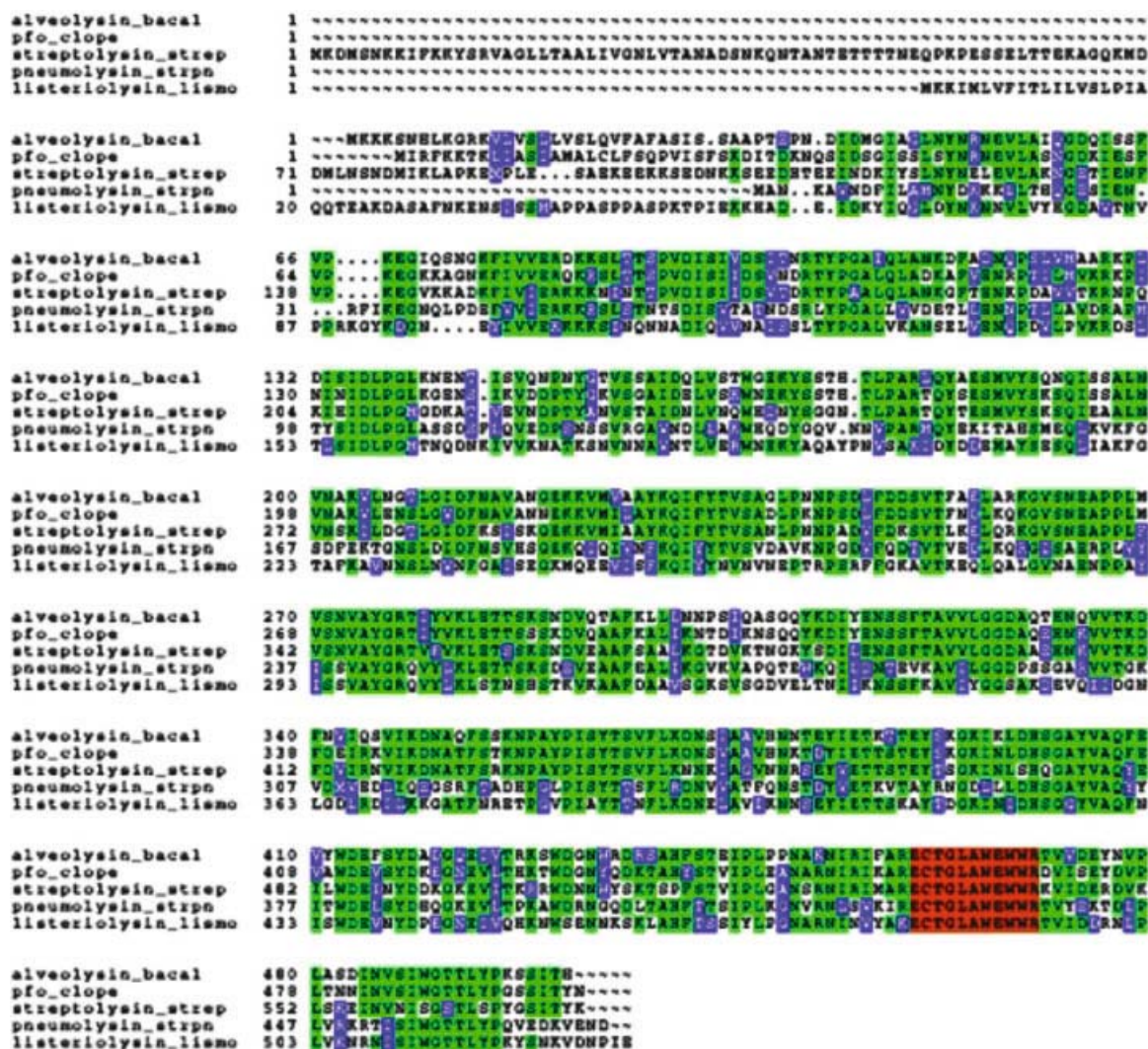
To date, the best characterized are perfringolysin O (PFO), a virulence factor of *Clostridium perfringens*, which causes gas gangrene (Rossjohn et al., 1997), streptolysin O, secreted by *Streptococcus pyogenes* (Kehoe et al., 1987), alveolysin, produced by Bacillus alvei (Geoffroy et al., 1990), and pneumolysin, the major causative agent of streptococcal pneumonia and meningitis (Rossjohn et al., 1998).

In addition to its role as a cytolysin, listeriolysin O (LLO), which is an essential virulence factor of *Listeria monocytogenes* (Gedde et al., 2000) has also been shown to induce lymphocyte apoptosis with rapid kinetics (Carrero et al., 2004).

These toxins share a similar mechanism of action, which consists of an interaction of monomeric toxin with target cells via cholesterol (their receptor), followed by oligomerization and insertion into the host cell membrane; this process ultimately results in serious membrane damage with formation of large pores with diameters exceeding 150 Å. All these toxins contain a common motif (boxed in Fig. 10), which is located approximately 40 amino acids from the carboxy terminus; this motif includes a Cys residue, which if oxidized abolishes the toxin's lytic activities. Lytic activity can be restored only upon addition of reducing agents such as thiols. However, despite their designation as "thiol-activated cytolysins," thiol activation is clearly not an important property of this group of toxins (Billington et al., 2000). Interestingly, the membrane-bound receptor, cholesterol, plays an important role in the oligomerization step as well as in membrane insertion and pore formation (Alouf and Geoffrey, 1991).

Crystallographic data are available only for the thiol-activated cytolysin (perfringolysin O; PFO; Fig. 1, panel 4) of *Clostridium perfringens* (Rossjohn et al., 1997). Nevertheless, given the high degree of sequence conservation (Fig. 10) detected within this class of protein toxins (ranging from the 43% identity of PFO and listeriolysin, to the 72% identity of PFO and alveolysin), this structure can be considered the prototype of the entire family (Fig. 11).

PFO is an unusually elongated rod-shaped molecule mainly composed of β-sheets; the monomer is made of four discontinuous domains, indicated with different colors in the picture. Domain 1 (green) has an α/β structure

Fig. 10. Multiple sequence alignment of proteins belonging to the class of large-pore-forming toxins. Green indicates identity, whereas blue stands for amino acid similarity.

containing a seven-stranded antiparallel β-sheet. Domain 2 (blue) consists mainly of four β-strands, while domain 3 (yellow) is comprised of an α/β/a structure. Finally, domain 4 (red) is folded into a compact β-sandwich consisting of multiple-stranded sheets.

The mechanism of membrane insertion is not clear; in fact, no canonical transmembrane domains can be identified along the primary structure and no significant patches of hydrophobic residues can be mapped on the surface of the molecule. Nevertheless, a model of the membrane-bound state, which takes into account the interaction with the cholesterol receptor as the first step for penetration of the hydrophobic bilayer core, has been proposed on the basis of electron microscopy and other experimental data. Several chemical modifications and mutagenesis studies have suggested the cholesterol-binding site to be located at the tip of domain 4 (Fig. 12), and in particular, it has been mapped within the highly conserved, Trp-rich segment (Michel et al., 1990; Hill et al., 1994). Proteolysis studies have further demonstrated that domain 4 is also the membrane-spanning domain, although the distribution of charged and hydrophobic residues on the β-sheet of this region is not compatible with an insertion into the lipid bilayer. From these studies, it has emerged that only the tip of the β-barrel domain D4 is responsible for membrane insertion and that a major conformational rearrangement takes place during pore formation (Shepard et al., 1998; Shatursky et al., 1999).

Taken together, these observations suggest a model of oligomer insertion. After the toxin binds to the cholesterol molecule, the aliphatic side chains neutralize the charged resi-
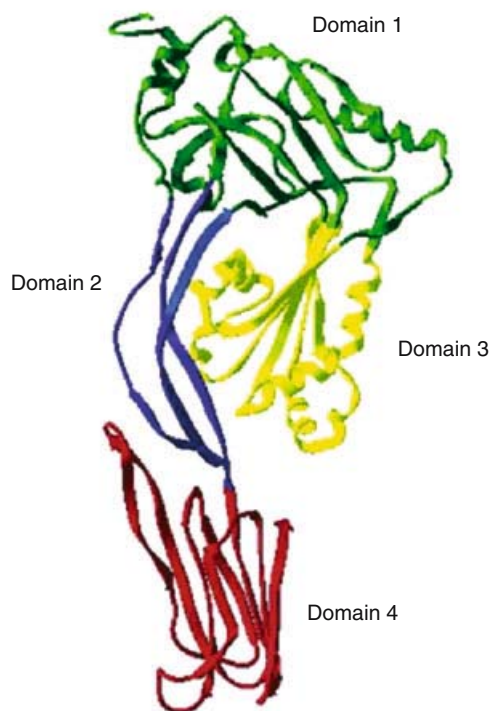
Fig. 11. Crystallographic structure and domain organization of perfringolysin O (PFO) produced by *Clostridium perfringens*.
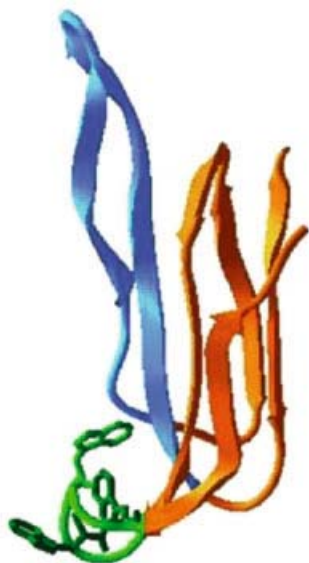

Fig. 12. Graphical representation of domain 4 of perfringolysin. The Trp-rich loop along with tryptophan side-chains are colored in green. In blue is the β-sheet probably involved in membrane insertion.
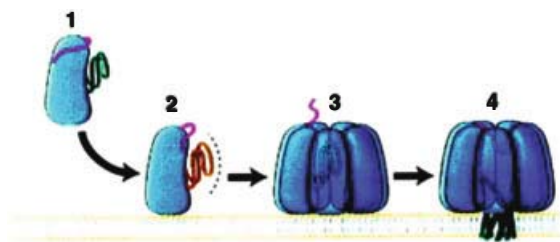

Fig. 13. General mechanism of assembly for small-pore-forming toxins: the stem region is initially folded against in the body of the water-soluble monomer; upon binding to the membranes and oligomerization, it subsequently undergoes conformational rearrangement and promotes insertion into the lipid bilayer.

could lead to and promote the final penetration step.

Furthermore, on the basis of recent data, a mechanism has been proposed whereby insertion into the bilayer occurs only after PFO monomers have assembled into a pre-pore state. Monomer-monomer interactions therefore not only promote insertion, but cooperative interactions between PFO monomers appear to be required to drive transmembrane insertion and β-barrel formation (Hotze et al., 2002). Recently, a protein belonging to this class of cytolysins has been identified in *Bacillus anthracis* and named "anthrolysin O" (ALO). This putative toxin is able to bind erythrocytes and could have a role in the virulence of anthrax (Shannon et al., 2003).

Small Pore-Forming Toxins

The family of small-pore-forming toxins acts by creating very small pores (1–1.5 nm of diameter) in the membrane of host cells, thus allowing their selective permeabilization to solutes with a molecular mass less than 2 kDa. Alpha toxin (α-hemolysin) is the prototype of a group of pore-forming toxins produced by most pathogenic strains of *Staphylococcus aureus* (Gray and Kehoe, 1984a; Song et al., 1996; Gouaux, 1998; Fig. 1, panel 5); other members of this family include leukotoxins, such as leukocidin F (LukF), leukocidin S (LukS), Panton-Valentine leukocidin (PVL) and γ-hemolysin (Prèvost et al., 1995; Tomita and Kamio, 1997; Olson et al., 1999; Pedelacq et al., 1999; Cooney et al., 1993) and the β-toxin of *Clostridium perfringens* (Steinthorsdottir et al., 2000; Tweten, 2001; Magahama et al., 2003). These staphylococcal and streptococcal proteins are secreted as water-soluble monomers and assemble on the surface of susceptible cells to form heptameric transmembrane channels of approximately 1 nm in diameter (Finck-Barbancon et al., 1993; Sugawara et al., 1997; Fig. 13).
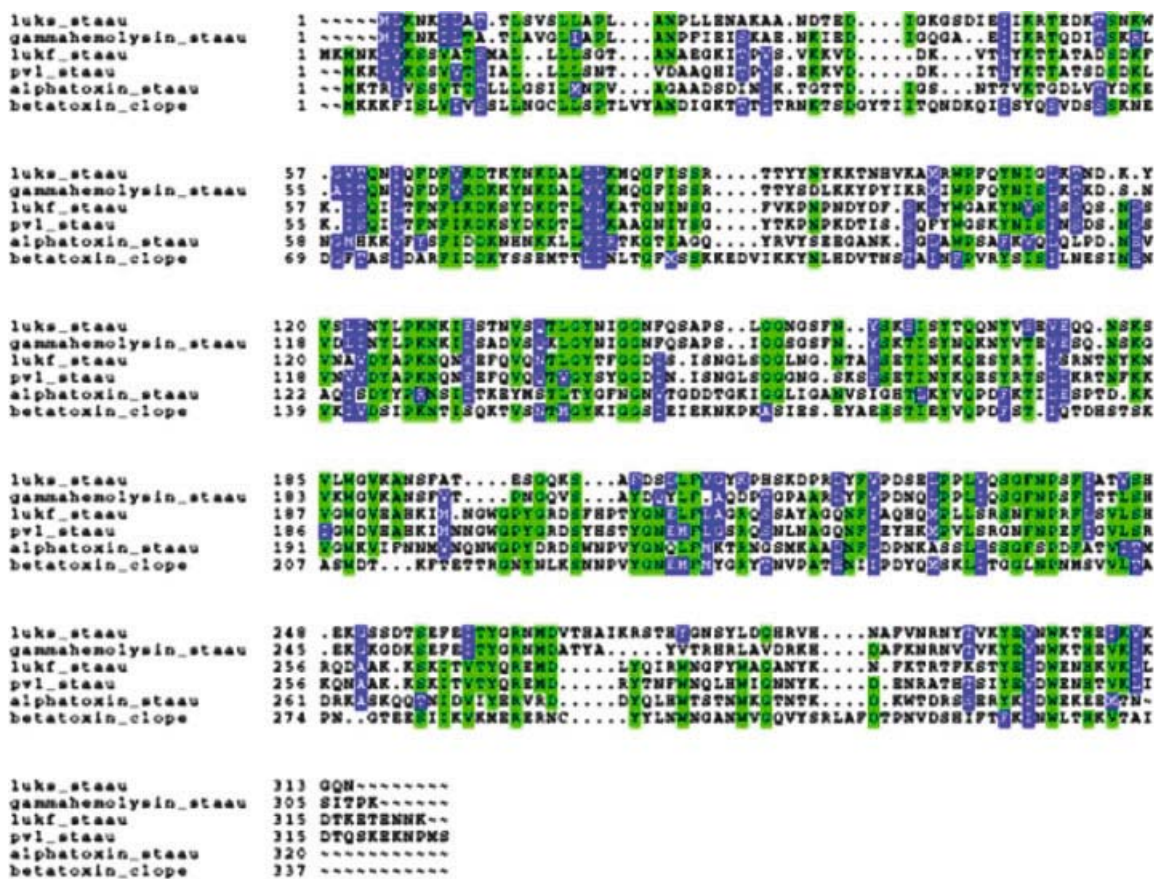
dues present on the β-sheet (blue) of domain 4 and then trigger membrane penetration. Consistent with this model is the hypothesis that the highly hydrophobic Trp-rich loop

```
luks_staau              1  ~~~~~~MKNKMMAL.TLSVSLLAPL...ANPLLENAKAA.NDTED....IGKGSDIEMIKRTEDKMSNKW
gammahemolysin_staau    1  ~~~~~~MMKNKMLTA.TLAVGLMAPL...ANPPIEISKAE.NKIED....IGQQA.EMIKRTQDIMSKPL
lukf_staau              1  MKMNKMKSSVAIMAL.LLLSGT...ANAEGKIMPMS.VKKVD....DK..VTMYETTATADSDKL
pvl_staau               1  ~~MKKMKSSVMMIAL.LLLSNT...VDAAQHIMPMS.EKKVD....DK..ITMYETTATSDSDKL
alphatoxin_staau        1  ~~MKTRMVSSVTMTLLLGSIMKMPV...AGAADSDINMK.TGTTD....IGS...NTTVKTGDLVMYDKE
betatoxin_clope         1  ~MKKKFISLMVMVSLLNGCLLSPTLVYANDIGKTMQTMTRNKTSDQYTIMTQNDKQIMSYQSMVDSMSKNE
```

```
luks_staau             57  ~VMMGNMQPDPMKDTKYNKDALMMMHQGFISMR....TTYYNYKKTNHVKAMRWPFQTNIGMMKDND.K.Y
gammahemolysin_staau   55  .AMMGNMQPDPMKDKHYNKDALMMMHQGFISMR....TTYSDLKKYPYIKRMIWPFQYNIS.KMPKD.S.N
lukf_staau             57  K..MMITMTPNPIKDKSYDKDTLVMMATGNIMIG...FVKPNPNDYDF..KMYMGAKYMWMSMSQS.NMS
pvl_staau              55  K..MMITMTPNPIKDKSYDKDTLMMMAAGNIYMG...YTKPNPKDTIS.QFYMGSKYNMEMMDS.NMS
alphatoxin_staau       58  NMNHKKMFPSPIDDKNHNHKKLLMMTKGTTAGQ....YRVYSEEGANK.GPAMDSAMKGQMQLPD.NMV
betatoxin_clope        69  DMFMBASMDARPIDDKYSSENTTMNLTGPMSEKKEDVIKKYNLHDVTHSMAMNPMVRSISMMLNESINMN
```

```
luks_staau            120  VSMMNTLPKNKIMSTNVSMTLGYNIGGNFQSAPS..LGGNGSFMM.MSKEISTGGNTVMSMEYGQ.NSKS
gammahemolysin_staau  118  VDLMNTLPKNKIMSADVSMKLGYNIGGNFQSAPS...IDGSGSFMM.MSKTISYMGKNVTRVMSQ.NSKG
lukf_staau            120  VNAMVDYAPKNQMMEFQVGMTLGYTFGGDMS.ISNGLSGGLNG.MTAMMETIMYKQESYAT.MSRNTNYKN
pvl_staau             118  VNMMVDYAPKNQMMEFQVGMTMGYSYGGD.N.ISNGLSGGGNG.SKSMMETIMYKQESTATSMMKATNFKK
alphatoxin_staau      122  AQMMSDYYMPKNSIMTKEYMSILTYGFNGNMTGDDTGKIMGLIGANVSIGHMKMVQPDMKIIMMSPTD.KK
betatoxin_clope       139  VKMMDSIPKNTMSQKTVSDMTMGYKIGGSMEIEKNKPKMSIRS.EYAEMSTIEMYVQPDMST.MQTDHSTSK
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luks_staau            185  VLMGVKANSFAT....ESDQKM...AMDSMLPMMYMPHSKDPRMYPMPDSEMMPLMQGGFNDSFMATVMSH
gammahemolysin_staau  183  VKMGVKANSFMT....DMGQVM...AYDMYLF.AQDPMGPAARMYPMPDNQMMPLMQGGFNDSFMTTLSM
lukf_staau            187  VGMGVEAHKIMM.NGWGPYGRDSFHPTYGNEMFLMMAGNGMSAYAGGNFMAQHQMMLLSRSNFNDRFMSVLSH
pvl_staau             186  VGMDVEAHKIMNNGWGPYGRDSYEHTYGNEMFLMMSMQMNLNAGGNFMMZYHKMMVLSRGNFNDEFMGVLSR
alphatoxin_staau      191  VGMKVIFNNMMMQNWGPYDRDSWNPVYGNQMFLF.KTENGSMKAAMNPMDPNKASSLLSHGFSDDFATVMMM
betatoxin_clope       207  ASMDT...KFTETTRGNYMNLKENNPVYGNMMMMYGKYMNVPATMNIMPDYQMSKLMTGGLNPNMSVVLMA
```

```
luks_staau            248  .EKMSSDTSEPEMMTYGRNMDVTHAIKRSTRMGNSYLDGHRVH....NAFVNRNYMVKYMMNWKTHEIMMK
gammahemolysin_staau  245  .EKMKGDKSEPEMMTYGRNMDATYA.....YVTHRRLAVDRKH....DAFKNRNVMVKYMMNWKTHEVKMML
lukf_staau            256  RQDMAAK.KSKMTVTYQREMD.....LYQIRWNGFYMAGANYK....N.FKTATFKSTYRMDWENEKVKMI
pvl_staau             256  KQNMAAK.KSKMTVTYQREMD.....RYTNFWNQLHWIGNNYK....D.ENRATHMSIYRMDWENETVKLMI
alphatoxin_staau      261  DRRMSKQQMNMDVIYERVRD.....DYQLHWTSTNMKGTNTK....D.KWTDRSMERAYKMDWEKEEMMN~
betatoxin_clope       274  PN..GTEEMSIEMKVKNERKRNC.....YYLNMNGANMVMQVYSRLAPMTPNVDSHIFTMGMWLTHKVTAI
```

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luks_staau            313  GGN~~~~~~~~
gammahemolysin_staau  305  SITPK~~~~~~
lukf_staau            315  DTKETENNK~~
pvl_staau             315  DTQSKEKNPMS
alphatoxin_staau      320  ~~~~~~~~~~~
betatoxin_clope       337  ~~~~~~~~~~~
```

Fig. 14. Multiple sequence alignment of proteins belonging to the family of small pore-forming toxins. Green and blue stand for amino acid identity and similarity, respectively.

The monomers have molecular weights of 33 kDa and are related in sequence and function (Fig. 14).

These toxins bind to human erythrocytes, monocytes, platelets, lymphocytes and endothelial cells, causing (at high concentrations) membrane rupture and cell lysis and death. Alpha-toxin has been recently shown to be the major mediator of caspase activation and apoptosis (Haslinger et al., 2003).

The structure of the transmembrane pore of staphylococcal α-toxin has been solved and has confirmed the heptameric structure of the oligomer (Song et al., 1996; Fig. 15). The complex is mushroom-shaped and measures 100 Å in height and up to 100 Å in diameter; the aqueous channel forms the transmembrane pore and spans the length of the entire complex ranging from 14 Å to 46 Å in diameter.

Each protomer (Fig. 16) is mainly composed of β-strand elements; two of these in particular constitute the stem domain, which contributes to the formation of the transmembrane pore in the heptameric form of the complex; a glycine-rich
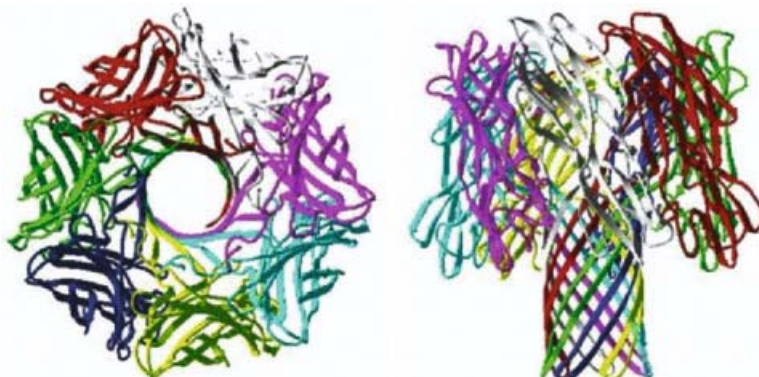

Fig. 15. Top and side views of the heptameric complex of a-toxin; each monomer is represented here with a different color (see Fig. 1, panel 5).
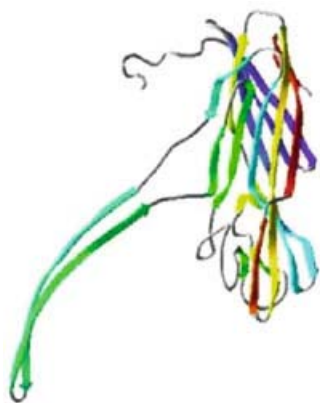
Fig. 16. Structure of the monomer of *S. aureus* α-toxin; the stem domain involved in pore formation protrudes outside of the core of the structure.
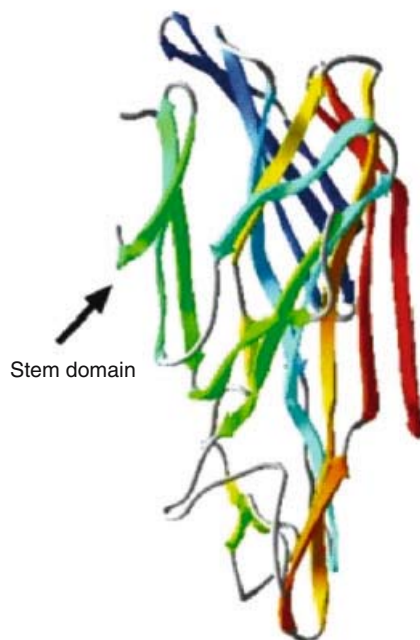

Fig. 17. Crystallographic structure determined for the protomer of toxin LukF; the glycine-rich, stem domain is in this case folded against the main body of the structure.

segment that is probably involved in solvent interaction characterizes this domain.

Leukotoxins and γ-hemolysin (HγII) should be grouped together, inasmuch as they form two types of bi-component complexes (LukF+LukS and LukF+HγII) that exhibit leukotoxic and hemolytic activity, respectively (Tomita and Kamio, 1997). Panton-Valentine leukocidin (PVL) is a closely related toxin carried by 2% of clinically isolated *S. aureus* strains and is also composed of type F and S components (Prèvost et al., 1995). The components of each protein class are produced as nonassociated, water-soluble proteins that undergo conformational changes and form oligomeric complexes after recognition of their cell targets, a process leading to transmembrane-pore formation and, ultimately, to cell death. The resultant transmembrane channels (estimated diameter 8 Å) are mainly permeable to divalent cations. Recently, fluorescence microscopy experiments have been performed to elucidate the mechanism of membrane insertion of the γ-hemolysin complex. This study shows that the three cooperative stages (dimer-dimer interaction, single pore assembly, and aggregation of pores) enhance the efficiency of assembly of oligomeric pores (Nguyen et al., 2003).

As representative of this class of bi-component toxins, consideration is given the X-ray structure of the Luk-F protomer (Olson et al., 1999; Fig. 17), which has been solved at a 1.90 Å resolution. The superposition of this monomer with that of α-toxin shows that the core structures are very similar despite the relatively low primary sequence identity (32%); nevertheless, a conformational change has affected the region of the glycine-rich stem domain, which appears in this case as a compact β-sheet folded against the body of the structure.

From a structural point of view, in contrast to a wide range of bacterial and insect toxins that utilize α-helices to perturb or penetrate the bilayer, these pore-forming toxins (members of an emerging family of proteins) can be defined by their use of bilayer-spanning antiparallel β-barrels instead.

Since the initial discovery of the first small pore-forming toxins, the number of these proteins has grown to include several members, among which are the recently identified hemolysin II (HlyII), and cytotoxin K (CytK) of *Bacillus cereus*, implicated in necrotic enteritis (Lund et al., 2000; Hardy et al., 2001; Miles et al., 2002).

RTX Toxins

Escherichia coli hemolysin (HlyA) is a 110-kDa protein, which can be considered as the prototype of a class of pore-forming toxins mainly produced by Gram-negative bacterial pathogens (Felmlee et al., 1985; Welch, 1991). This well-represented family includes a large number of calcium-dependent cytolysins known as RTX toxins, which are produced by different genera of Enterobacteriaceae and Pasteurellaceae. Characterized by the presence of a conserved repeated glycine- and aspartate-rich motif of nine amino acids, these cytolysins have multiple calcium-binding sites essential for function (Felmlee and Welch, 1988).

The toxin is encoded by four genes, one of which, *hlyA*, encodes the 110-kDa hemolysin.
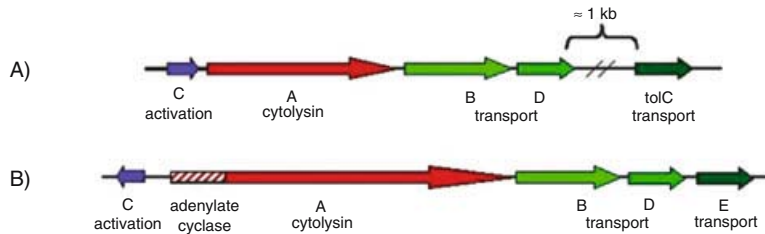
Fig. 18. Schematic representation of the genetic organization of RTX determinants; the genes encoding the Hly, Lkt, Aalt and Hpp proteins are organized in the same fashion, as illustrated in panel A, whereas the genes involved in synthesis and secretion of adenylate cyclase/hemolysin of *B. pertussis* display a somewhat different organization (panel B).

The other genes are required for its posttranslational modification (*hlyC*) and secretion (*hlyB* and *hlyD*). The four genes are found in a very limited number of *E. coli* clonal types, and can be sometimes located on transmittable plasmids (Smith and Halls, 1967). To give an idea of the level of toxicity associated with *hlyA* gene product, when non-hemolytic strains of *E. coli* are transformed with recombinant plasmids encoding the hemolysin, the transformants (in rodent models of peritonitis) are 10-fold to a 1000-fold more virulent than the parental strains. The receptor-binding domain of HlyA has been recently mapped (Cortajarena et al., 2003).

Other members of this class of RTX proteins include the adenylate cyclase/hemolysin of *Bordetella pertussis* (CyaA; Glaser et al., 1988), the ApxI-II and III hemolysins from Actinobacillus pleuropneumoniae (Maier et al., 1996), and the leukotoxins of *A. actinomycetemcomitans* (LtxA; Korostoff et al., 1998; Henderson et al., 2003) and of *Pasteurella haemolytica* (LktA; Chang et al., 1987; Wang et al., 1998).

Although a remarkable level of primary structure similarity can be detected among this group of toxins (20–60% identity), nevertheless they differ in host cell specificity and seem to adopt diverse mechanisms for cellular damage (Frey et al., 2002).

The synthesis and secretion of RTX toxins involve the participation of at least five different gene products; the organization of the five genes is very similar (Fig. 18, panel A), with the exception of B. pertussis bifunctional adenylate cyclase/hemolysin, where all five (*cyaC, A, B, D* and *E*) are found together (Glaser et al., 1988; Barry et al., 1991; Fig. 18, panel B); for the other family members, in fact, four of the genes are encoded within a single operon, whereas the fifth gene is located approximately 1 kb downstream (Welch and Pellett, 1988; Wandersman and Delepelaire, 1990).

The activation process performed by HlyC on HlyA ultimately results in the acquired capacity of HlyA to bind target cells; this activation involves proteolytic processing and posttranslational acylation, as well as binding of Ca^{+2} ions to the repeated domain.

Membrane-Perturbing Toxins

δ-Toxin or δ-hemolysin is secreted into the medium by *S. aureus* strains at the end of the exponential phase of growth. It is a 26-amino-acid peptide (MAQDIISTIGDLVKWI-IDTVNKFTKK) that has the general structure of soap with a nonpolar segment followed by a strongly basic carboxy-terminal peptide. The peptide has no structure in aqueous buffers but acquires an a-helical structure in low-dielectric-constant organic solvents and membranes. The α-helix has a typical amphipathic structure, which is necessary for the toxin to interact with membranes. The toxin binds nonspecifically parallel to the surface of any membrane without forming transmembrane channels. At high concentration, the peptide self-associates and increases the perturbation of the lipid bilayer that eventually breaks into discoidal or micellar structures. Interestingly, mellitin, which is also a 26-amino-acid lytic peptide produced by *S. aureus*, has no sequence homology with δ-toxin but has identical distribution of charged and nonpolar amino acids. These toxins are active in most eukaryotic cells. Cells first become permeable to small solutes and eventually swell and lyse, releasing cell intracellular content.

Recent data have demonstrated that δ-hemolysin insertion is strongly dependent on the peptide-to-lipid ratio, suggesting that association of a critical number of monomers on the membrane is required for activity. The peptide appears to cross the membrane rapidly and reversibly and cause release of the lipid vesicle contents during this process.

Other Pore-Forming Toxins

Additional members of this class of β-barrel, channel-forming toxins include aerolysin of *Aer-*
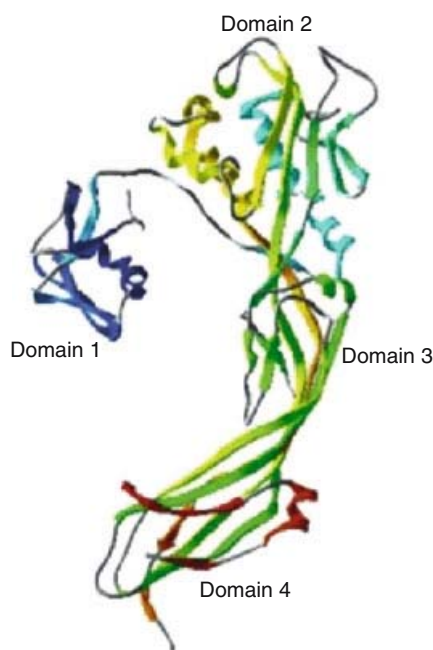
Fig. 19. X-ray structure of aerolysin toxin of *Aeromonas hydrophila*; the four domains are indicated; in particular, domain 1 clearly protrudes outside of the main body of the structure.

Fig. 20. Structure of the dimer of aerolysin and interaction between the two first domains.

omonas hydrophila (Parker et al., 1994; Rossjohn et al., 1998), and the closely related a-toxin of *Clostridium septicum* (Ballard et al., 1995), the anthrax toxin protective antigen PA of *Bacillus anthracis* (Petosa et al., 1997; Wesche et al., 1998), and the HlyE pore-forming toxin produced by pathogenic *E. coli*.

AEROLYSIN AND ALPHA-TOXIN (AT). Aerolysin (Fig. 1, panel 7) is mainly responsible for the pathogenicity of Aeromonas hydrophila, a bacterium associated with diarrheal diseases and wound infections (Altwegg and Geiss, 1989; Fivaz et al., 2001). It is secreted as a 52-kDa protoxin that is proteolytically cleaved into a 25-residue carboxy-terminal peptide and a 48-kDa active protein. Like other functionally related toxins, aerolysin changes its topology in a multistep process from a completely water-soluble form to a membrane-soluble heptameric transmembrane channel (ca. 1.5 nm in diameter) that destroys sensitive cells by breaking their permeability barriers.

Proaerolysin is a dimer in solution as well as in the crystal form (van der Goot et al., 1993; Parker et al., 1994); four structural domains characterize the monomer (Fig. 19).

In the structure of the dimer, the position of domain 1 appears to be stabilized by contacts with domain 1 of the other monomer, resulting in a very strict interaction of the two (Fig. 20).

Domain 4 is characterized by an amphipatic β-barrel structure, which is responsible for mem-

brane insertion of the final complex. In fact, oligomerization is an essential step in channel formation and it seems to precede membrane insertion. A model has been suggested for the entire process; it assumes that proaerolysin approaches the target cell as a water-soluble, hydrophilic dimer which, once concentrated on the surface of the target cell, binds to the receptor; subsequent proteolytic cleavage would cause dimer dissociation and oligomerization. This would ultimately result in an exposure of the hydrophobic region of the toxin and thus in membrane penetration.

Clostridium septicum AT is a channel-forming protein that is an important contributor to the virulence of the organism. Recent data have proved that this toxin, like aerolysin, binds to glycosylphosphatidylinositol (GPI)-anchored protein receptors. Furthermore, AT is also active against *Toxoplasma gondii* tachyzoites. Toxin treatment causes swelling of the parasite endoplasmic reticulum thus providing the first direct evidence that α-toxin is a vacuolating toxin (Ballard et al., 1995; Gordon et al., 1999). Recently, based on the available crystal structure of aerolysin, a molecular model of the membrane spanning domain of AT has been generated (Melton et al., 2004).

ANTHRAX PROTECTIVE ANTIGEN (PA). Anthrax protective antigen (PA; Fig. 1, panel 17) is one of the three components of the anthrax toxin complex secreted by *Bacillus anthracis*, which also includes the edema factor (EF) and the lethal factor (LF; Brossier et al., 2000; Collier and Young, 2003). Whereas EF and LF are responsible for the toxic activity, PA can be considered as the receptor-binding domain for two distinct A subunits, which are in turn EF and LF. The three subunits are encoded on a plasmid and are synthesized and secreted independently. Once on the host cell surface, PA needs a proteolytic activation to form a membrane-inserting heptamer through which EF and LF can be translocated (Klimpel et al., 1992; Milne and Collier, 1993; Milne et al., 1994; see Fig. 37 for the mechanism of action). The monomer is mainly constituted by antiparallel
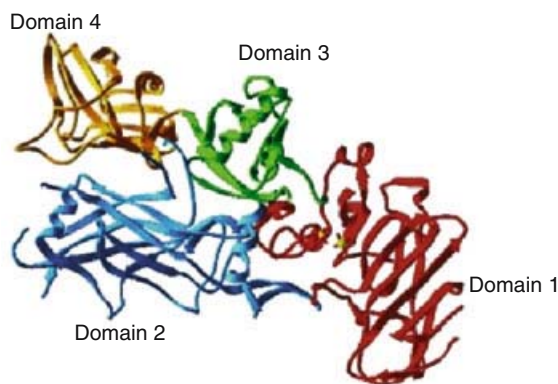
Fig. 21. X-ray structure of *Bacillus anthracis* protective antigen PA. The four structural domains are indicated by different colors. The two cysteines present in domain 1 are colored in yellow.
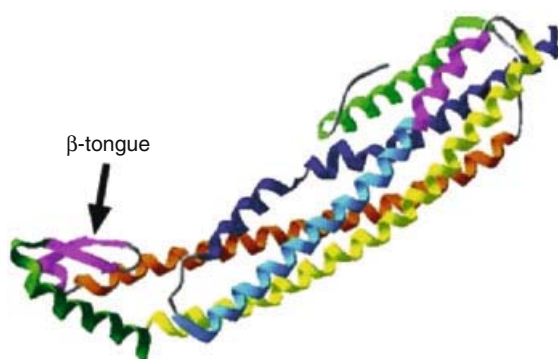

Fig. 22. X-ray structure of *E. coli* HlyE cytolysin. Two hydrophobic domains are present at the extremities of the α-helical bundle (colored in magenta).

β-sheets and contains four functional domains (Fig. 21). The crystallographic structure has revealed how PA can be assembled into heptamers and has suggested how some of the domains can undergo pH-driven conformational change.

Domain 1 (red) contains two Ca^{+2} ions (yellow) and the cleavage site for proteolytic activation; domain 2 (cyan) is the heptamerization domain and is implicated in membrane insertion; domain 3 (green) has an unknown function, whereas domain 4 (yellow) is for receptor-binding. Given its ability to promote the translocation of many heterologous proteins, PA is being evaluated as a general protein delivery system (Leppla et al., 1999).

ESCHERICHIA COLI HLYE. *Escherichia coli* produces a novel pore-forming toxin HlyE (Fig. 1, panel 8), which is completely unrelated to the *E. coli* hemolysin HlyA of the RTX family (Reingold et al., 1999; Wallace et al., 2000). Nevertheless, sequence comparison studies confirm the presence of highly homologous toxins in other pathogenic organisms such as *Salmonella typhi* and *Shigella flexneri* (these orthologs display 92–98% identity to HlyE). This observation suggests that HlyE could be the prototype of a new family of HlyE-like hemolysins specific for Gram-negative bacteria.

This new class of pore-forming toxins form cation-selective water-permeable pores (25–30 Å in diameter); the channel formation could be either part of a mechanism for iron acquisition by the bacterial cell, or it may promote bacterial infection by killing immune cells and causing tissue damage (Ludwig et al., 1999).

The crystal structure of HlyE has been solved (Wallace et al., 2000; Fig. 22).

The toxin has an elongated shape characterized by a four-helix (A–D) bundle topology with each helix approximately 70–80 Å long. Two predicted hydrophobic domains have been identified on the primary sequence: both are located at the extremities of the molecule, one being mainly composed of a short β-hairpin (β-tongue) folded between the third and fourth helices of the main bundle, and the other consisting of the C-terminal end (magenta) of helix B (cyan).

The precise mechanism of HlyE oligomerization to form the final transmembrane pore is at the moment unknown; nevertheless, the first step involves a process of dimerization of two HlyE molecules that pack in a head-to-tail fashion burying the two hydrophobic patches against each other. Electron microscopy experiments have led to a model of channel formation in which the possible oligomer topology is that of an octameric complex, and the β-tongue domain is primary responsible for interaction with the membrane.

Insecticidal Toxins

The class of insecticidal proteins, also known as δ-endotoxins, includes a number of toxins produced by species of *Bacillus thuringiensis*. These exert their toxic activity by making pores in the epithelial cell membrane of the insect midgut (Hofte and Whiteley, 1989; Knowles, 1994).

δ-Endotoxins form two multigenic families, *cry* and *cyt*; members of the cry family are toxic to insects of Lepidoptera, Diptera and Coleoptera orders (Hofmann et al., 1988), whereas members of the cyt family are lethal specifically to the larvae of Dipteran insects (Koni and Ellar, 1994). The insecticidal toxins of the cry family are synthesized by the bacterium as protoxins with molecular masses of 70–135 kDa; after ingestion by the susceptible insect, the protoxin is cleaved by gut proteases to release the active toxin of 60–70 kDa (Drobniewski and Ellar, 1989). In this form, they bind specifically and with high affinity to protein
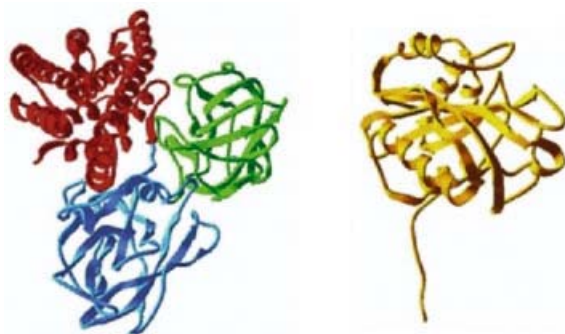
Fig. 23. Comparison of X-ray structures determined for representatives of the cry and cyt families of insecticidal δ-endotoxins: CryIA (left panel) is organized in three structural domains, whereas CytB (right panel) is a single-domain globular protein.

receptors and create channels 10–20 Å wide in the cell membrane. This subgroup includes several toxins (CryIA, CryIIIA, CryIV, CryV, etc.), whereas the only proteins so far characterized that belong to the cyt are CytA and CytB (Koni and Ellar, 1993; 1994).

Three-dimensional (3D) structures determined for members of the two families show that the folding of these toxins is entirely different. As representative of the two families, consideration is given to the structures of CryIA (Fig. 1, panel 9; Grochulski et al., 1995) and CytB (Li et al., 1996; Figs. 1 [panel 10] and 23), which share more than 39% sequence identity, suggesting an overall similar folding of the corresponding 3D structures.

The CryIA toxin is a globular protein composed of three distinct (but closely packed) domains connected by single linkers: domain 1 is totally α-helical, domain 2 consists of three antiparallel β-sheets and two short α-helices, and domain 3 is a β-sandwich. On the other hand, CytB (also a globular protein) is composed of a single domain with α/β architecture. The molecular mass of the protoxin is in this case only 30 kDa.

The region of CryIA, which has been associated with receptor–binding, maps within a loop of domain 2, whereas domain 1 has been shown to be responsible for membrane insertion and pore formation (Martens et al., 1995); this notion is strongly supported by the high structural similarity between the domain 1 of CryIA and that of CryIIIA to the pore-forming domains of colicin A and diphtheria toxin, both composed of helical bundles (Cabiaux et al., 1997; Duche et al., 1999). Conversely, in the case of the CytB/A, the model that has been proposed for the channel formation is based on a β-barrel structure.

Because they are toxic to several species of insects, δ-endotoxins have been formulated into

commercial insecticides, and these insecticides have been used for more than three decades. Recently, Lepidoptera-specific toxin genes have also been used to engineer insect-resistant plants (Christov et al., 1999).

Very recently, a novel crystal protein produced by *B. thuringiensis* has been identified. This toxin (BT) is noninsecticidal and nonhemolytic, but has strong cytocidal activity against various human cells. Its amino acid sequence has little homology with the other known insecticidal toxins, suggesting that BT might belong to a new group of *Bacillus thuringiensis* crystal toxins (Ito et al., 2004).

Toxins Acting on Intracellular Targets

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

The group of toxins with an intracellular target (A/B toxins) contains many toxins with different structures that have only one general feature in common: they are composed of two domains generally identified as "A" and "B." The A domain is the active portion of the toxin; it usually has enzymatic activity and can recognize and modify a target molecule within the cytosol of eukaryotic cells. The B domain is usually the carrier for the A subunit; it binds the receptor on the cell surface and facilitates the translocation of A across the cytoplasmic membrane (Fig. 2, panel 2). Depending on their target, these toxins can be divided into different groups that act on protein synthesis, signal transduction, actin polymerization, and vesicle trafficking within eukaryotic cells.

Toxins Acting on Protein Synthesis

These toxins are able to cause rapid cell death at extremely low concentrations. Two ADP-ribosylating bacterial proteins (see also the section ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity) are actually known to belong to this class of toxins: diphtheria toxin (DT) of *Corynebacterium diphtheriae* (Pappenheimer, 1977; Collier et al., 1982) and *Pseudomonas aeruginosa* exotoxin A (PAETA; Gray et al., 1984b; Wick et al., 1990). Both display their toxic activity by transferring the ADP-ribose moiety to a posttranslationally modified histidine residue of the cytoplasmic elongation factor 2 (EF2) of eukaryotic cells (Brown and Bodley, 1979; Van Ness et al., 1980). This reaction leads to the formation of a completely inactive EF2-ADP-ribose complex, which ultimately results in inhibition of protein

synthesis and cell death. From the biochemical point of view, the two toxins have a similar size, a signal peptide and disulfide bridges, and both are produced in iron-depleted medium. Nevertheless, they show a completely different amino acid composition and bind different cell receptors. In addition, Shiga toxin is another protein that exerts its toxic activity by interfering with protein synthesis.

DIPHTHERIA TOXIN. This toxin (DT; Fig. 1, panel 11) is a 535-amino acid polypeptide that is secreted into the growth medium by strains of toxinogenic *Corynebacterium diphtheriae*, and the polypeptide sequence is encoded by a lysogenic bacteriophage. Biosynthesis is regulated by an iron-binding protein, and proceeds only in the absence of iron (Qiu et al., 1995; Ding et al., 1996). The toxin is synthesized as a single polypeptide chain that is subsequently cleaved into two fragments, A and B of 21 kDa and 37 kDa, respectively (Pappenheimer, 1977).

From the functional point of view, three separate domains (C, T and R) are seen in the crystallographic structure of DT. The catalytic domain (C) entirely corresponds to the A subunit, whereas the translocation domain (T) and the carboxy-terminal, receptor-binding domain (R) are contained in fragment B (Choe et al., 1992; Bennett and Eisenberg, 1994).

From the structural point of view, the C domain (residues 1–191) has an $\alpha+\beta$ structure, the receptor-binding domain is a flattened β-barrel with a jelly-roll-like topology, whereas the translocation domain T (residues 201–384) consists in nine helices, two of which may participate in the pH-triggered membrane insertion. The molecule contains four cysteines and two disulfide bridges: one joins fragment C to fragment T and the other is contained within fragment R (Fig. 24).

Although the toxicity of DT is entirely due to the enzymatic activity carried on by fragment A (Fig. 25), fragment B is absolutely required for the cell intoxication process.

After secretion from *Corynebacterium diphtheriae*, the toxin binds to the DT receptor and is internalized by receptor-mediated endocytosis. In the endosome, the acidic environment triggers a conformational change of the B subunit that exposes the hydrophobic regions of the T domain allowing the interaction with the endosomal membrane and the translocation of the amino-terminal catalytic domain C across the membrane to the cytosol. According to a recent model, the A subunit of DT is able to cross the endosomal membrane making use of a metastable transmembrane domain, which has also been identified (Wolff et al., 2004). The toxin receptor is the heparin-binding, epidermal growth factor (EGF)-like precursor (Naglich et al., 1992;
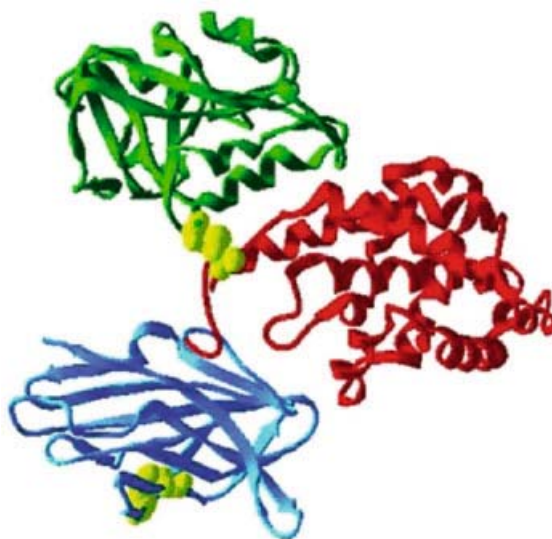

Fig. 24. X-ray structure of diphtheria toxin. The three functional domains are indicated with different colors: the catalytic domain C is green, the translocation domain T is red and the receptor-binding domain R is cyan. The two disulfide bridges are colored yellow.
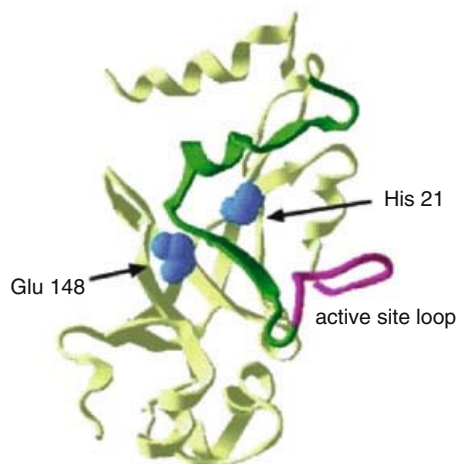

Fig. 25. Crystal structure of the isolated catalytic domain of diphtheria toxin. The scaffold of the enzymatic cleft is green, and the two described catalytic residues are blue. The "active-site loop" is represented here in the "closed" conformation.

Hooper and Eidels, 1995) that is present in most mammalian cells; nevertheless, the receptors of murine cells contain a few amino acid substitutions that make rodents insensitive to DT.

Diphtheria toxin is one of the most potent bacterial toxins: in vitro experiments have shown that a single molecule of the enzymatically active fragment A is by itself able to kill one eukaryotic cell (Yamaizumi et al., 1978).

Biochemical and mutagenesis studies have greatly contributed to the understanding of structure-function relationships and to the mapping of the catalytic residues. In particular,

His-21 has been mutagenized with a number of different residues and has been found to be essential for catalysis (Papini et al., 1989; Blanke et al., 1994); in fact, some activity was maintained only when Asn replaced His. In a similar manner, Glu-148 was identified as an active-site residue by photoaffinity labeling experiments with nicotinamide adenine dinucleotide (NAD; Carroll et al., 1985) and subsequent site-directed mutagenesis studies; in this case, not even a conservative substitution with Asp could be possible without complete loss of activity (Tweten et al., 1985). Whereas the possible function for His-21 could be that of maintaining the integrity of the active-site pocket, Glu-148 is likely to be involved in the interaction with the upcoming substrate molecule. Later, crystallographic data confirmed and extended the experimental observations, and added a number of other important residues to the list of the catalytic ones.

A very important step in the elucidation of the mechanism of enzymatic activity has been the determination of the crystal structure for the complex of diphtheria toxin with NAD (Bell and Eisenberg, 1997). Upon the addition of NAD to nucleotide-free DT crystals, a significant structural change affects the region encompassing residues 39–46. This portion of the C domain constitutes a mobile loop that becomes disordered after the formation of the complex. The best hypothesis to explain this observation is that NAD enters the cavity upon displacement of the mobile loop, which is then made available for the recognition and binding of the acceptor substrate EF-2. This would explain why DT recognizes EF-2 only after NAD has bound (see the section A Common Structure of the Catalytic Site in this Chapter).

Detoxified diphtheria toxin has been used in the formulation of a vaccine against toxinogenic strains of *Corynebacterium diphtheriae* (Porro et al., 1980; Rappuoli, 1983).

PSEUDOMONAS AERUGINOSA EXOTOXIN A. This exotoxin (PAETA; Fig. 1, panel 12) is a 66-kDa single-chain protein that inhibits protein synthesis (by a mechanism of action identical to that of DT) in eukaryotic cells by catalyzing the transfer of the ADP-ribosyl moiety of oxidized NAD onto elongation factor 2 (Brown and Bodley, 1979; Van Ness et al., 1980; Gray et al., 1984b; Wick et al., 1990; see the section ADP-ribosylating Toxins in this Chapter). Exotoxin A is the most toxic of the proteins secreted by the opportunistic pathogen *Pseudomonas aeruginosa*, having an LD_{50} of 0.2 mg upon intraperitoneal injection into mice. Secreted in the supernatant as an enzymatically inactive proenzyme; this toxin must undergo structural alteration to be able to perform its ADP-ribosylating activity.

According to X-ray crystallography (Allured et al., 1986; Li et al., 1995; 1996b), the molecule can be divided into three functional domains. The receptor-binding domain I binds to the ubiquitous α2-macroglobulin receptor of eukaryotic cells, thus initiating receptor-mediated endocytosis. This domain is composed primarily of anti-parallel β-structure and is arranged in two noncontiguous regions that encompass residues 1–252 (Ia) and 365–399 (Ib), respectively. Domain II maps within amino acids 253–364, is composed mostly of hydrophobic α-helices, and mediates the translocation of the enzymatically active carboxy-terminal domain III (residues 400–613) to the cytosol of infected cells. Furthermore, it has been shown that for domain III to be functional, a specific proteolytic cleavage at residue 280 of domain II is needed.

Genetic studies based on the expression of mutated forms of the exotoxin A gene in *E. coli* have confirmed these functional assignments. In fact, whereas deletion of domain Ia results in nontoxic, enzymatically active molecules that cannot bind the cells, deletions in domain II give rise to molecules that bind to the cells, are enzymatically active, but are not toxic; finally, deletions or mutations in domain III result in enzymatically inactive molecules (Siegall et al., 1989). To become active, the PAETA toxin requires an intracellular furin-mediated proteolytic cleavage to generate a 37-kDa C-terminal fragment that is then translocated to the cytoplasm to reach the EF2 target (Inocencio et al., 1994).

By using a fluorescence resonance energy transfer approach, the mechanism of interaction between ExoA and its substrate EF has been studied, showing that the binding is strongly dependent on the pH. Furthermore, the finding that EF-2 bound to GDP or GTP is still recognized by ExoA shows how adaptable this toxin is in ADP-ribosylating its substrate.

In particular, mutational analysis affecting the last five residues at the carboxy-terminus of the enzymatic domain resulted in complete loss of cytotoxicity; this segment (Arg-Glu-Asp-Leu-Lys, REDLK) closely resembles the KDEL motif that is a well-defined endoplasmic reticulum retention sequence and that has also been found at the C-terminus of other ADP-ribosyl-transferases such as cholera toxin and heat-labile enterotoxin of *E. coli* (Chaudhary, 1990). It has been postulated that the sequence REDLK may be a recognition signal required for entry of the ADP-ribosylation domain of PAETA into the cytosol. Four disulfide bonds are present in the structure, but all of them are confined to the portion of exotoxin A that is not required for enzymatic activity.

Photoaffinity labeling experiments have identified Glu-553 as an active-site residue; substitu-

Glu 553   His 440
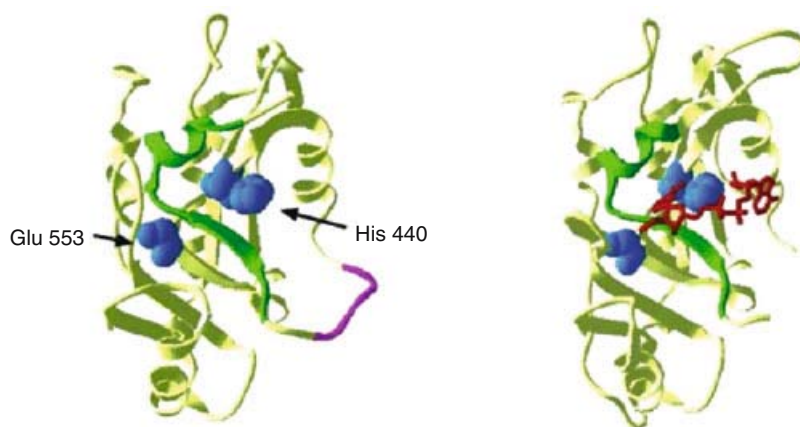
tion of this residue with any other amino acid, including the closely related Asp, decreased the enzymatic activity by a factor of 1000 (Douglas and Collier, 1990). In a similar manner, experiments of site-directed mutagenesis on His-440 led to molecules with a severely reduced cytotoxic activity, thus suggesting an important role for this residue in the reaction (Han and Galloway, 1995).

The crystal structure of the catalytic domain has been recently solved both in the isolated conformation and in the presence of an NAD analog (β-methylene thiazole-4-carboxamide adenine dinucleotide; β-TAD; Li et al., 1995; 1996b; Fig. 26). Comparison of the two structures shows that the major difference resides in the new conformation of the loop 458–463, which appears to be displaced by ligand binding; displacement of this loop from the active-site cleft could be an essential step allowing entrance and correct positioning of the NAD molecule during the enzymatic reaction.

Given the potent lethal activity, the catalytic domain of exotoxin A has been widely used for the construction of fusion proteins with cell-binding domains specific for tumor cells or other types of dangerous cells. So far, nucleotides encoding domain I have been replaced by sequences encoding interleukin (IL) 2, IL-6 and T-cell antigen CD4. These fusion molecules are promising candidates for the treatment of arthritis and allograft rejection (PAETA-IL2), acquired immune deficiency syndrome (PAETA-CD4), and other diseases (Chaudhary et al., 1987, 1988; Siegall et al., 1988; Ogata et al., 1989; Baldwin et al., 1996; Mori et al., 1997).

SHIGA TOXIN. This toxin (SHT; Fig. 1, panel 13), also known as "verotoxin," is the key virulence factor produced by *Shigella dysenteriae*, the pathogen responsible for the most severe forms of dysentery in humans (Kozlov et al., 1993). Shiga toxin is the prototype of a family of closely related bacterial protein toxins (Shiga-like toxins), also produced by certain strains of *E. coli* responsible for hemorrhagic colitis (Karmali et al., 1988).

From its 3D structure (Fraser et al., 1994), it is possible to recognize this protein as belonging to the class of A/B bacterial toxins, which consist of an enzymatic A subunit associated with a B domain binding to specific cell-surface receptors. The A subunit bears the enzymatic activity and is thus responsible for toxicity; like *Pseudomonas aeruginosa* exotoxin A and diphtheria toxin of *Corynebacterium diphtheriae*, SHT has an effect on protein synthesis, and in particular, by means of its *N*-glycosidase activity, it is able to depurinate a specific adenosine of ribosomal RNA and stop protein synthesis in the target cell (Endo et al., 1988). The catalytic subunit is composed of two regions, A1 and A2, and like many other bacterial protein toxins, it needs to be activated by proteolytic cleavage. Fragment A2 has an α-helical structure and is noncovalently linked to the B domain (Fig. 27). Interestingly, its primary structure displays a notable similarity to chain A of ricin, a plant toxin that also shares the same enzymatic function acting on the same substrate (Katzin et al., 1991).

This domain displays an overall organization which is very similar to that of the corresponding receptor-binding subunits of the ADP-ribosyltransferases cholera toxin and heat-labile enterotoxin LT of *E. coli*, all formed by five identical protomers which assemble into the final ring-like structure of the B oligomer (Fig. 28). The B-subunit of Shiga toxin has been demonstrated as a powerful vector for carrying attached peptides into cells for intracellular transport studies and for medical research (Hagnarelle et al., 2003).

Upon binding of verotoxin to its receptor (globotrialosylglyceramide, Gb) on the surface of a eukaryotic cell (Cohen et al., 2000), the toxin is internalized by receptor-mediated endocytosis and is transported to the Golgi and to the endoplasmic reticulum, from which the A subunit is

translocated to the cytoplasm, where it can gain access to the ribosomal target. Numerous recent studies have shown that Shiga toxins trigger programmed cell death signaling cascades in intoxicated cells. The mechanisms of apoptosis induction by these toxins are newly emerging, and the toxins may signal apoptosis in different cells types via different mechanisms (Cherla et al., 2003).

Toxins Acting on Signal Transduction

Signal transduction is an essential mechanism for the survival of any living organism. In eukaryotic cells, signals received from the outside stimulate receptors on the cell surface and are subsequently transmitted across the cell membrane mainly using two types of mechanism: 1) tyrosine phosphorylation of the cytoplasmic portion of the receptor which initiates a cascade of intracellular signaling events; and 2) modification of a receptor-coupled GTP-binding protein that transduces the signal to various enzymes which respond with the release of secondary messengers such as cyclic AMP (cAMP), inositol triphosphate, and diacylglycerol; accumulation of these products alter the normal equilibrium of the cell and thus provoke malfunction and death.

Pertussis Toxin

This toxin (PT; Fig. 1, panel 14) is a protein of 105 kDa released into the extracellular medium by *Bordetella pertussis*, the etiological agent of whooping cough. It belongs to the A/B class of ADP-ribosylating toxins and is composed of five distinct subunits, named "S1" through "S5," where S4 is present in two copies in the final oligomer. The genes encoding for the five monomers of pertussis toxin are organized into an operon structure (Locht et al., 1986; Fig. 29) and contained within a chromosomic DNA fragment of approximately 3200 base pairs.

Interestingly, the genes coding for S2 and S3 share a 75% similarity (67%, if calculated from S2 and S3 gene products at the amino acid level), suggesting a common evolutionary origin for the two sequences, possibly because of gene duplication.

The five subunits are independently secreted into the periplasmic space, where the toxin is assembled and then released in the culture
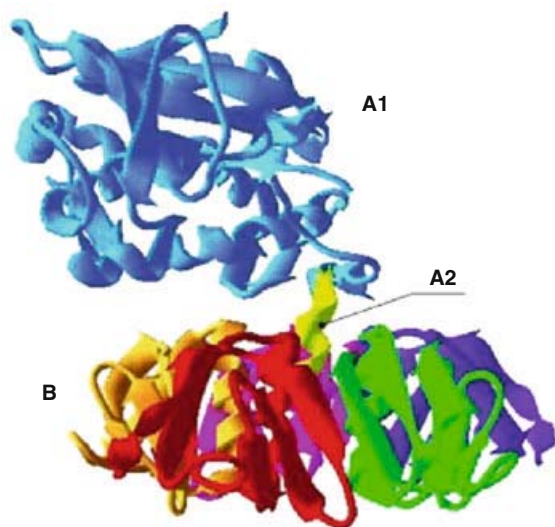

Fig. 27. Three-dimensional structure of Shiga holotoxin. The A subunit is distinguished between A1 (blue) and A2 (yellow), whereas the receptor-binding domain B has different colors for the five monomers.
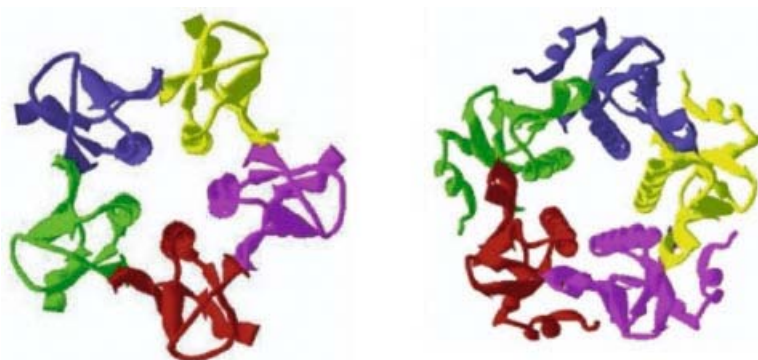

Fig. 28. Bottom view of the B subunit of Shiga toxin (left panel) in comparison to the B subunit of *E. coli* LT (right panel).
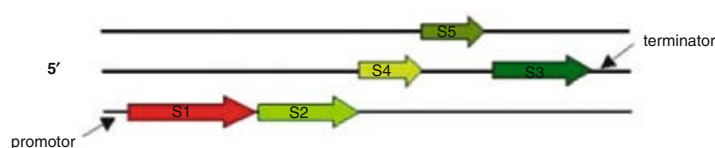

Fig. 29. Schematic representation of the genetic organization of the open reading frames (ORFs) coding for the five subunits of pertussis toxin.

Fig. 30. Three-dimensional structure of the pertussis holotoxin. Left panel: side view of the intact holotoxin; right panel: bottom view of the receptor-binding domain. Each subunit is colored accordingly to the corresponding genes as represented in Fig. 29.
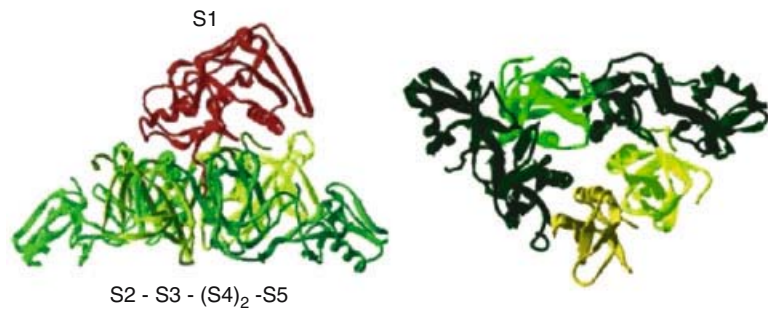
medium by a specialized type IV secretion apparatus (Covacci and Rappuoli, 1993a; Weiss et al., 1995). Subunit S1 represents the enzymatically active domain A, which is totally responsible for the toxicity, whereas the pentamer S2-S3-(S4)$_2$-S5 constitutes the receptor-binding domain B (Fig. 30).

The A domain acts on eukaryotic cells by ADP-ribosylating their GTP-binding proteins, and specifically it transfers an ADP-ribose group to a cysteine residue located in the carboxy-terminal region of the α-subunit of many G proteins such as G$_i$, G$_o$ and transducin (Katada et al., 1983; West et al., 1985); G$_s$ which has a tyrosine residue in place of the cysteine is not a valid substrate for PT. The consequence of ADP-ribosylation is the uncoupling of G-proteins from their receptors which results in an alteration of the response of eukaryotic cells to exogenous stimuli and thus in a variety of in vivo phenotypes, such as leukocytosis, histamine sensitization, and increased insulin production (Sekura, 1985). Conversely, the most interesting activity displayed by PT in vitro is the observed change in cell morphology in Chinese hamster ovary (CHO) cells (Hewlett et al., 1983).

The B domain is a nontoxic oligomer that binds the receptors on the surface of eukaryotic cells and allows the toxic subunit S1 to reach its intracellular target proteins through a mechanism of receptor-mediated endocytosis, likely following a mechanism of retrograde transport through the Golgi apparatus. The importance of the Golgi localization of pertussis toxin for the S1-dependent ADP-ribosylation of G-proteins was investigated employing Brefeldin A (BFA) treatment to disrupt Golgi structures. This treatment completely blocked the pertussis toxin ADP-ribosylation activity of cellular G-proteins, therefore indicating that retrograde transport to the Golgi network is a necessary prerequisite for cellular intoxication (el Baya et al., 1997). In CHO cells, the PT receptor has been shown to be a high-molecular weight glycoprotein that binds the B oligomer through a branched-mannose core containing N-acetylglucosamine (Sekura, 1985). In contrast to the other ADP-

ribosyltransferases, where the enzymatically active domain A mediates all the toxic activities, PT possesses other nonlethal activities (such as a mitogenic activity on T cells), which are mediated exclusively by the receptor-binding domain B (Tamura et al., 1983). The active site of pertussis toxin is structurally homologous to the active sites of other ADP-ribosylating toxins. This aspect will be described in the section ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity in this Chapter.

Pertussis toxin plays a central role in the pathogenesis of whooping cough and in the development of protective immunity against reinfection. For this reason, the role of many residues of S1 has been tested by site-directed mutagenesis to produce nontoxic mutants of the toxin to be used as vaccines. The minimal region still enzymatically active is constituted by amino acids 4–179 of S1 subunit (Pizza et al., 1988; Cieplak et al., 1988; Fig. 31), and it is within this fragment that many mutations have been

Fig. 31. Crystal structure of the wildtype S1 subunit of pertussis toxin. The scaffold of the enzymatic cleft is represented as a green ribbon, whereas the rest of the molecule is in pale yellow carbon trace representation. Residues proved to be essential for activity by means of site-directed mutagenesis are represented with side chains and are colored in blue.
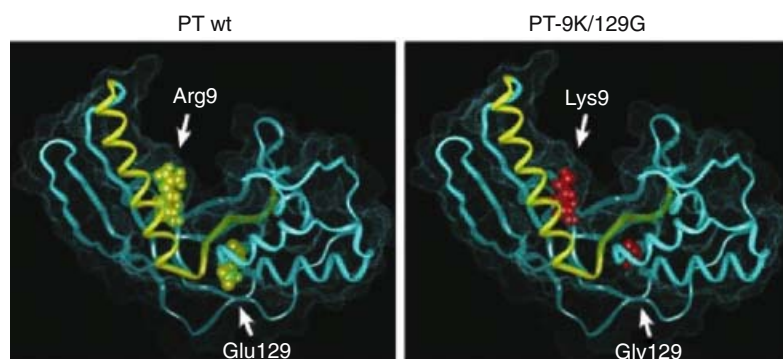
Fig. 32. X-ray representation of the wildtype pertussis toxin (left panel) and of the double mutant 9K/129G (right panel). The catalytic cleft is colored in yellow, whereas the mutated residues are in red.

designed and analyzed for activity. In particular, Arg-9, Asp-11, Arg-13, Trp-26, His-35, Phe-50, Glu-129 and Tyr-130 were found to be essential for enzymatic activity and, when replaced with other residues, the toxicity was reduced to levels of about 1%; nevertheless, none of the single-amino acid mutants were completely devoid of toxicity.

The most successful mutant contains in fact two amino acid substitutions: Arg-9/Lys and Glu-129/Gly (PT-9K/129G; Fig. 32). This mutant is structurally identical to the wildtype but is completely nontoxic and has been used for the construction of an acellular vaccine against pertussis. This vaccine has been extensively tested and has been shown to induce protection from disease (Pizza et al., 1989; Rappuoli, 1997).

Cholera Toxin and Heat-Labile Enterotoxin

Cholera toxin (CT) and *E. coli* heat-labile enterotoxins (LT-I and LT-II) share an identical mechanism of action and homologous primary and 3D structures (Dallas and Falkow, 1980; Spicer et al., 1982; Sixma et al., 1991; Figs. 1 [panel 15] and 33). The CT is produced by *Vibrio cholerae* (the etiological agent of cholera), whereas LT-I and LT-II are produced by enterotoxigenic strains of *E. coli* (ETEC) isolated from humans with traveler's diarrhea, from pigs (LT-I), or from food (LT-II; Seriwatana et al., 1988). The two toxins belong to the class of ADP-ribosylating toxins and are organized in an AB_5 architecture, where the B domain is a pentamer which binds the receptor on the surface of eukaryotic cells, and domain A bears the enzymatic activity and is thus responsible for toxicity (Holmgren, 1981; Moss and Vaughan, 1988). Both the A and B subunits of CT and LT are synthesized intracellularly as precursor proteins which, after removal of the leader peptide and translocation across the cytoplasmic membrane, assemble in the periplasmic space to form the final AB_5 complex. While *V. cholerae* exports the CT toxin into the culture medium, LT remains
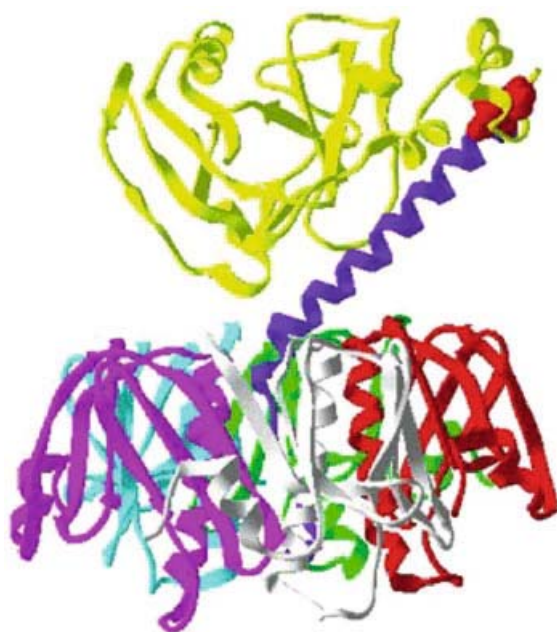

Fig. 33. X-ray structure of heat-labile enterotoxin LT of *E. coli*. The catalytic domain A1 is yellow, the linker domain A2 is blue, and the five monomers of the B subunit are all represented in different colors.

associated to the outer membrane bound to lipopolysaccharide (LPS; Horstman et al., 2002). The corresponding genes of CT and LT are organized in a bicistronic operon and are located on a filamentous bacteriophage and on a plasmid, respectively (So et al., 1978).

The A subunit (Fig. 34, left panel) is a 27-kDa monomer composed of a globular structure and linked to the B domain by a trypsin-sensitive loop and a long α-helix, which inserts inside the core of the B pentamer thus anchoring the two subunits. For full activity, the A subunit needs to be proteolytically cleaved and reduced at the disulfide bridge between cysteines 187 and 199 to give two fragments: the enzymatic subunit A1 and the linker fragment A2 (Lai et al., 1981).
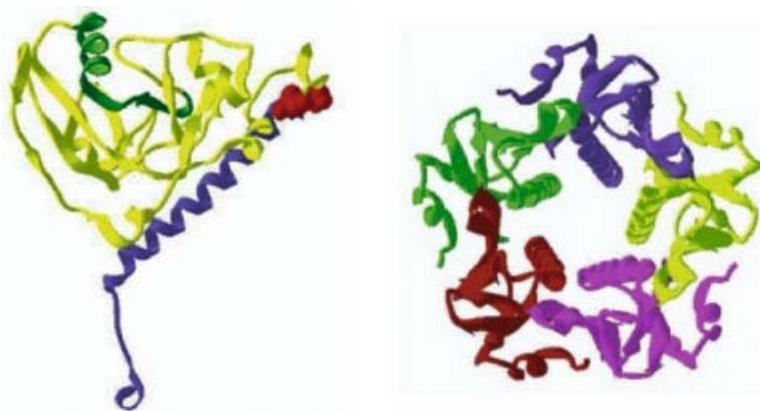
Whereas in cholera toxin the proteolytic process is performed during biosynthesis by an endoprotease (Booth et al., 1984), in the case of LT, it occurs by extracellular processes; in both cases, the reduction is thought to take place at the surface of the target cell.

The enzymatically active domain A binds NAD and transfers the ADP-ribose group to an Arg residue located within the central portion of several GTP-binding proteins such as G_s, G_t and G_{olf}. Upon ADP-ribosylation of G_s, in particular, the adenylate cyclase is permanently activated, causing an abnormal intracellular cAMP accumulation, which in turn alters ion transport and thus is the main reason for the toxic effects (Field et al., 1989a, 1989b).

A peculiar feature of CT and LT is that the basal ADP-ribosyltransferase activity is enhanced by interaction with 20-kDa guanine-nucleotide binding proteins, known as "ADP-ribosylation factors" (ARFs; Tsai et al., 1988; Moss and Vaughan, 1991). After receptor binding, the holotoxins are internalized and undergo retrograde transport through the Golgi to the endoplasmic reticulum (ER). Recent studies show that both A and B subunits move together from the cell surface into the ER, and this depends on the B-subunit binding to ganglioside GM1. The KDEL motif in the A2 chain does not appear to affect retrograde transport, but slows recycling of the B-subunit from ER to distal Golgi stacks. Specificity for GM1 in this trafficking pathway is shown by the failure of the *E. coli* type II toxin LTIIb that binds ganglioside GD1a to concentrate in lipid rafts, enter the ER, or induce toxicity. These results show that the B subunit carries the A1 chain from cell surface into the ER where they dissociate, and that a membrane lipid with strong affinity for lipid rafts provides the dominant sorting motif for this pathway (Fujinaga et al., 2003). In the ER, the A1-chain of the CT unfolds and enters the cytosol by a process termed "retro-translocation."

Upon entering the cytosol, the A1-chain rapidly refolds, binds ARF and induces toxicity (Lencer et al., 1995). The B subunits persist in the Golgi and are subsequently degraded.

The exact localization of the ARF-binding site is still unknown, but it has emerged from recent studies that the two domains (the NAD-binding and ARF-binding) are independent and located in different regions of the A domain (Stevens et al., 1999).

When the toxins are released in the intestine during infection, the major consequence is intestinal fluid accumulation and watery diarrhea (also typical symptoms of the diseases; Holmgren, 1981).

The B domain (Fig. 34, right) is composed of five identical subunits (each 11.5 kDa) that are arranged in a symmetric shape around a central pore inside which the C-terminal portion of the catalytic domain (A2) is inserted (Sixma et al., 1991, 1993). Their secondary structure consists predominantly of two three-stranded antiparallel β-sheets, a short N-terminal helix, and a long central helix. Although still well conserved in terms of quaternary structure, CT and LT B domains have a lower degree of primary sequence homology than the corresponding A domains. Interestingly, the B subunit of LT-II, although maintaining a conserved structure, lacks any sequence homology with the corresponding B domains of CT and LT-I (Domenighini et al., 1995).

In addition to their function as receptor-binding domains and as carriers of the toxic moieties, the B subunits possess specific biological activities such as induction of apoptosis of CD8+ and CD4+ T cells (Truitt et al., 1998) and the property to function as potent mucosal adjuvants (Xu-Amano et al., 1994). This feature has been extensively used to develop mucosal vaccines against cholera and ETEC infection, and to induce a mucosal response also against the other antigens used.
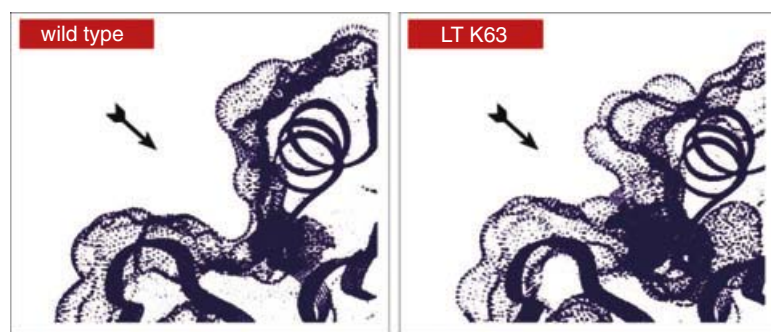
Fig. 35. Three-dimensional structure of the enzymatic cavity of the wildtype LT (left) and of the mutant LT-K63 (right). The arrows point out how much a single amino acid substitution can affect the dimension of the pocket and thus the entrance of NAD.

To produce molecules suitable for these pharmacological applications but completely devoid of toxic activity, more than fifty mutant derivatives have been constructed both for the A and B subunits. Among those which completely inactivate the toxin, the best characterized are LT-K63 (Fig. 35), LT-K97 and LT-K7, all in the vicinity of the catalytic domain, and for which the 3D structures have also been determined (Merritt et al., 1995; Van den Akker et al., 1995, 1997). In the case of LT-K63 (and the corresponding CT-K63), where the wildtype Ser in position 63 is substituted with a Lys, the mutated proteins are enzymatically inactive and nontoxic, either in vitro and in vivo, but are otherwise indistinguishable from the wildtype. In fact, they are still able to bind the receptor and the ARFs (Stevens et al., 1987), and the crystal structure and that of wildtype LT are almost perfectly superimposable except for the catalytic site, where the bulky side-chain of Lys-63 fills the catalytic pocket thus making it unsuitable for NAD entrance and binding (Giannelli et al., 1997; Douce et al., 1998).

Another interesting mutant is LT-K97, where the substitution Val/Lys introduces a salt bridge between Lys-97 and the carboxylate of Glu-112, thus making it unavailable to further interactions. This observation suggests a dominant role of this glutamic acid in the enzymatic reaction.

Mutations affecting the B domain lead often to products that can no longer bind to eukaryotic receptors, as is the case of LTB-D33, which contains a glycine-to-aspartic acid substitution in position 33. These types of mutants have been found to be almost completely nonimmunogenic at mucosal surfaces, suggesting that an intact receptor-binding site is necessary not only for binding but also for immunogenicity and adjuvanticity (Guidry et al., 1997).

Clostridium perfringens Alpha-Toxin

This toxin (Fig. 1, panel 16) is the most important toxin produced by *Clostridium perfringens* and is responsible for gas gangrene or clostridial myo-necrosis (Stevens et al., 1987; Florez-Diaz et al., 2003). It plays a key role in the spread of the infection either by suppressing host immune responses, triggering the release of inflammatory mediators, or causing changes in intracellular calcium levels. Specific mutants of *C. perfringens* that do not produce the toxin are unable to cause disease, and vaccination with a genetically engineered toxoid has been shown to induce protection against gas gangrene (Williamson and Titball, 1993).

This virulence factor is a 370-amino acid zinc metalloenzyme that also displays phospholipase C (PLC) activity (Leslie et al., 1989); nevertheless, not all the bacterial PLCs act as virulence determinants, therefore this enzymatic activity is not sufficient for toxicity.

Alpha-toxin is capable of binding to mammalian cell membrane and cleaving membrane-bound phosphatidylcholine (or sphingomyelin) to produce phosphocholine and diacylglycerol (or ceramide). The reaction product diacylglycerol, which is a leukotriene precursor, is believed to be the responsible of the subsequent lethal effects.

The crystal structure of α-toxin has been recently solved (Naylor et al., 1998; Fig. 36), indicating the presence of two distinct domains in the molecule. Whereas the N-terminus is mainly organized as a globular α-helical domain that contains the active site, the β-sandwich C-terminal subunit is involved in membrane binding and shows strong structural analogy to eukaryotic calcium-binding C2 domains. A flexible linker containing a series of highly mobile residues connects the two domains.

In addition, the C-terminal subunit displays hemolytic and sphingomyelinase activities and primarily contributes to the toxin's lethal effect, even if it is completely devoid of toxic activity when used alone. Nevertheless, immunization with this domain affords full protection from disease in mouse models, thus indicating that the protective epitopes are located in this portion of the molecule (Titball et al., 1993; Nagahama et al., 2002).
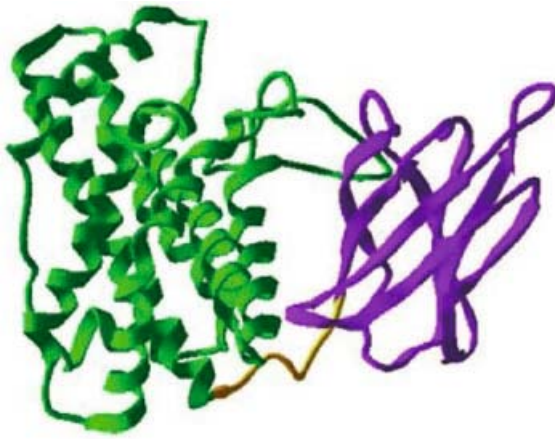
Fig. 36. Three-dimensional structure of *Clostridium perfringens* α-toxin. The N-terminal and C-terminal domains are green and violet, respectively, and the flexible linker is orange.

Recently, other bacterial PLCs, like those from *L. monocytogenes* and *Mycobacterium tuberculosis*, have been implicated in the pathogenesis of a number of diseases (Wadsworth et al., 1999; Raynaud et al., 2002).

Clostridium difficile Toxins A and B

Enterotoxin A (TcdA) and cytotoxin B (TcdB) of *Clostridium difficile* are the two virulence factors responsible for the induction of antibiotic-associated diarrhea. These toxins have molecular masses of 308 and 270 kDa, respectively, and belong to the class of large clostridial cytotoxins (Lyerly et al., 1986; Knoop et al., 1993).

The toxin genes *tcdA* and *tcdB* together with three accessory genes (*tcdC–E*) constitute the pathogenicity locus (PaLoc) of *C. difficile* (Cohen et al., 2000). Primary sequence homology between *tcdA* and *tcdB* gene products is higher than 60% identity (von Eichel-Streiber et al., 1994).

Upon binding to eukaryotic cells and translocation across membranes via receptor-mediated endocytosis, TcdA and TcdB monoglucosylate small GTP-binding proteins such as Rho, Rac and Cdc42 at a threonine residue (Just et al., 1995a, 1995b; Ciesla and Bobak, 1998). In most cells, *C. difficile* toxins induce depolymerization of the actin cytoskeleton, leading to a morphology similar to that induced by C3-like transferases. While toxin B has potent cytotoxic activity in vitro, the enterotoxic activity of *C. difficile* in animals has been mainly attributed to toxin A.

From the structural point of view, they are composed of two portions: the N-terminal nonrepetitive two thirds corresponding to the catalytic subunit, and the C-terminal third char-

acterized by a highly repetitive domain called the "clostridial repetitive oligopeptide" (CROP), identified as the site of interaction with a carbohydrate structure as well as the ligand to which neutralizing antibodies bind (von Eichel-Streiber, 1994). A central hydrophobic region contains several predicted transmembrane segments and is believed to function as the translocation unit.

Bordetella pertussis Adenylate Cyclase

Adenylate cyclase (CyaA) is a toxin produced by *Bordetella pertussis*, *B. bronchiseptica* and *B. parapertussis* (Weiss and Hewlett, 1986). It is essential in the early stages of bacterial colonization of the respiratory tract and can induce apoptosis of lung alveolar macrophages (Goodwin and Weiss, 1990; Khelef et al., 1993).

Organized as a bifunctional protein, CyaA (177 kDa) is composed of an N-terminal cell-invasive and calmodulin-dependent adenylate cyclase domain (residues 1–400) fused to a pore-forming hemolysin (residues 401–1706; Glaser et al., 1988; Bejerano et al., 1999; see also the section Pore-Forming Toxins: RTX Hemolysins). Unlike most of the other members of the RTX family that are secreted into the supernatant, CyaA remains associated to the bacterial surface, through interactions with filamentous hemagglutinin (FHA). This toxin forms small cation-selective channels in lipid bilayer membranes and delivers into the cytosol of target cells the adenylate cyclase (AC) domain, which, upon binding to calmodulin, catalyzes an uncontrolled conversion of ATP to cAMP, thus causing intoxication and disruption of cellular functions (Ladant and Ullmann, 1999). Calcium has been shown to play a fundamental role in channel formation (Knapp et al., 2003). Furthermore, it was also demonstrated that the ability of the AC domain to form pores and translocate across the membrane is strictly linked to the correct folding of an amphipathic α-helix spanning residues 509–516. Substitution of Glu-509 with a helix-breaker proline residue, in fact, significantly reduced the capacity of the toxin to undergo translocation (Osickova et al., 1999).

A very similar function and mechanism of action is that of ExoY, an adenylate cyclase produced by *Pseudomonas aeruginosa* and injected into the cytoplasm of eukaryotic cells by the type III secretion apparatus (see Table 1, and the section Toxins Injected into Eukaryotic Cells in this Chapter). However, differently from CyaA, ExoY is not activated by calmodulin. In vivo, following infection with ExoY-expressing strains, CHO cells showed a rounded morphology, which correlated with increased cAMP levels (Yahr et al., 1998).

Anthrax Edema and Lethal Factors

Lethal factor (LF) and edema factor (EF) proteins, produced by *Bacillus anthracis*, combine with the protective antigen PA to give the lethal (PA+LF) and edema (PA+EF) toxins (Brossier et al., 2000; Collier and Young, 2003; Fig. 1, panel 17). In both complexes, the PA has the pore-forming, receptor-binding activity (see the section Pore-Forming Toxins in this Chapter), whereas EF and LF display, in turn, the toxic activities.

The EF and LF genes are located on a large plasmid (Mikesell et al., 1983) and encode precursors of approximately 800 residues. Cleavage of the N-terminal signal peptides yields mature EF and LF proteins with molecular masses of 88.8 kDa and 90.2 kDa, respectively. These virulence factors enter cells by binding to proteolytically activated, receptor-bound, oligomeric PA; following receptor-mediated endocytosis, the low pH causes a conformational change in PA, allowing the translocation of EF-LF across cell membrane (Collier, 1999). The EF-LF is then endocytosed and translocated from endosomes directly to the cytosol of cells, where both toxins perform their toxic activities (Fig. 37). The binding sites of EF and LF on PA have been recently mapped (Cunningham et al., 2002).

Once inside the cells, EF binds calmodulin and catalyzes an unregulated production of the second messenger cAMP, thereby perturbing the normal cell regulatory mechanisms (Goyard et al., 1989). Calcium influx is required for inducing cyclic AMP toxicity in target cells (Kumar et al., 2002).

Whereas the PA-binding domain displays a strong sequence homology to lethal factor LF, the catalytic domain is more similar to the other known adenylate cyclase CyaA toxin of *Bordetella pertussis* (Escuyer et al., 1988). On the other hand, LF cleaves the amino-terminus of the cellular mitogen-activated protein kinase kinases (MAPKK1 and MAPKK2), thus causing inhibition of the MAPK signal transduction pathway, which is key to cellular proliferation and signal transduction processes in the cell (Duesbery et al., 1998; Vitale et al., 1999).

Recently, the 3D structures of LF and EF have been solved (Fig. 38). LF comprises four domains: domain I binds the membrane-translocating component of anthrax toxin (PA); domain II resembles the ADP-ribosylating toxin from *Bacillus cereus*; domain III is inserted into domain II, and seems to have arisen from a repeated duplication of a structural element of domain II. Domain IV is distantly related to the zinc metalloprotease family, and contains the catalytic center (Pannifer et al., 2001). The catalytic portion of EF is made by three globular domains. The active site is located at the inter-
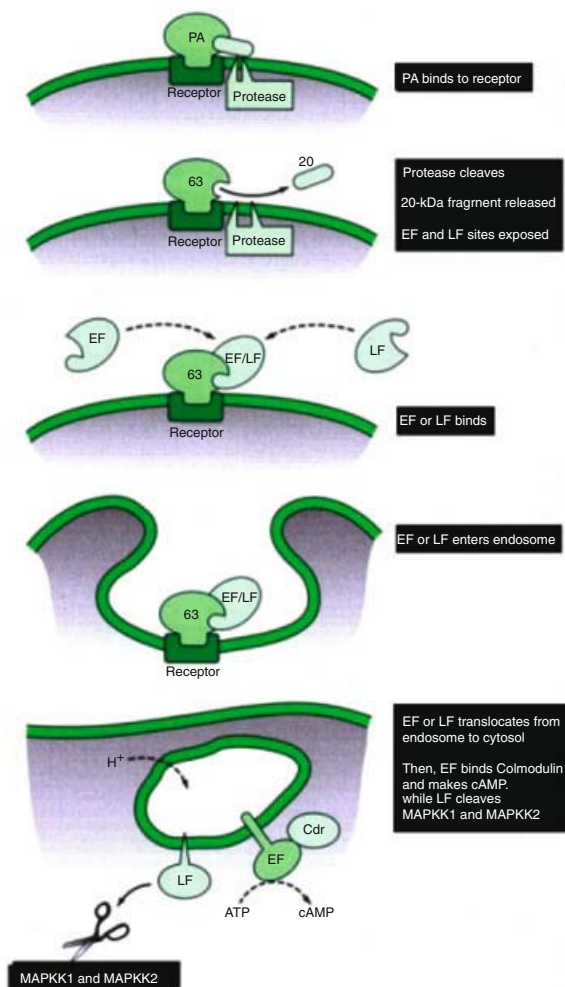

Fig. 37. Mechanism of PA-mediated entry and intoxication of anthrax LF and EF toxins.

face of two domains (C_A and C_B), which together form the catalytic core, containing the catalytic residue His351. EF has been crystallized both alone and in complex with calmodulin. The differences between the two forms are induced by calmodulin, which acts by stabilizing the conformation of the substrate-binding-site of EF (Drum et al., 2002). Interestingly, a remarkable level of primary sequence similarity can be detected between EF and the N-terminal, calmodulin-binding domain of *Bordetella* adenylate cyclase CyaA. In particular, His351 is conserved between the two proteins.

Once in the cytoplasm, LF acts as a zinc-metalloprotease disrupting normal homoeostatic functions. The macrophage is a uniquely sensitive cell type that seems to be a vital global mediator of toxin-induced pathologies. Removal of macrophages from mice renders them insensitive to LF challenge (Hanna, 1999).

In addition, LF, but not EF, is able to cause apoptosis in human endothelial cells. As a con-

Domain I

Domain II

Domain IV

Domain III

PANEL A
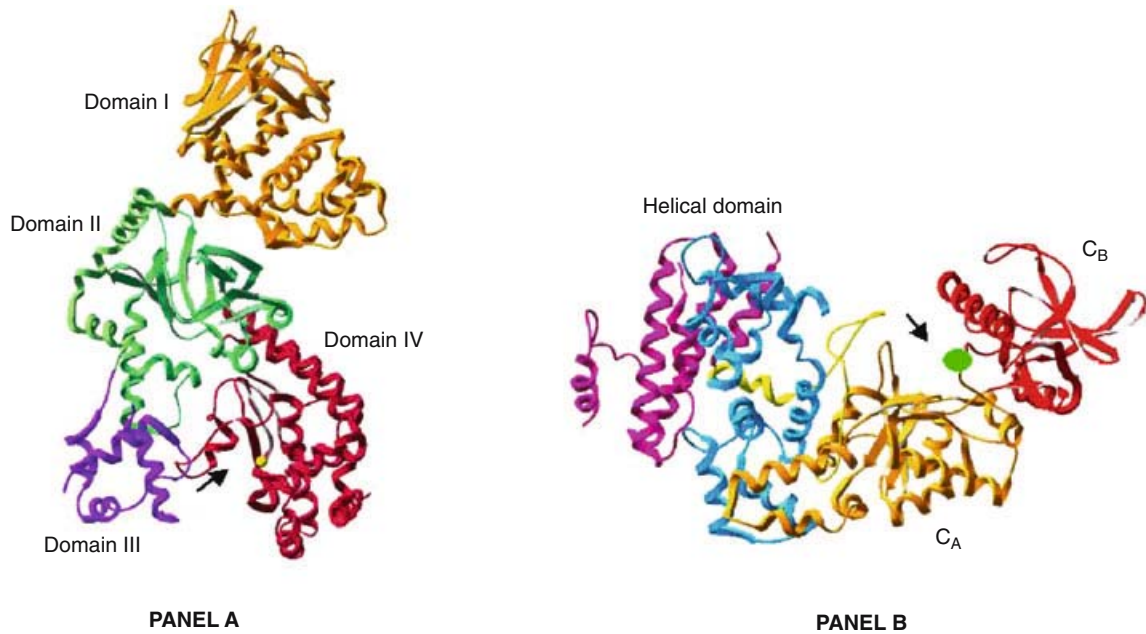
Helical domain

C_B

C_A

PANEL B

Fig. 38. Crystal structures of the catalytic portion of anthrax lethal factor (panel A) and edema factor in complex with calmodulin (panel B). Panel A. The four domains are in different colors. The zinc atom complexed by domain IV is indicated by an arrow.

sequence, the observed endothelial toxicity contributes to vascular pathology and hemorrhage during systemic anthrax (Kirby, 2004).

E. coli Cytotoxin Necrotizing Factors

Cytotoxin necrotizing factors (CNF1 [Fig. 1, panel 18] and CNF2), single-chain proteins of 115 kDa produced by a number of uropathogenic and neonatal meningitis-causing pathogenic *E. coli* strains (Caprioli et al., 1984; De Rycke et al., 1987), are immunologically related and share 85% identity. They also share some similarity with the dermonecrotic toxin of *Pasteurella multocida* and *Bordetella pertussis* (Schmidt et al., 1999). Both CNF1 and CNF2 toxins are encoded by a single structural gene with a low G+C content (35%). However, whereas *cnf1* is chromosomally encoded, cnf2 is carried on a large transmissible F-like plasmid called "Vir" (Oswald and De Rycke, 1990; Falbo et al., 1992).

These toxins induce ruffling, stress fiber formation, and cell spreading in cultured cells by activating the small GTP-binding proteins Rho, Rac and Cdc42, which control assembly of actin stress fibers (Oswald et al., 1994). CNF1 induces only a transient activation of Rho GTPase and a depletion of Rac by inducing the addition of an ubiquitin chain, which is known to drive to specific degradation by the proteasome. Reduction of Rac GTPase levels induces cell motility and

Fig. 39. Crystal structure of the active site of *E. coli* CNF1. The catalytic site composed by Cys866-His881 is colored in blue.

cellular junction dynamics allowing efficient cell invasion by uropathogenic bacteria (Doye et al., 2002). The catalytic region of CNF1 has been crystallized (Buetow et al., 2001; Fig. 39). The active site contains a catalytic triad, which is positioned in a deep pocket, thus explaining the restricted access to unspecific substrates and therefore its specificity. Very likely, some type of conformational rearrangement is required also to accomodate Rho in this narrow cavity.

Recently, a CNF1-like toxin (CNFY) has been identified also in *Yersinia pseudotubercolosis* (Lockman et al., 2002). Differently from the *E. coli* CNFs, CNFY has been shown to selectively activate RhoA (Hoffman et al., 2004).

Bordetella Dermonecrotic Toxin

Dermonecrotic toxin (DNT) is produced by *Bordetella* species as a single-chain polypeptide chain of 1464 amino acids, which is composed of a C-terminal portion that contains the catalytic site, and of an N-terminal receptor-binding domain. DNT shares about 30% identical residues in the catalytic domain with *E. coli* CNF1, including the catalytic Cys and His residues. DNT is a transglutaminase, which catalyzes the deamidation or polyamination at Gln63 of Rho and of the corresponding residues of Rac and Cdc42 (Horiguchi, 2001). This activity causes alteration of cell morphology, reorganization of stress fibers, and focal adhesions on a variety of animal models. Recently, it has been demonstrated that the initial 54 amino acids of DNT are sufficient for cell surface recognition. However, the receptor is still unknown.

Cytolethal Distending Toxins

The cytolethal distending toxin (CDT) produced by *Haemophilus ducreyi* (HdCDT) is the prototype of a growing family of bacterial toxins that act by inducing cell enlargement followed by cell death (Cortes-Bratti et al., 1999; Frisan et al., 2003). HdCDT is a complex of three proteins (CdtA, CdtB and CdtC) encoded by three genes that are part of an operon. Members of this family have been identified in *E. coli*, *Shigella*, *Salmonella*, *Campylobacter*, *Actinobacillus* and *Helicobacter hepaticus* (Okuda et al., 1995; Lara-Tejero and Galan, 2001; Hoghjoo et al., 2004; Pickett et al., 2004; Shenker et al., 2004; Young et al., 2004). The overall sequence similarity varies among the different members of this family of toxins. HdCDT intoxicates eukaryotic cells by causing a three- to fivefold gradual distension and induces cell cycle arrest in the G_2 phase. It has also been shown to induce DNA double-strand breaks and formation of actin stress fibers via activation of the small GTPase RhoA. Recently it has been shown that CdtB is the active subunit of the CDT toxin and acts as a nuclease. All the amino acids predicted to be important for nuclease activity are conserved in the CdtB of different bacteria, suggesting that the mechanism of action is the same for all CDT toxins. On the other hand, CdtA and CdtC are able to bind to the surface of HeLa cells, therefore playing a role in the delivery of the active domain to target cells (Lee et al., 2003).

Toxins Acting on the Cytoskeleton Structure

The cytoskeleton is a cellular structure that consists of a fiber network composed of microfilaments, microtubules, and the intermediate filaments. It controls a number of essential functions in the eukaryotic cell and participates in all kinds of cellular movement and transport; furthermore, the cytoskeleton is involved in processes like exo- and endocytosis, vesicle transport, cell-cell contact, and mitosis (Kabsch and Vandekerckhove, 1992).

The group of cytoskeleton-affecting bacterial toxins comprises not only virulence factors that directly act on particular elements of the cytoskeleton, but also proteins that perform an indirect action by affecting regulatory components, which control its organization (Aktories, 1994; Richard et al., 1999). Most of them do it by modifying the regulatory, small G proteins, such as Ras, Rho, Rac and Cdc42, which control cell shape. These toxins, which have a dramatic but indirect effect on the cytoskeleton and are described in the section Toxins Acting on Signal Transduction, are *E. coli* CNF and *C. difficile* enterotoxins A and B. Other toxins acting on regulatory G proteins are exoenzyme S, C3 and YopE, which are described below as toxins that are directly injected into the eukaryotic cells. Other bacterial molecules that cannot be strictly considered toxins but that have a powerful ability to polymerize actin are ActA and IcsA of *Listeria* and *Shigella,* respectively. These are described elsewhere in this volume (see Listeria and Relatives in Volumn 4 and The Genus Shigella in Volumn 6). Another toxin acting indirectly on the cytoskeleton is the zonula occludens toxin (Zot) produced by *V. cholerae*, a toxin with an unknown mechanism of action that modifies the permeability of tight junctions (Zot is described in the paragraph Toxins with Unknown Mechanism of Action in this Chapter). In the following section we consider only toxins that have the cytoskeleton as a direct target. The only toxin shown to affect directly the cytoskeleton is the C2 toxin of *C. botulinum*, which ADP-ribosylates monomeric actin, making it unable to polymerize. A second protein that has recently been described as being able to bind actin and stabilize the fibers supporting the ruffles induced by the *Salmonella* type III secretion system is SipA (described in the section Toxins Injected into Eukaryotic Cells in this Chapter).

Representatives of both subgroups can be identified among the class of ADP-ribosylating factors that ultimately display their toxic effect on the cytoskeleton of eukaryotic cells. In fact, whereas the family of *Clostridium botulinum* toxin C2, clostridial toxin C3 (and related pro-

teins), and *Pseudomonas aeruginosa* exoenzyme S (Exo S) act on small GTP-binding proteins that regulate the correct functioning of the cytoskeleton, and thus have an indirect toxic effect (Coburn et al., 1999).

Clostridium botulinum Toxin C2 and Related Proteins

Clostridium botulinum toxin C2 is the main representative of a class of binary cytotoxins produced by clostridial species that predominantly act on polymerized actin microfilaments of 7–9 nm in diameter (Aktories et al., 1986; Aktories and Wegner, 1992). C2 ADP-ribosylate monomeric G-actin at an arginine residue (Aktories et al., 1986; see the section ADP-Ribosylating Toxins in this Chapter). Because this arginine (Arg-177) is a contact site between actin monomers, the binding of the ADP-ribose moiety prevents actin's polymerization.

Other members of this family are *C. perfringens* iota toxin (Stiles and Wilkins, 1986; Perelle et al., 1993) and the related *C. spiroforme* and *C. difficile* ADP-ribosylating toxins (Popoff and Boquet, 1988a; Just et al., 1994), which are generally classified as iota-like toxins. These binary toxins are constructed according to the A/B model architecture, but in this case the two domains reside in separate molecules that interact to cause the toxic effect. Therefore, these toxins have an enzymatically active and toxic domain (A) and a binding component (B), which is essential for the binding at the cell surface and for the translocation inside the cell.

Clostridium botulinum toxin C2 is an extremely toxic agent, which induces hypotension, increase in intestinal secretion, vascular permeability, and hemorrhaging in the lungs. In contrast to botulinum neurotoxins, C2 does not seem to display any neurotoxic effect. The two molecules that constitute its toxic moiety are classified as C2-II (for the binding component) and C2-I (for the enzymatic component). The C2-II is a 100-kDa protein that must be proteolytically cleaved to a 75-kDa fragment before it can bind to the surface receptor; upon this interaction, a binding site for the 50-kDa C2-I component is activated and the toxic domain is taken up by receptor-mediated endocytosis (Ohishi, 1987). Substrates of the C2-I toxin are β/γ-non-muscle actin and γ-smooth muscle actin, but not α-actin isoforms. Conversely, the related iota toxin of *Clostridium perfringens* has been found to ADP-ribosylate all actin isoforms (Mauss et al., 1990). The iota toxin is a binary toxin produced by *Clostridium perfringens* type E, which has been implicated in fatal calf, lamb and guinea pig enterotoxemias (Madden et al., 1970). Structurally, it has two independent
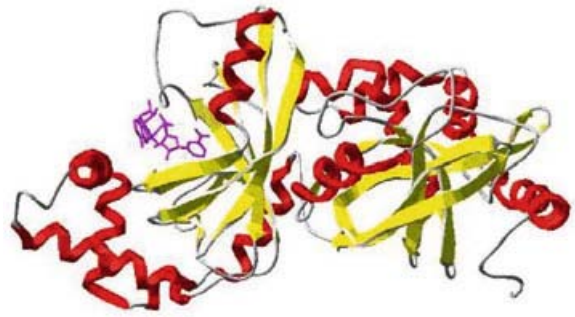

Fig. 40. Crystal structure of the catalytic domain C2I of *C. perfringens* C2 toxin (red and yellow) in complex with NADH (pink).

domains: Ia, which is the ADP-ribosyltransferase, and Ib, which is involved in the binding and internalization of the toxin by the cell (Stiles and Wilkins, 1986). The crystallization of the C2-I component in complex with its substrate NADH has recently been achieved (Tsuge et al., 2003; Fig. 40), showing a close relationship of iota toxin with insecticidal protein VIP2 of *Bacillus cereus*.

Clostridium difficile induces its pathogenic effects by secreting a number of potent cytotoxins; one, in particular, has been found to possess ADP-ribosyltransferase activity (CDT). CDT acts on the cytoskeleton structure by disaggregating actin filaments and thus provokes an increase of globular actin (G-actin; Popoff et al., 1988b; Gulke et al., 2001).

Another member of the group of iota-like toxins is the *Clostridium spiroforme* toxin, composed of a toxic subunit Sa and a binding subunit Sb (Popoff et al., 1989). The level of primary sequence homology detected among the enzymatic and binding components of this class of ADP-ribosylating toxin ranges from 32% to 80% identity, the binding domains being the better conserved. The C2 toxin is the one with the lower degree of sequence conservation, and this correlates with the fact that it does not appear to be crossreactive with the other iota-like toxins.

Experiments of site-directed mutagenesis have helped to define for these toxins an active site very similar to those described for the better studied members of the family of ADP-ribosyltransferases (Barth et al., 1998; see the section ADP-Ribosyltransferases: A Common Structure of the Catalytic Site in this Chapter).

Escherichia coli Lymphostatin

Lymphostatin is a very recently identified protein in enteropathogenic strains of *E. coli* (EPEC; Klapproth et al., 2000).

A leading cause of diarrhea among infants in developing countries, EPEC is also one of the few known bacterial causes of chronic diarrhea. These strains are characterized by their ability in host cells to induce cytoskeletal rearrangements that result in the formation of adhesion pedestals. This mechanism known as "the attaching and effacing effect" (Moon et al., 1983; Khoshoo et al., 1988) ultimately allows the bacterium to colonize the host for prolonged periods.

Lymphostatin also has been identified as one of the primary factors that selectively block the production of interleukin-2 (IL-2), IL-4, IL-5 and γ interferon by human peripheral cells and inhibit proliferation of these cells, thus interfering with the cellular immune response (Klapproth et al., 1995).

Lymphostatin, a very large toxin with a predicted molecular weight of 366 kDa, shares significant homology with the catalytic domain of the large clostridial cytotoxins, including toxins A and B of *Clostridium difficile*, lethal toxin of *C. sordelii*, and a toxin of *C. novyi*. Its corresponding gene, *lifA*, with 9669 bp, is the largest reported gene in *E. coli*. Some *lifA* mutants of EPEC have been constructed to verify the lymphocyte inhibitory factor (LIF) activity of its gene product; lysates of this mutant lacked the ability of wildtype EPEC lysates to inhibit expression of IL-2, IL-4 and γ interferon mRNA and protein in mitogen-stimulated lymphocytes, while the expression of IL-8 was unaffected (Klapproth et al., 2000). Experiments of colony hybridization performed using an internal fragment of the *lifA* gene identified a similar gene present in most of the EPEC and enterohemorrhagic *E. coli* (EHEC) strains able to produce the attaching and effacing lesions on host epithelial cells, but this gene was not found in other *E. coli* and related organisms (Klapproth et al., 2000).

Toxins Acting on Intracellular Trafficking

Vesicle structures are essential in the eukaryotic cell for a number of vital processes such as receptor-mediated endocytosis and exocytosis; these are used either to internalize portions of the plasma membrane and address them to the specialized compartment, or to transport to the cell surface molecules synthesized in the ER and modified in the Golgi apparatus.

One example of exocytic pathway is that involving the release of neurotransmitters that are contained within small synaptic vesicles packed at synaptic terminals; the majority of these vesicles are bound to the cytoskeleton and are not directly available for immediate release, but some of them are present at the cytosolic face of the presynaptic membrane and are ready to release their content. However, at low calcium concentrations, only an occasional vesicle fuses to the presynaptic membrane, giving rise to a depolarization event. This event leads to the opening of calcium channels and thus to an increase of calcium concentration, which finally triggers the fusion of the neurotransmitter vesicles with the plasma membrane.

Recently, this field was greatly advanced by the identification of the eukaryotic molecules responsible for vesicle docking and membrane fusion. Three of these proteins (namely vesicle-associated membrane protein [VAMP]/synaptobrevin, synaptosome-associated protein [SNAP-25], and syntaxin) are the specific targets of a number of neurotoxins produced by bacteria of the genus *Clostridium* (CNTs; Montecucco and Schiavo, 1994; Fig. 41).
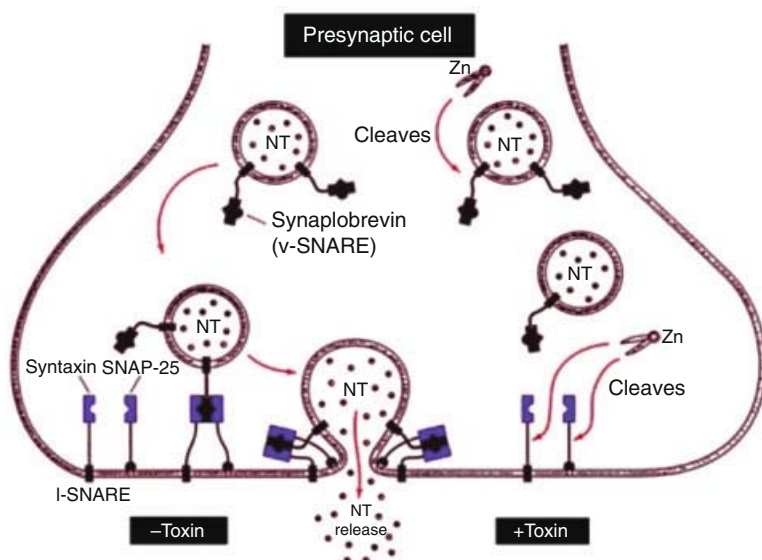

Fig. 41. Mechanism of action of clostridial neurotoxins.

The CNT family is composed of tetanus neurotoxin (TeNT) and seven serotypes of botulinum neurotoxins (BoNT/A–BoNT/G), which are specific zinc-dependent proteases whose action finally causes the block of neuroexocytosis (Schiavo et al., 1992; Pellizzari et al., 1999; Lalli et al., 2003). The degree of sequence homology detected among this group of toxins is high, ranging from 30% to more than 50% identity.

Clostridium tetanii Neurotoxin

Tetanus neurotoxin (TeNT; Fig. 1, panel 19) is the unique causal agent of the pathological condition of spastic paralysis known as tetanus. This is one of the most potent toxins known so far, with a 50% lethal dose (LD_{50}) in humans of 0.1–1.0 ng/kg.

The TeNT is produced by *Clostridium tetanii* as a single chain polypeptide of 150 kDa that, following proteolytic cleavage, is divided into fragments H (heavy) and L (light) held together by a disulfide bridge. Its overall structure is similar to that of A/B toxins, where the toxic subunit A is represented here by the light chain L, and subunit B is constituted by the H_C and H_N domains. The heavy chain is composed of fragments HC, which has recently been found to bind di- and trisialylgangliosides on neuronal cell membranes, and HN, which is involved in the transmembrane translocation of the L chain to the cytosol (Schiavo et al., 1990; Shapiro et al., 1997). The L chain is a 50-kDa fragment containing the –HExxH– motif typical of metalloproteases. It binds zinc and specifically cleaves VAMP/synaptobrevin, a eukaryotic factor essential for membrane fusion (Rossetto et al., 1995).

The first step of intoxication is the specific binding of domain H_C of TeNT to both high and low affinity receptors exposed on the presynaptic neuronal membrane at neuromuscular junctions (Montecucco, 1986); the second step is internalization of TeNT into the peripheral motoneuron and then retrograde axonal transport. The TeNT is released through the postsynaptic membrane into the synaptic space where it enters into the inhibitory interneurons of the central nervous system through receptor-mediated endocytosis (Halpern and Neale, 1995). At this point, while the H_C domain is in the vesicle, the translocation domain H_N helps the catalytic light chain L to cross the vesicle membrane and gain access to the cytosolic compartment where L performs its toxic activity on VAMP/synaptobrevin (Montal et al., 1992).

Interestingly, domain H_C retains the unique transport properties of the intact holotoxin and is capable of eliciting a protective immunological response against the full-length tetanus neurotoxin. A single zinc atom is bound to the L chain
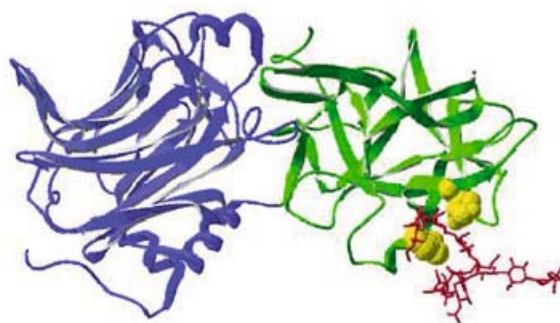

Fig. 42. Crystal structure of the receptor-binding domain HC of tetanus neurotoxin in complex with a ganglioside analogue (in red). The N-terminus and C-terminus are colored in blue and green, respectively. The residues probably involved in ganglioside binding are yellow (see Fig. 1, panel 18).

of TeNT and is essential for toxicity. This specific metallo-dependent proteolytic activity is common to the other clostridial toxins and to the lethal factor (LF) of *Bacillus anthracis*.

The crystal structure of the receptor-binding fragment HC of tetanus neurotoxin has been recently determined at 2.7 Å resolution (Umland et al., 1997; Fig. 42) revealing an N-terminal jelly-roll domain and a C-terminal β-trefoil domain.

To determine which amino acids in tetanus toxin are involved in ganglioside binding, homology modeling was performed using recently resolved X-ray crystallographic structures of the tetanus toxin HC fragment. On the basis of these analyses, the amino acids tryptophan 1288, histidine 1270, and aspartate 1221 were found to be critical for binding of the HC fragment to ganglioside GT1b (Fotinou et al., 2001; Louch et al., 2002).

Although the overall sequence homology detected among clostridial neurotoxins is significant, this similarity weakens in the region encompassing the C-terminal domain (Murzin et al., 1992); the fact that each toxin possesses its own unique receptor and is immunologically distinct from the others has been attributed to sequence divergence of this domain which, therefore, could be responsible for receptor specificities (Lacy et al., 1999).

Clostridium botulinum Neurotoxins

These neurotoxins (BoNT/A-G; Fig. 1, panel 20) are the causative agents of the flaccid paralysis typical of clinical botulism intoxication (Hatheway, 1995). All of them are zinc-dependent proteases that show a strong tropism for the neuromuscular junction (Simpson, 1980; Rossetto et al., 1995), where they bind to still unidentified receptors in a strictly serotype-specific manner. This binding step is followed by the entry of the toxin into the cytoplasm of the motoneurons and

by specific proteolytic cleavage of intracellular targets belonging to the family of soluble *N*-ethylmaleimide-sensitive, fusion factor attachment protein receptors (SNARE). Four out of the seven botulinum neurotoxins (BoNT/B, D, F and G) cleave VAMP/synaptobrevin, another two act specifically on SNAP/25, whereas the last one, BoNT/C, cleaves both syntaxin and SNAP/25 substrates. In all cases, the ultimate effect is the total block of acetylcholine release (Montecucco and Schiavo, 1995).

These toxins are generally produced as large complexes of 300–900 kDa containing additional proteins such as hemagglutinin (300 kDa) and nontoxic peptides, which are believed to act as stabilizing agents of the neurotoxins in the gut environment (Sakaguchi, 1983).

The BoNTs are synthesized as inactive polypeptide chains of 150 kDa, which (following proteolytic cleavage) divide into two chains of 50 and 100 kDa that remain linked by a disulfide bridge. The catalytic function is carried by the 50-kDa fragment, the light chain L (residues 1–437), whereas the 100-kDa subunit (heavy chain, H) contains both the translocation (residues 448–872) and the receptor-binding domains (residues 873–1295; Krieglstein et al., 1994). The crystal structure determined for the full-length polypeptide of BoNT serotype A (Lacy et al., 1998; Fig. 43) reveals a number of remarkable features, particularly related to the peculiar structure of the translocation domain. This contains, in fact, a central pair of α-helices 105 Å long and a 50-residue loop that wraps around the catalytic domain in a belt-like fashion, partially occluding the active-site pocket. This unusual loop bears the site of the proteolytic cleavage, which is required for

activation of the toxin; the fact that in the protoxin, the translocation domain shields the active site explains why the catalytic activity in test tube experiments is greatly enhanced by reduction of the disulfide bond. The fold of the translocation domain suggests a mechanism of pore formation different from that displayed by other pore-forming toxins. The helices are antiparallel and amphipathic and twist around each other in a coiled-coil-like structure. In addition, the domain has two strand-like segments that lie parallel to the helical axis and are predicted to be directly involved in membrane spanning. Very recently, the X-ray structure obtained for the recombinant form of chain L of BoNT-A has shed light on a possible novel mode of substrate binding and catalytic mechanism (Segelke et al., 2004).

The highest degree of homology detected among this family of clostridial neurotoxins is concentrated in the light chain L (30–60% identity; particularly its N-terminus), probably involved in substrate recognition, and in the central portion that contains the catalytic zinc-binding motif –HexxH– characteristic of zinc endopeptidases. The zinc atom coordinated by this pocket is required for the in vivo toxicity of BoNTs.

Years ago, medical experiments demonstrated that injection of BoNT/A is very effective in strabismus; since then, the therapeutic applications of these neurotoxins have been extended to a variety of diseases which benefit from a functional paralysis of the neuromuscular junction, and all the BoNTs are under clinical testing (Jankovich and Hallett, 1994).

Helicobacter pylori Vacuolating Cytotoxin Vac A

Highly pathogenic strains of *Helicobacter pylori*, the etiological agent of peptic ulcer and gastritis (Cover and Blaser, 1992), produce vacuolating cytotoxin A (VacA; Papini et al., 1994; Fig. 1, panel 21). This toxin is responsible for massive growth of vacuoles within epithelial cells and, when administered to mice, VacA causes loss of gastric gland architecture, cell necrosis, and gastric ulceration (Telford et al., 1994). Synthesized as a 140-kDa precursor, VacA is secreted from the bacterium through its 45-kDa carboxy-terminal domain, using a mechanism similar to that of neisserial IgA proteases (Schmitt and Haas, 1994; Fiocca et al., 1999). When purified from the culture supernatant of Type I *H. pylori* strains, the protein has a molecular weight of approximately 600–700 kDa, suggesting the idea of a multimeric complex; electron microscopy studies have in fact demonstrated the flower-shaped structure of the toxin (Lupetti et al., 1996; Fig. 44) resulting from the aggregation of either six or seven monomers, each

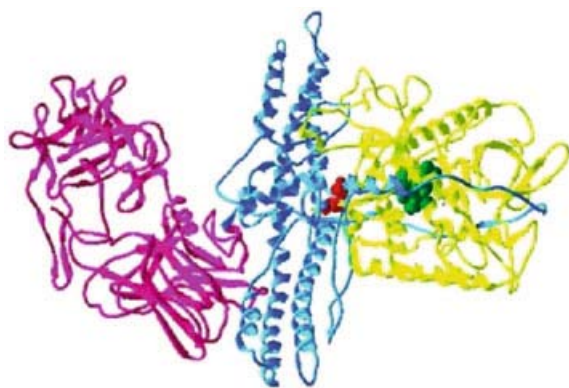Fig. 43. X-ray structure of *Clostridium botulinum* neurotoxin serotype A. The 50-kDa catalytic domain (L) is colored in yellow, with the zinc-binding domain in green. The N-terminal portion of the 100-kDa subunit involved in translocation is blue, whereas the C-terminal receptor-binding moiety is in magenta. The disulfide bond linking the two 50- and 100-kDa fragments is colored in red (see Fig. 1, panel 20).

Fig. 44. Vacuolating cytotoxin structure: heptameric and hexameric forms of VacA as observed in electron micrographs of quick-freeze, deep-etched preparations. The oligomers are approximately 30 nm in diameter with a 10–12 nm central cavity.
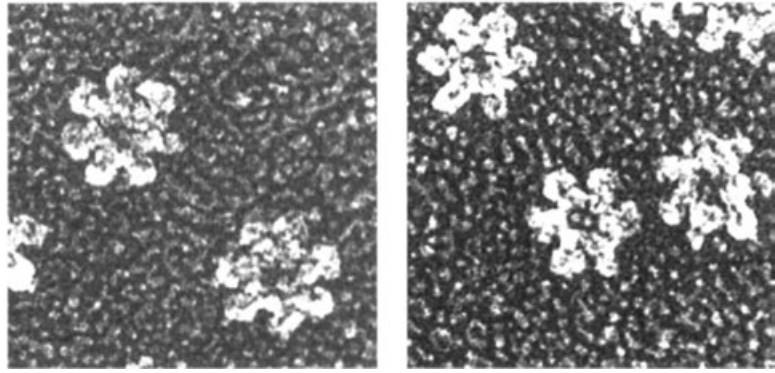
comprising the 95-kDa amino-terminal region of the VacA precursor. Recently, a model has been proposed to show how VacA can insert into membranes forming hexameric, anion-selective pores (Kim et al., 2004).

Each monomer can be cleaved at a protease-sensitive site into two fragments of 37 kDa and 58 kDa (p37 and p58 moieties) that may represent the A and B moieties of AB-like bacterial toxins. The 37-kDa, amino-terminal portion is highly conserved at the sequence level and is able to induce vacuoles when the *vacA* gene is placed under the control of a strong eukaryotic promoter and transfected into epithelial cells. This evidence suggests that the active site could be located in this region of the molecule, whereas the carboxy-terminal portion is likely to be devoted to receptor recognition and binding. Although VacA is exported over the outer membrane and is released from the bacteria, recent data have been presented to show that a portion of the toxin remains associated with the bacterial surface. Surface-associated toxin is biologically active and organized into distinct toxin-rich domains on the bacterial surface. Upon bacterial contact with host cells, toxin clusters are transferred to the host cell surface via a contact-dependent mechanism, followed by uptake and intoxication (Ilver et al., 2004).

The mechanism of toxicity exploited by this virulence factor has not yet been completely elucidated. What is known is that VacA causes an alteration of the endocytic pathway, which results in the selective swelling of late endosomes or prelysosomal structures. The small GTP-binding protein Rab7 is necessary for vacuole formation (Papini et al., 1994, 1997). Even though it is unknown, the target of VacA action is strongly believed to be a fundamental effector in membrane trafficking.

Streptococcus pyogenes
NAD$^+$ Glycohydrolase

NAD$^+$ glycohydrolase is an important virulence factor produced by group A streptococci (GAS),

which is thought to enhance pathogenicity by facilitating the spread of the microorganism through host tissues. This enzyme catalyzes the hydrolysis of the nicotinamide-ribose bond of NAD to yield nicotinamide and ADP-ribose. Differently from ADP-ribosylating toxins, NAD$^+$ glycohydrolases possess a much higher rate of NADase activity and do not require an ADP-ribose acceptor. Interestingly this GAS virulence factor is functionally linked to streptolysin O (SLO), a pore-forming toxin, which has been shown to be required for efficient translocation of NAD$^+$ glycohydrolase into epithelial cells. In contrast to the wildtype GAS, isogenic mutants deficient in the expression of SLO, NAD$^+$ glycohydrolase, or both proteins resulted in reduced cytotoxicity and keratinocyte apoptosis. These results suggest that NAD$^+$ glycohydrolase modulates host cell signaling pathways and contributes to the enhancement of streptolysin O cytotoxicity (Bricker et al., 2002).

Toxins Injected into Eukaryotic Cells

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

In the classical view, toxins were believed to be molecules that cause intoxication when released by bacteria into the body fluids of multicellular organisms. This definition failed to explain the pathogenicity of many virulent bacteria such as *Salmonella*, *Shigella* and *Yersinia*, which did not release toxic proteins into the culture supernatant. Today we know that these bacteria also intoxicate their hosts by using proteinaceous weapons. These bacteria intoxicate individual eukaryotic cells by using a contact-dependent secretion system to inject or deliver toxic proteins into the cytoplasm of eukaryotic cells (Fig. 2, panel 3). This is done by using specialized secretion systems that in Gram-negative bacteria are called "type III" or "type IV," depending on whether they use a transmem-

brane structure similar to flagella or conjugative pili, respectively.

Mediators of Apoptosis

Pathogens use different mechanisms to induce or prevent apoptosis in host cells. Virulence factors produced by the pathogen can interact directly with effector molecules of apoptosis or interfere with factors involved in cell survival (Weinrauch and Zychlinsky, 1999).

They include: pore-forming toxins which induce cell death by altering host cell permeability, bacterial toxins (such as DT, PAETA, Shiga and Shiga-like toxins) which induce cell death by inhibition of host protein synthesis, and type III secreted proteins of *Shigella*, *Salmonella* and *Yersinia* which are directly delivered into host cell compartment and trigger apoptosis by altering the signal transduction pathway. This latter class of toxins will be described here in more detail.

IpaB

Shigella, the causative agent of bacillary dysentery produces IpaB. *Shigella* invades the epithelial cells by causing the cell cytoskeleton to reorganize during bacterial entry. The bacteria are phagocytosed by macrophages and rapidly escape from phagosomal compartment to the cytosol where they induce apoptosis of the macrophages. Invasion and cytotoxicity require *Shigella* invasion plasmid antigen (Ipa) proteins, which are secreted by a type III secretion apparatus. Invasion and escape from the phagosome are dependent upon the expression and secretion of the IpaB, IpaC and IpaD. Only IpaB is required to initiate cell death by interaction with the interleukin-1β converting enzyme, or caspase I, which is one of the effector molecules of apoptosis. The IpaB-induced apoptosis results in an inflammation that has the effect not only of clearing and possibly localizing the infection but also promoting bacterial spread in the intestinal epithelium (Hilbi et al., 1998). Protein domains directly involved in pathogenicity have recently been mapped (Guichon et al., 2001).

SipB

An analog of *Shigella* invasin IpaB, *Salmonella* invasion protein (SipB) is produced by *Salmonella* and is delivered to the host cells by a type III secretion system. In contrast to *Shigella*, *Salmonella* does not escape from the phagosome, but it survives and multiplies within the macrophages. *Salmonella* virulence genes responsible for invasion and killing of macrophages are encoded by a chromosomal operon named *sip*

containing five genes (sipEBCDA; Hermant et al., 1995). The sip genes show high sequence homology with the *ipa* operon of *Shigella*, and the Sip proteins show functional similarities with Ipa proteins. Both proteins have a predominant alpha-helical structure and contain two helical transmembrane domains, which insert deeply into the bilayer (Hume et al., 2003). Similarly to IpaB, SipB also induces apoptosis by binding interleukin-1β-converting enzyme.

Necessary for *Salmonella*-induced macrophage apoptosis, SipB acts through a caspase-I-activating mechanism similar to that used by IpaB (Hersh et al., 1999). Also, SipB can complement IpaB mutants, enabling them to invade cells and escape macrophage phagosomes.

YopP, YopJ and Related Proteins

Yersinia enterocolitica and *Yersinia pestis* produce YopP and YopJ, respectively (Straley et al., 1986; Mills et al., 1997). Following contact with the host cell, *Yersiniae* deliver into the cytoplasm of eukaryotic cells, through a type-III secretion system, plasmid-encoded proteins named "Yersinia-outer-membrane proteins" (Yop). These proteins are able to induce alteration of cytoskeleton (YopE and YopT), inhibition of phagocytosis (YopH), and in the case of YopP and YopJ, induction of apoptosis.

The mechanism by which *Yersinia* induces apoptosis is probably different from that described for *Shigella*, inasmuch as *Yersinia* induces apoptosis from the outside of host cells. The binding of YopJ directly to the superfamily of MAPKKs blocks both their phosphorylation and subsequent activation. These activities of YopJ are responsible for the inhibition of extracellular signal-regulated kinase, downregulation of TNF-α and suppression of the nuclear factor kappa B (NF-κB) signaling pathways, preventing cytokine synthesis and promoting apoptosis (Orth et al., 1999). The YopJ-related proteins that are found in a number of bacterial pathogens of animals and plants, such as AvrRxv from *Xanthomonas campestris* (Whalen et al., 1993), AvrA from *Salmonella* (Hardt et al., 1997), and y410 from *Rhizobium* (Freiberg et al., 1997) may function to block MAPKKs so that host signaling responses can be modulated upon infection. Whereas no function is known for AvrA and y410, AvrRxv is a plant pathogen virulence protein involved in the programmed cell death pathway.

Toxins Interfering with Inositol Phosphate Metabolism: SopB and IpgD

The SopB protein, secreted by *Salmonella dublin*, is a virulence factor essential for

	Motif 1	Motif 2
SopB	VVTFNFGVNELALKM	AWNCKSGKDRTGMMSDE
IpgD	VAAFNVGVNELALKL	CWNCKSGKDRTGMQDAE
PTPaseI	PVLFNVGINEQQTLA	FTSCKSAKDRTAMSVTL
PTPaseII	PVLFNVGINEQQTLA	FTCCKSAKDRTSMSVTL

Fig. 45. Alignment of conserved motifs.

enteropathogenicity. The toxin hydrolyzes phosphatidylinositol triphosphate (PIP$_3$), which is a messenger molecule that inhibits chloride secretion, thus favoring fluid accumulation and diarrhea (Norris et al., 1998). Furthermore, SopB, mediates actin cytoskeleton rearrangements and bacterial entry in a Rac-1 and Cdc42-dependent manner. Consistent with an important role for inositol phosphate metabolism in *Salmonella*-induced cellular responses, a catalytically defective mutant of SopB failed to stimulate actin cytoskeleton rearrangements and bacterial entry (Zhou et al., 2001).

SopB is homologous to the *Shigella flexneri* virulence factor IpgD, suggesting that a similar mechanism of virulence is also present in *Shigella*. Both proteins contain two regions of sequence similarities (motifs 1 and 2, Fig. 45) with human inositol polyphosphatases types I and II. Motif 2 contains a consensus sequence (Cys-X5-Arg) characteristic of Mg^{+2}-independent phosphatases in which the cysteine is the residue essential for catalysis. Recent studies have shown that IpgD acts as a potent inositol 4-phosphatase and is responsible for dramatic morphological changes of the host cell, ultimately leading to consistent actin filament remodeling (Niebuhr et al., 2002).

Toxins Acting on the Cytoskeleton

PSEUDOMONAS AERUGINOSA EXOENZYME S. This toxin is one of several products of *Pseudomonas aeruginosa* that contributes to its pathogenicity (Woods et al., 1989; Kulich et al., 1993; Fig. 1, panel 22). It belongs to the group of ADP-ribosylating factors that lack both the receptor-binding and translocation domains, and are directly injected by bacteria into the cytoplasm of eukaryotic cells. In this case, bacteria intoxicate individual eukaryotic cells by means of a contact-dependent type III secretion system (Yahr et al., 1996).

The 49-kDa ExoS protein ADP-ribosylates the small GTP-binding protein Ras at multiple sites but preferably at Arg-41 (Ganesan et al., 1998; see the section ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity in this Chapter). To become enzymatically active, ExoS requires the interaction with a cytoplasmic activator named "FAS" or "14.3.3"

(Fu et al., 1993). When cells are transfected with the *exos* gene under the control of a eukaryotic cell promoter, a collapse of the cytoskeleton and a change of the morphology of the cells can be observed as primary consequences.

Pseudomonas aeruginosa ExoS is a bifunctional cytotoxin where the ADP-ribosyltransferase domain is located within its C-terminus portion. Recent studies showed, in fact, that when transfected or microinjected into eukaryotic cells, the N-terminus part of ExoS (amino acid residues 1–234) stimulates cell rounding. The N-terminus of ExoS (1–234) does not influence nucleotide exchange of Rho, Rac and Cdc42 but increases GTP hydrolysis. It has also been shown that Arg-146 of ExoS is essential for the stimulation of GTPase activity of Rho proteins (Goehring et al., 1999). The GTPase activating domain (GAP) of ExoS has been crystallized (Wurtele et al., 2001). In addition to these toxic effects performed on the cytoskeleton, other activities have been demonstrated for Exo S, such as the adhesive property on buccal cells (Baker et al., 1991) and the induction of human T lymphocyte proliferation (Mody et al., 1995). From sequence analysis, it has been possible to identify the regions of Exo S, which could be involved in NAD binding and thus constitute the common structure of the catalytic site.

CLOSTRIDIUM BOTULINUM EXOENZYME C3 AND RELATED PROTEINS. Produced by certain strains of *Clostridium botulinum* types C and D, exoenzyme C3 is a 251-amino acid protein that specifically ADP-ribosylates *rho* and *rac* gene products in eukaryotic cells (Moriishi et al., 1993; Fig. 1, panel 23). These substrates belong to the group of small GTP-binding proteins and seem to have a fundamental role in cell physiology and cell growth. The ADP-ribosylation process occurs at asparagine residues (Asn-41) located in the putative effector binding domains of *rho* and *rac* and thus alter their functions (Sekine et al., 1989). The enzymatic activity is identical to that of all ADP-ribosylating enzymes; however, the recently solved 3D structure has shown that the C3 exoenzyme structure can be distinguished by the absence of the elongated α-helix, which generally constitutes the ceiling of the active site cleft in the ADP-ribosylating toxins crystallized so far. Seemingly, this feature does not impair the ability of C3 either to accommodate the NAD substrate or to carry out the enzymatic reaction (Han et al., 2001; Fig. 46).

This exoenzyme is the prototype of the group of A-only toxins because it apparently lacks the receptor-binding B domain and thus is unable to enter the cells; for this reason, C3 cannot be considered a real virulence factor, and still unknown is whether C3 alone is able to intoxicate the cells.

Nevertheless, when microinjected into cells, it causes complete disruption of actin-stress fibers, rounding of the cell body, and formation of arborescent extensions.

Other members of this family of C3-related exoenzymes have been isolated from Gram-positive bacteria, such as certain strains of *Staphylococcus aureus* (Sugai et al., 1992), *Clostridium limosum* (Just et al., 1992) and *Bacillus cereus* (Just et al., 1995c). Whereas *C. botulinum* C3 and *C. limosum* exoenzyme are about 70% homologous and immunologically related, the epidermal cell differentiation inhibitor (EDIN) produced by *S. aureus* is only 35% homologous with C3 and shows no immunological crossreactivity (Fig. 47). However, crystal data recently obtained for *S. aureus* C3 exotoxin (EDIN-B) have disclosed a very similar structure (Evans et al., 2003). *Bacillus cereus* exoenzyme exhibits the same substrate specificity as the other C3-like transferases (it was found to act specifically on rho proteins). Nevertheless some differences can be observed for this toxin, such

as the higher molecular weight (28 kDa) and, more importantly, the lack of immunological relationship to any other member of this family (Just et al., 1995a).

SALMONELLA SOPE AND SIPA. *Salmonella typhimurium* achieves entry into cells by delivering effector proteins into the cytosol through a type III secretion system. These effectors stimulate signal pathways leading to reorganization of the cell's actin cytoskeleton, membrane ruffling and stimulation of nuclear response to promote efficient bacterial internalization. One of the proteins that stimulate the cellular response is SopE, which is able to activate signaling pathways through Rho GTPases by stimulating GTP/GDP nucleotide exchange on proteins such as Cdc42 and Rac (Hardt et al., 1998).

These signaling events lead to the recruitment of cellular proteins such as actin and T-plastin (an actin-binding protein that bundles actin), which finally induce actin cytoskeleton rearrangement and membrane ruffling. In addition, SopE stimulates nuclear responses that induce the synthesis of proinflammatory cytokines that contribute to the induction of diarrhea.

These cytoskeletal rearrangements are further modulated by SipA, which binds directly to actin, stabilizes actin filaments inhibiting depolymerization, and forms a complex with T-plastin thus increasing its actin-bundling activity (Zhou et al., 1999a, 1999b). SipA activities result in localized actin cytoskeleton reorganization and more pronounced extension of membrane ruffles, which facilitate bacterial uptake. The actin-cytoskeleton reorganization induced by *Salmonella* is reversible and infected cells are able to recover their normal architecture after bacterial internalization.

Crystal structures are available for SipA (Lilic et al., 2003) and for the catalytic fragment of SopE in complex with its host cellular target
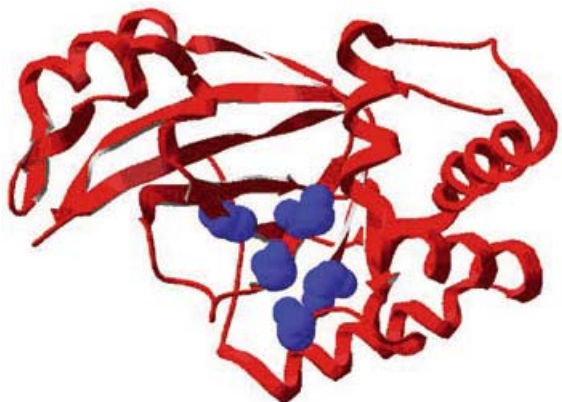

Fig.46. Crystal structure of exoenzyme C3 of *C. botulinum*. The residues which constitute the catalytic site are in blue.
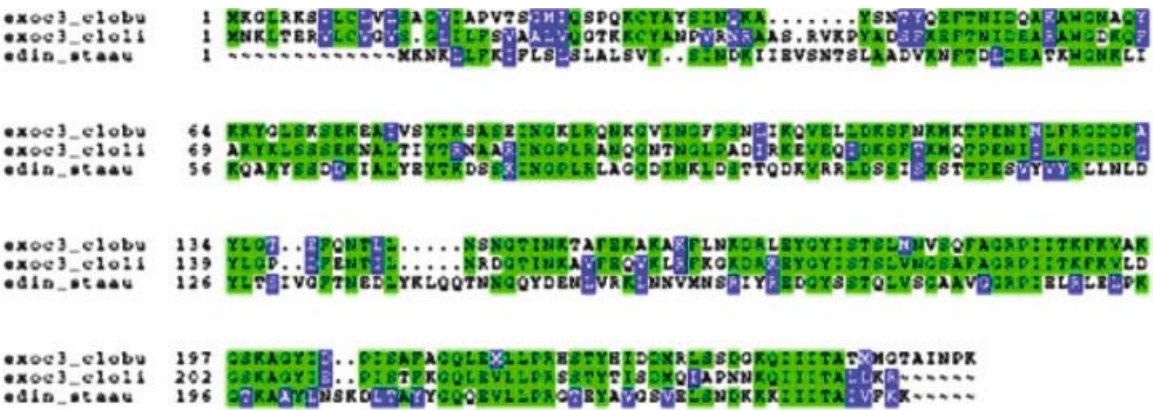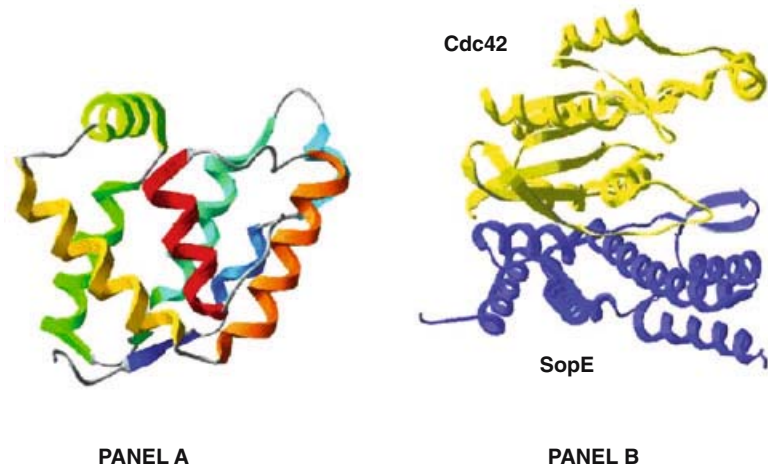

Fig. 47. Multiple sequence alignment of protein toxins belonging to the group of exoenzyme C3-like ADP-ribosyltransferases.

Fig. 48. Crystal structures of SipA (panel A) and of SopE in complex with Cdc42 (panel B).

PANEL A

PANEL B

Cdc42 (Buchwald et al., 2002; Figs. 1 [panels 24 and 25] and 48).

SHIGELLA IPAA. The entry of *Shigella* into epithelial cells requires the Ipa proteins, which are secreted upon cell contact by the type III apparatus and act in concert. The IpaB and IpaC proteins form a complex that binds B1 integrin and CD44 receptors and induces actin polymerization at the site of bacterium-cell contact, allowing the formation of membrane extension that probably requires also the action of Cdc42, Rac and Rho GTPases (Nhieu and Sansonetti, 1999).

The translocation of IpaA into the cell cytosol probably favors *Shigella* entry. The IpaA protein binds with high affinity to the N-terminal residues 1–265 of vinculin, a protein involved in linking actin filaments to the plasma membrane. The vinculin-IpaA complex interacts with F-actin inducing subsequent depolymerization of actin filaments. Presumably, these interactions further modulate the formation on the membrane of adhesion-like structures required for efficient invasion.

Shigella internalization still occurs at low levels in the absence of IpaA, suggesting that IpaA acts in concert with other bacterial effectors to promote cell entry. Binding of the *Shigella* protein IpaA to vinculin induces F-actin depolymerization (Bourdet-Sicard et al., 1999). The IpaA and vinculin rapidly associate during bacterial invasion. Although defective for cell entry, an *ipaA* mutant is still able to induce foci of actin polymerization but differs from wildtype *Shigella* in its ability to recruit vinculin and α-actinin. It has been postulated that IpaA-vinculin interaction initiates the formation of focal adhesion-like structures required for efficient invasion (Tran Van Nhieu et al., 1997).

YERSINIA YOPE. A protein secreted by *Yersinia* through a type III secretion system,

YopE contributes to the ability of *Yersinia* to resist phagocytosis (Rosqvist et al., 1990). Following infection of epithelial cells with *Yersinia*, the microfilament structure of the cells changes leading to a complete disruption of the actin microfilaments, which finally results in cell rounding and detachment from the extracellular matrix (Rosqvist et al., 1991). The effector YopE was recently shown to possess GAP activity towards the Rho GTPases RhoA, Rac and CDC42 in vitro (Aili et al., 2003; Fig. 1, panel 26). Further experimentation has shown that in vivo YopE is able to inhibit Rac- but not Rho- or Cdc42-regulated actin structures. Furthermore, the structure of this toxin has recently been solved, showing a close relationship with the analogous ExoS Gap domain (Evdokimov et al., 2002).

YERSINIA YOPT. YopT is the prototype of a new family of 19 cysteine proteases with potent
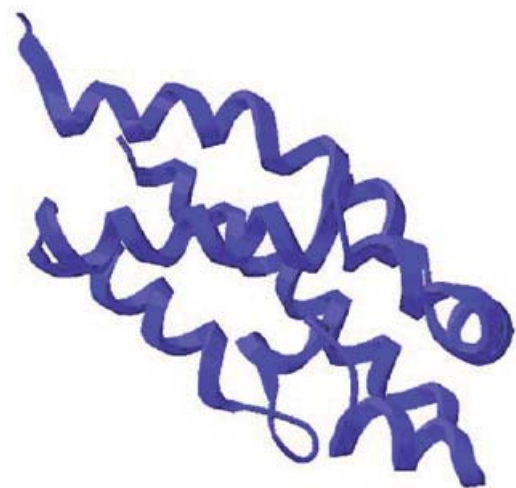

Fig. 49. Crystal structure of YopE catalytic domain.

effects on host cells. These include the AVr protein of the plant pathogen *Pseudomonas* and possibly Yop-J of *Yersinia*. YopT cleaves the posttranslationally modified cysteine located at the C-terminal end of Rho GTPases (DKG-CASS), causing the loss of the prenyl group from RhoA, Rac and cdc42, and releasing them from the membrane (Shao et al., 2003). The inability of Rho to be located to the membrane causes disruption of the cytoskeleton. While the C terminus of YopT is crucial for activity, the N terminus of YopT is crucial for substrate binding (Sorg et al., 2003).

SHIGELLA VIRA. The invasiveness of *Shigella* is an essential pathogenic step and a prerequisite of bacillary dysentery. VirA is a *Shigella* effector protein, which is delivered into the host cell by a specialized type III secretion system. This protein can interact with tubulin to promote microtubule destabilization and membrane ruffling (Yoshida et al., 2002). With this mechanism, *Shigella* is able to remodel the cell surface and thus promote its entry into the host. Recent data have shown that *VirA* deletion mutants displayed decreased invasiveness and were unable to stimulate Rac1.

Toxins Acting on Signal Transduction

YERSINIA YPKA AND YOPH. Phosphorylation is central to many regulatory functions associated with the growth and proliferation of eukaryotic cells. Bacteria have learned to interfere with these key functions in several ways. The best-known system is that of *Yersinia*, where a protein kinase (YpkA; Barz et al., 2000) and a protein tyrosine phosphatase (YopH; Zhang, 1995; Fig. 1, panel 27) are injected into the cytoplasm of eukaryotic cells by a type III secretion system to paralyze the macrophages before they can kill the bacterium.

YpkA is a Ser/Thr protein kinase that also displays autophosphorylating activity in vitro. In vivo experiments have shown that this protein is essential for virulence: in fact, challenge with a *YpkA* knockout mutant causes a nonlethal infection, whereas all mice challenged with wildtype *Y. pseudotuberculosis* die. Recently, natural eukaryotic substrates of YpkA have been identified by using a two-hybrid assay. These belong to the class of small GTPases and comprise RhoA and Rac-1, but not Cdc42.

YopH is a modular protein where the tyrosine phosphatase domain shows a structure and catalytic mechanism very similar to those of eukaryotic enzymes. YopH acts by dephosphorylating cytoskeletal proteins thus disrupting phosphotyrosine-dependent signaling pathways necessary for phagocytosis. Host protein targets include Crk-associated substrate, paxillin, and
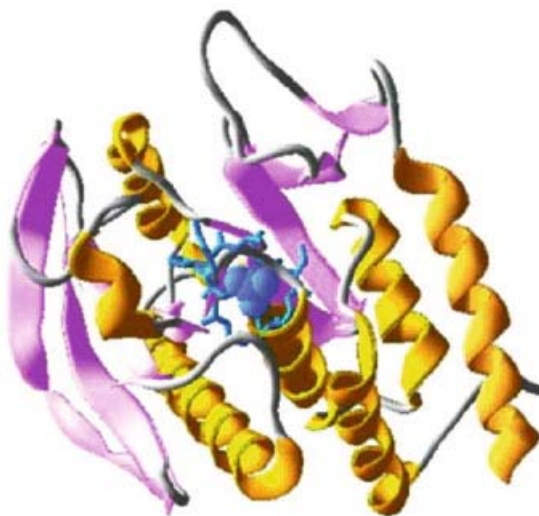

Fig. 50. X-ray structure of YopH. Colors have been assigned on the basis of secondary structure (yellow for helix and pink for β-sheet). The PTPase phosphate-binding loop and Cys-403 are in blue.

focal adhesion kinase. In vivo, YopH inhibits phagocytosis by polymorphonuclear leukocytes (PMNs) and macrophages (Fallman et al., 1995; Ruckdeschel et al., 1996). The protein has a molecular weight of 51 kDa and is composed of an N-terminal domain important for translocation and secretion (Sory et al., 1995) and a C-terminal domain homologous to eukaryotic PTPases (Guan and Dixon, 1990; Bliska, 1995).

The three-dimensional structure of YopH has been solved (Stuckey et al., 1994; Su et al., 1994) revealing the presence of a catalytic domain which, despite its low level of sequence identity to the human PTP1B, still contains all of the invariant residues present in eukaryotic PTPases. Its tertiary fold is a highly twisted α/β structure with an eight-stranded β-sheet flanked by seven α-helices. Residues 403–410 form the PTPase phosphate-binding loop with the invariant Cys-403 thiol centered within the loop (Fig. 50).

EPEC TIR. A 78-kDa protein produced by enteropathogenic *E. coli* (EPEC) strains, Tir mediates the attachment of bacteria to eukaryotic cells and is essential for EPEC virulence. The Tir protein is tyrosine phosphorylated upon injection into eukaryotic cells by a type III secretion system. While in the host cell, it becomes an integral part of the eukaryotic cell membrane and functions as receptor for intimin, the major EPEC adhesin (Kenny et al., 1997). It is believed that, once in the host, Tir adopts a hairpin-like structure using its two putative transmembrane domains (TMDs) to span the host cell membrane. The region between the two TMDs constitutes the extracellular loop that functions as the intimin-binding domain. Following tyrosine

phosphorylation, the protein mediates actin nucleation, resulting in pedestal formation and triggering tyrosine phosphorylation of additional host proteins, including phospholipase C-γ. Tir is essential for EPEC virulence and was the first bacterial protein described to be tyrosine phosphorylated by host cells (Crawford and Kaper, 2002).

HELICOBACTER PYLORI CAGA. Cytotoxin-associated gene A (CagA) is an immunodominant protein produced by most virulent strains of *Helicobacter pylori*, with a size that can vary from 128 kDa to 146 kDa and which is commonly expressed in peptic ulcer disease (Covacci et al., 1993b). CagA is characterized by a central region containing an EPIYA motif, which can be repeated up to six times increasing the molecular weight of the protein. The gene is encoded within a pathogenicity island, which also encodes the type IV secretion system necessary to inject the protein into eukaryotic cells. Once injected into the host cell, the protein is tyrosine phosphorylated at the EPIYA motif by the kinase C-Src and Lyn. The signal is proportional to the number of EPIYA motives present (Stein et al., 2000). The tyrosine phosphorylated CagA (CagA-P) activates SHP-2, inactivates C-Src leading to cortactin dephosphorylation triggering a signal transduction cascade (which results in cellular scattering proliferation, a phenotype indistinguishable from that induced by the hepatocyte growth factor [HGF]).

The long-term chronic infection and the continuous stimulation increase the risk of cancer of people infected by CagA+ *H. pylori*. CagA is the first bacterial protein linked to cancer in humans and the *cagA* gene can be considered the first bacterial oncogene.

YERSINIA PESTIS YOPM. YopM is an effector protein delivered to the cytoplasm of infected cells by the type III secretion mechanism of *Yersinia pestis*. YopM is a highly acidic protein, which is essential for virulence, but whose mechanism of action is still elusive. Differently from other effectors, this toxin has been shown to accumulate not only in the cytoplasm but also in the nucleus of mammalian cells. Recently, McDonald and colleagues have found that YopM interacts with two kinases, protein kinase C-like 2 (PRK2) and ribosomal S6 protein kinase 1 (RSK1). These two kinases associate only when YopM is present, and expression of YopM in cells stimulates the activity of both kinases. These results indicate that PRK2 and RSK1 are the first intracellular targets of YopM (McDonald et al., 2003).

The X-ray structure determined for YopM has shown a modular architecture constituted by leucine-rich repeats, mainly organized in an extended β-sheet structure (Evdokimov et al.,
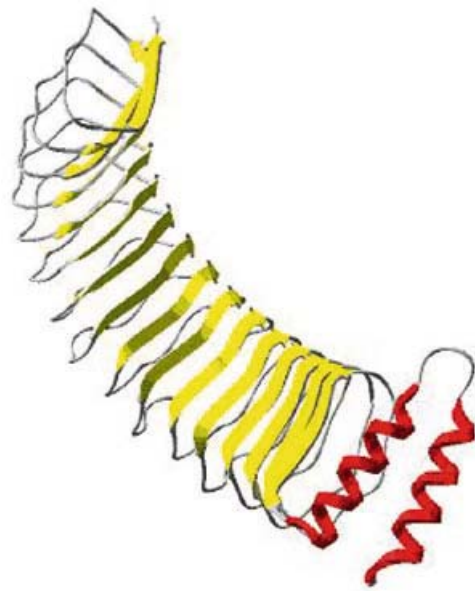
Fig. 51. Crystal structure of YopM effector protein of *Yersinia pestis*.

2001; Figs. 1 [panel 28] and 51). This organization is very similar to that found for other important proteins, such as rab geranylgeranyltransferase and internalin B produced by *Listeria*.

SALMONELLA SPTP. *Salmonella* protein tyrosine phosphatase (SptP) is an effector protein secreted by the type III secretion apparatus of *Salmonella enterica*. SptP is a modular protein composed of two functional domains, a C-terminal region with sequence similarity to *Yersinia* tyrosine phosphatase YopH, and an N-terminal domain showing homology to bacterial cytotoxins such as *Yersinia* YopE and *Pseudomonas* ExoS (Murli et al., 2001). Recently, it was demonstrated that this domain possesses strong GTPase activating domain protein (GAP) activity for Cdc42 and Rac1. The crystal structure of SptP-Rac1 complex has shown that SptP is strongly stabilized by this interaction (Stebbins and Galan, 2000; Fig. 52).

PSEUDOMONAS AERUGINOSA EXOU. Several extracellular products secreted by the *P. aeruginosa* type III secretion system are responsible for virulence. Among these, the 70-kDa protein, ExoU, is responsible for causing acute cytotoxicity in vitro and epithelial lung injury. Recent studies demonstrated that ExoU has lipase activity, and that the cytotoxicity of ExoU is dependent on its patatin-like phospholipase domain. The results suggest that ExoU requires the presence of a catalytically active site Ser(142) and that a yet unknown eukaryotic cell factor(s) is necessary for its activation (Tamura et al., 2004).

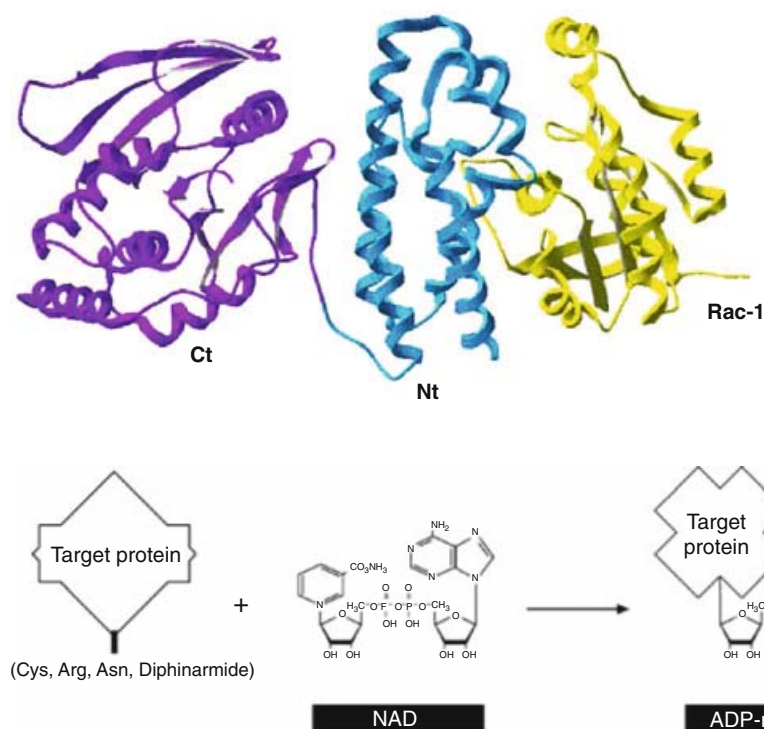Fig. 52. Crystal structure of SptP in complex with Rac-1 (yellow). The N-term and C-term domains of SptP are colored in cyano and purple, respectively.

Fig. 53. Mechanism of ADP-ribosylation reaction catalyzed by ADP-ribosyltransferases.

ADP-Ribosyltransferases: A Family of Toxins Sharing the Same Enzymatic Activity

ADP-Ribosylating Toxins: Main Features

The ADP-ribosylating toxins are a class of bacterial proteins that characterized by an enzymatic domain with ADP-ribosyltransferase activity (Ueda and Hayaishi, 1985; Althaus and Richter, 1987). During ADP-ribosylation (Fig. 53), these toxins bind NAD and transfer the ADP-ribose moiety to a specific substrate molecule, which is thus forced to undergo a dramatic functional modification. The toxic effect is totally dependent upon the enzymatic activity.

On the basis of their overall structure, ADP-ribosyltransferases can be separated into A/B toxins, binary toxins, and A-only toxins, where A is the subunit with the enzymatic activity, and B is the carrier domain involved in the recognition of the specific surface receptor and in the translocation of the toxic moiety into the eukaryotic cell. Most of the best characterized ADP-ribosylating toxins belong to the class with an A/B architecture: pertussis toxin (PT; Locht et al., 1986; Nicosia et al., 1986), cholera toxin (CT; Mekalanos et al., 1983), and *E. coli* heat-labile enterotoxin (LT; Spicer and Noble, 1982; Yamamoto et al., 1984) are typical examples of this family where the A domain (called "S1" in PT) bears the enzymatic core and the B domain is an oligomer that helps the translocation across the cell membrane; the two subunits are linked together by noncovalent bonds. The genes coding for CT and LT are highly homologous (Dallas and Falkow, 1980) and are organized into operons located on the chromosome of *Vibrio cholerae* and on a plasmid of *E. coli* (So et al., 1978).

Diphtheria toxin (DT; Pappenheimer, 1977; Collier et al., 1982) and *Pseudomonas aeruginosa* exotoxin A (PAETA; Gray et al., 1984b; Wick et al., 1990) are A/B toxins with a three-domain structure: the catalytic domain C, contained in fragment A, and the transmembrane domain T and receptor-binding domain R, both within the B subunit.

The binary (as opposed to the A/B) toxins have a fairly similar organization, but in this case the A and B domains are separately secreted in the culture supernatant where the B domain initially binds the receptor on the surface of the target cell and only then is able to bind the A subunit and help its translocation into the cytosol. Examples of this family of ADP-ribosyltransferases are the C2 toxin of *Clostridium botulinum* (Aktories et al., 1986), the iota toxin of *C. perfringens* (Perelle et al, 1995), the toxin of *C. spiroforme* (Popoff and Boquet, 1988a), the mosquitocidal toxin (MTX) of *Bacillus sphaeri-*

cus (Thanabalu et al., 1993), and the *C. difficile* transferase (Just et al., 1994).

Finally, the "A-only" toxins include Exo S of *Pseudomonas aeruginosa* (Kulich et al., 1994) and other toxins such as C3 of *Clostridium botulinum* (Nemoto et al., 1991), EDIN of *Staphylococcus aureus* (Sugai et al., 1990), and the toxins of *Bacillus cereus* (Just et al., 1995b) and of *Clostridium limosum* (Just et al., 1992). All the A-only toxins possess a still unknown mechanism of cell entry, with the notable exception of Exo S, which has been shown to be directly injected into eukaryotic cells by a specialized secretion system (Yahr et al., 1996).

With the exception of actin, all the eukaryotic proteins that are ADP-ribosylated by these toxins are GTP-binding proteins (G-proteins); these proteins are molecular switches involved in a number of essential cell functions including protein synthesis and translocation, signal transduction, cell proliferation, and vesicular trafficking (Hamm and Gilchrist, 1996).

ADP-Ribosylating Toxins: A Common Structure of the Catalytic Site

Bacterial enzymes with ADP-ribosyltransferase activity include a variety of toxins with different structural organizations; the better-represented class is that comprising proteins with an A/B structure (PAETA, DT, CT, LT and PT), where subunit A is responsible for enzymatic activity and subunit B is involved in receptor binding.

Other toxins, termed "binary toxins" (*Clostridium botulinum* toxin C2 and related proteins) are still composed of the two functional domains A and B. However, they reside on different molecules and need to interact to acquire activity. Finally, there is a group of ADP-ribosylating toxins that do not possess the receptor-binding domain B at all and are thus named "A-only toxins." This group includes *Clostridium botulinum* exoenzyme C3 and related proteins, which are unable to invade the cells, and toxins which are directly injected into eukaryotic cells (ExoS) by means of a specialized secretion apparatus.

From primary sequence analysis, it is possible to identify two main groups of homology (Fig. 54): the DT-like group, mainly composed of DT and PAETA, and the CT-like group comprising the remaining ADP-ribosyltransferases.

Although some homology is present among the members of the CT group, no overall significant and extended sequence similarity can be detected to justify the observed common mech-
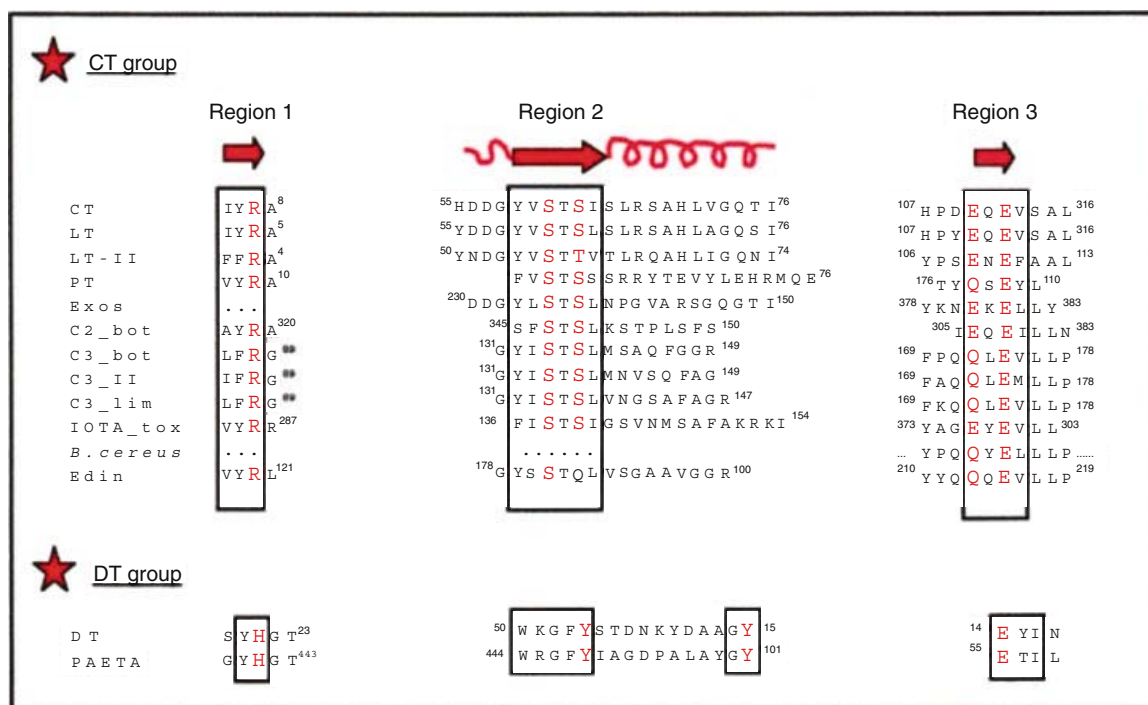
Fig. 54. Sequence alignment of protein segments containing Regions 1, 2 and 3 of bacterial ADP-ribosylating enzymes. The two groups of homology (DT-like and CT-like groups) are distinguished. Catalytic residues of Regions 1 and 3, and most relevant and conserved residues of Region 2 are colored in red; extended consensus sequences detected in the three regions are boxed, whereas other partially conserved residues are in boldface. Predicted and observed secondary structure folding is indicated for each region: Regions 1 and 3 are β-strands (arrows), while Region 2 is characterized by a short coil (solid line), followed by a β-strand and by an α-helix.

anism of catalysis; nevertheless, biochemical experiments of photoaffinity labeling and studies of site-directed mutagenesis had previously demonstrated for most of the toxins that the presence of a glutamic acid is so important for catalytic activity, even a conservative substitution with an aspartate could not be tolerated without loss or drastic decrease of toxicity (Douglas and Collier, 1987; Wilson et al., 1990; Lobet et al., 1991; Antoine et al., 1993).

On the basis of these experimental data and on the crystallographic structures which are now available for LT (Sixma et al., 1991), CT (Zhang et al., 1995), PT (Stein et al., 1994), DT (Choe et al., 1992) and PAETA (Allured et al., 1986), a common catalytic site could be identified which, despite the low level of sequence homology, is almost perfectly superimposable for all them (Domenighini et al., 1994).

In terms of tertiary structure, the active site is a cleft formed by a β-strand followed by a slanted α-helix that has a different length in the various toxins (spanning from 12 residues for DT, PAETA and LT, to 21 in the case of PT). The β-strand and the α-helix represent, respectively, the lower and upper face of the cavity in which the nicotinamide ring of NAD is anchored during the enzymatic reaction (Region 2 of Fig. 54).

Although all the toxins share this similar folding in the region of the active site, at the amino acid level, the only residue which is well conserved among all the representatives of the CT- and DT-groups is a glutamic acid (Glu-148 of DT, Glu-553 of PAETA, Glu-112 of CT and LT, and Glu-129 of PT), which corresponds to the core of Region 3 (Fig. 54). These residues retain an equivalent spatial position and orientation residing in a short β-strand flanking the external side of the cavity (Fig. 55). With the exception of the conserved glutamate, the consensus sequence generated for Region 3 differs between the two groups of toxins. In the DT family, in fact, it is composed of the catalytic Glu followed by an aromatic and a hydrophobic residue, whereas in the CT-group, the consensus can be extended to a few neighboring residues (Fig. 45). On the basis of alignment of C2-I with iota toxin and with the other ADP-ribosyltransferases, the catalytic glutamate was identified (Glu-389 of C2) and its function experimentally confirmed by site-directed mutagenesis (Barth et al., 1998). In the case of *Pseudomonas aeruginosa* Exo S, the equivalent Glu has been mapped at position 381 (Liu et al., 1996).

Another well-conserved residue is His-21 of DT that can be aligned to His-440 of PAETA, and with the conserved Arg-7 of CT and LT, and Arg-9 of PT (Burnette et al., 1988, 1991; Papini et al., 1990; Lobet et al., 1991; Han and Galloway, 1995). The segment comprising this residue is

Fig. 55. Superimposition of the three-dimensional structures of the NAD-binding cavities (Region 2) of the bacterial toxins LT (green), PT (yellow), DT (red) and PAETA (blue). The catalytic residues carried by Region 1 (Arg/His) and by Region 3 (Glu) and common to the two regions of homology are shown. In addition, the two essential tyrosines of the DT group are colored in red.

termed "Region 1" (Fig. 54). These amino acids are once again located in essentially identical positions within the active site, lying opposite to the glutamic acid on an antiparallel β-strand close to the internal face of the catalytic cleft.

Although several models have been proposed to explain the possible function of the conserved histidine/arginine of Region 1, that this residue does not play a direct role in catalysis seems now widely accepted; very likely it may have a function in maintaining the integrity of the active-site pocket upon formation of structurally stabilizing hydrogen bonds (Johnson and Nicholls, 1994). Nevertheless, mutations at the His-440 position of PAETA, though affecting the enzymatic activity, have little or no effect on NAD-binding (Han and Galloway, 1995); this suggests that His-440 may not be exactly homologous to His-21 of DT or to the arginines of the CT-group. In the case of the C2-I component of clostridial toxin C2, site-directed mutagenesis of Arg-299 induced a dramatic reduction of transferase activity, thus suggesting an equivalent role for this residue in the conformation of the active site (Perelle et al., 1995).

Region 2 includes a number of amino acids that, while maintaining the same secondary structure in both DT- and CT-families (Fig. 55), result in a major sequence difference (Fig. 54). This is mainly a structural region corresponding to the core of the active site cleft, which is devoted to the docking of NAD. The consensus sequence generated for the DT group is characterized by two conserved tyrosines spaced by ten amino acids, and located on the middle portion

of the β-strand and on the internal face of the α-helix, respectively. Tyr-54 and Tyr-65 of DT, and Tyr-470 and Tyr-481 of PAETA have been shown to play an important role in catalysis inasmuch as they anchor the nicotinamide ring during the reaction by creating a p pile of three aromatic rings which strengthen the overall binding of NAD and stabilize the complex (Carroll and Collier, 1984; Li et al., 1995). This consensus motif can be extended to four other residues which precede the first Tyr, and to a glycine residue which is located upstream of the second Tyr.

In PT, a similar role is likely to be played by Tyr-59 and Tyr-63, which have a similar spatial orientation and distance from each other. This observation is supported by the fact that in CT and LT, where the stacking interactions produced by the two tyrosines are lacking, the affinity for NAD is 1000-fold lower (Galloway and van Heyningen, 1987).

In the case of the CT-group, Region 2 is centered on a consensus core domain characterized by the motif Ser-Thr-Ser that is observed and predicted to fold in a β-strand representing the floor of the cavity. Experiments of site-directed mutagenesis have confirmed the importance of these residues in maintaining the shape of the cavity. Substitutions of Ser-61 and Ser-63 of LT with Phe and Lys, respectively, have been shown to produce nontoxic mutants (Harford et al., 1989; Fontana et al., 1995). The core sequence of Region 2 can be extended to give the more general consensus aromatic-hydrophobic-Ser-Thr-Ser-hydrophobic.

Another amino acid that has been proposed as being important in catalysis is His-35 of PT (Xu et al., 1994) located near the beginning of the β-strand which forms the floor of the cavity, in a position equivalent to that of His-44 of LT and CT (Yamashita et al., 1991); a functional homologue, His is also present in the mosquitocidal toxin SSII-1 from *Bacillus sphaericus* (Thanabalu et al., 1991) but is absent in DT and PAETA. In the 3D structure, this residue appears to be sufficiently close to the oxygen atom of the ribose ring of NAD to interact with it and increase the electrophilicity of the adjacent anomeric carbon atom. The absence of an equivalent residue in DT and PAETA again supports the idea that the two groups of toxins perform the same enzymatic activity in a slightly different fashion.

An additional feature that is common to all ADP-ribosylating toxins is the need for a conformational rearrangement to achieve enzymatic activity.

In the native structure, in fact, the NAD-binding site of LT and CT is obstructed by a loop (amino acids 47–56) that needs to be displaced to obtain a functional NAD-binding cavity. A

functionally homologous region is also present in PT where the loop comprises residues 199–207. In the case of DT, where the crystallographic data of the complex are available, the observation that the active-site loop consisting of amino acids 39–46 changes structure upon NAD-binding, suggests that these residues may be important for the recognition of the ADP-ribose acceptor substrate, EF-2 (Weiss et al., 1995; Bell and Eisenberg, 1996).

The recent publication of the crystallographic data of the DT-NAD complex, and the presence of common features within all ADP-ribosylating toxins, permits speculation on a possible common mechanism of catalysis (Fig. 56). The best hypothesis is that NAD enters the cavity, which is then made available for the recognition of the
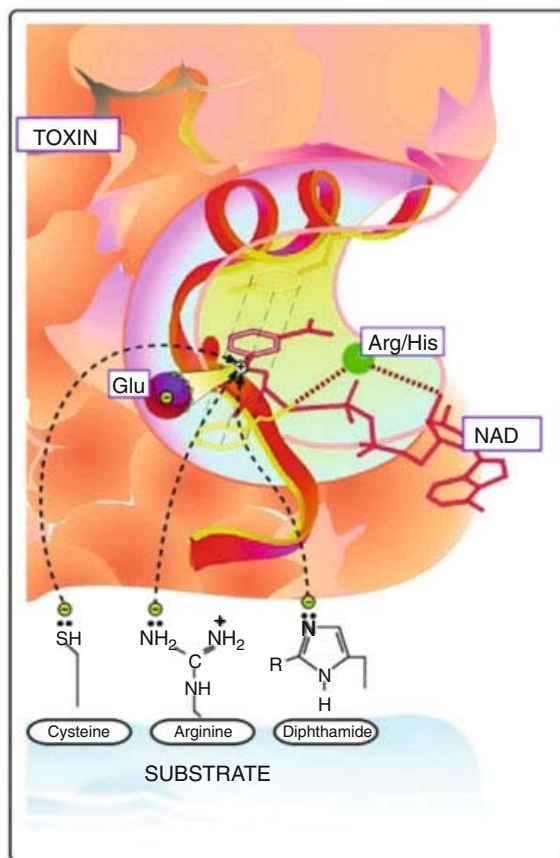

Fig. 56. Schematic representation of a possible common mechanism of catalysis: the nicotinamide adenine dinucleotide (NAD) molecule (red) is docked inside the cavity by means of stacking interactions provided by the two aromatic rings (yellow) that protrude from the scaffold of Region 2. The catalytic glutamic acid (purple) and its possible interactions with the acceptor residues of the various substrates are also reported. The Arg/His residue (green) provides stabilizing interactions with the backbone of the cavity and seems to be also responsible for the correct positioning of NAD inside the pocket.

substrate, upon displacement of the mobile loop. Then, NAD docks at the bottom of the pocket where a small residue (the conserved serine in Region 2 of the CT-group, the threonine-56 of DT, and the alanine-472 of PAETA) is required to allow good positioning. The nicotinamide moiety of NAD is then blocked in a suitable position by means of stacking interactions provided by a couple of aromatic rings (Tyr-54 and Tyr-65 of DT, Tyr-470 and Tyr-481 of PAETA, and possibly, Tyr-59 and Tyr-63 of PT). In this context the conserved arginine/histidine might display its key role in maintaining the correct shape of the active site pocket via hydrogen bonds formed with the backbone of the structure and possibly one with the ribose moiety. The enzymatic reaction is then catalyzed by the essential glutamic acid, which is likely to stabilize a positively charged oxocarbonium intermediate of NAD, to favor its subsequent interaction with the nucleophilic residue of the incoming substrate (diphthamide in the case of DT and PAETA, arginine in the case of LT and CT, and cysteine in the case of PT).

Novel ADP-Ribosylating Toxins Detected by Genome-Mining

With the advent of the Genomic Era, identification of bacterial factors possibly involved in virulence is an easier challenge. In fact, given the vast amount of information that we now possess on toxins—including sequence data—and thanks to the growing number of sequenced bacterial genomes, it is possible to proceed by homology criteria to predict novel members of important classes of bacterial toxins.

Several examples exist where computer-based methodologies have been instrumental to the identification of novel potential bacterial toxins in sequenced genomes. Among them, we will mention here the case of mono ADP-ribosyltransferases.

Mono-ADP-ribosyltransferases (mADPRTs) constitute a class of potent toxins in bacteria, which generally play an important role in the pathogenesis of related microorganisms. Despite the poor overall conservation at the primary structure level, the catalytic subunits of these toxins show a remarkable similarity within the enzymatic cavity, so that these portions of the proteins are quite well conserved.

For these reasons, and encouraged by the availability of a growing number of sequenced bacterial genomes, a series of studies have been directed towards the computer-based identification of novel members of this family of enzymes by means of sequence-homology criteria in finished and unfinished genome sequences. As a result, more than twenty novel putative ADP-ribosyltransferases have been identified both in Gram-positive and Gram-negative organisms, including five from *Pseudomonas syringae*, five from *Burkholderia cepacea*, two from *Enterococcus faecalis*, and one each from *Salmonella typhi*, *Streptococcus pyogenes*, *Mycoplasma pneumoniae*, *Streptomyces coelicolor*, *Bacillus halodurans* and *Vibrio parahaemolyticus* (Pallen et al., 2001). With the exception of the protein detected in *Salmonella*, which is adjacent to an ORF protein similar to the S2 subunit of pertussis toxin, all the other genome-derived putative ADPRTs lack a predicted translocation domain. So far, none of these bacterial proteins has been tested either for their ADP-ribosyltransferase activity or for the capability of entering eukaryotic cells; however, sequence data indicate a possible role of these proteins in the pathogenesis of the corresponding microorganisms. Very recently, a new protein has been added to the list of ADP-ribosyltransferases detected by computer analysis (Masignani et al., 2003). This novel factor has been identified by means of primary and secondary structure analysis in the genomic sequence of a virulent isolate of *Neisseria meningitidis* and has been named "NarE" (*Neisseria* ADP-ribosylating enzyme). As predicted by "in silico" studies, biochemical analysis has demonstrated that NarE is capable of transferring an ADP-ribose moiety to a synthetic substrate.

Toxins with Unknown Mechanism of Action

See Tables 1 and 2 for a summary of the principal features of toxins described in this section.

The zonula occludens toxin (Zot) is produced by bacteriophages present in toxinogenic strains of *Vibrio cholerae*. Zot is a single polypeptide chain of 44.8 kDa, which localizes in the outer membranes. After internal cleavage, a carboxy-terminal fragment of 12 kDa is excreted and this is probably responsible for the biologic effect. Zot has the ability to reversibly alter the tight junctions of intestinal epithelium, thus facilitating the passage of macromolecules through mucosal barriers (Di Pierro et al., 2001). Zot has also been shown to act as mucosal adjuvant and to induce protective immune response in the animal model (Marinaro et al., 2003).

Hemolysin BL (HBL) is an enterotoxin produced by *B. cereus*, which is composed of three proteins (B, L1 and L2), each with a molecular mass of 40 kDa, and whose corresponding genes are located on the same operon. HBL has hemolytic as well as dermonecrotic and vascular permeability activities and is able to cause fluid accumulation in ligated rabbit ileal loops (Beecher et al., 1997; Beecher and Wong, 2000).

The bile-salt hydrolase (BSH) is a protein elaborated by *Listeria monocytogenes*, which is absent from the genome of the nonpathogenic *L. innocua*. The *bsh* gene encodes an intracellular enzyme and is positively regulated by PrfA, the transcriptional activator of known *L. monocytogenes* virulence genes (Dussurget et al., 2002). Furthermore, *bsh* deletion mutants show reduced virulence and liver colonization, thus demonstrating that BSH is a toxin specifically involved in the intestinal and hepatic phases of listeriosis.

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