# THE BACTERIAL TOXIN TOOLKIT

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Pathogenic bacteria and higher eukaryotes have spent a long time together, leading to a precise understanding of one another's way of functioning. Through rapid evolution, bacteria have engineered increasingly sophisticated weapons to hit exactly where it hurts, interfering with fundamental host functions. However, toxins are not only useful to the bacteria — they have also become an essential asset for life scientists, who can now use them as toolkits to explore cellular processes.

AMPHIPATHIC
A molecule that contains both hydrophobic and hydrophilic parts.

β-BARREL
A configuration in which β-strands are organized into a sheet that is rolled up into a barrel-like structure. When imbedded in a lipid bilayer, the interior of the barrel is exposed to solvent, in contrast to the outer wall.

GLYCAN
A polymer consisting of several monosaccharide residues (polysaccharide). In the case of GPI-anchored proteins, the basic unit is composed of a glucosamine and three mannose residues.

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One outcome of the evolutionary race between host and microbial pathogens is the development of sophisticated and specific virulence factors, which give a selective advantage to the organism that produces them[1]. Over the past decade, our understanding of the mechanism of action of bacterial toxins has increased enormously[2]. These studies have contributed to the molecular definition — and, sometimes, to the discovery — of important pathways in cell biology. A striking example is the finding that tetanus toxin is an endopeptidase, specific for a membrane protein that is localized on synaptic vesicles and secretory granules. This finding was crucial in the definition of the molecular mechanisms that underlie regulated secretion[3]. Similarly, the importance of guanine-nucleotide-binding (G) proteins in signal transduction was uncovered by studies into the action of the cholera and pertussis toxins, which modify different classes of G proteins[4].

Now that the mode of action of many toxins has been unravelled, they can be used as highly specific and efficient tools in cell biology. Here, we have made a subjective selection of what might be beneficial to an experimental cell biologist, dividing bacterial toxins into areas of interest (FIG. 1) and highlighting, where appropriate, the possibility of using them in new, exciting applications.

## Plasma membrane permeabilization

A recurrent theme in cell biology is the need to design systems that make it possible to gain access to the cytoplasm while keeping the cell alive during the course of the experiment. Such a semi-intact cell system can be obtained by treating cells with pore-forming toxins[5] (TABLE 1). These toxins are secreted as water-soluble proteins and, when added to target cells, they bind to cell-surface components acting as receptors. Many pore-forming toxins must then oligomerize into an AMPHIPATHIC, circular ring-like structure that inserts into biological membranes to form a pore[6]. These structures can be formed from just a few monomers (up to seven), generating small pores, or they may be composed of up to 50 units, creating pores large enough to permit the passage of proteins. The membrane-spanning region of these oligomeric complexes is thought to be formed by β-BARRELS, as shown by the crystal structure of the *Staphylococcus aureus* α-toxin[7].

The *S. aureus* α-toxin and *Aeromonas* sp. aerolysin[5,8] (FIG. 2) are the best-characterized members of this family. These two toxins are suitable for creating hydrophilic pores of fixed size (approximately 1.5 nm in diameter) that allow exchange of small ions as well as nucleotides. The α-toxin has the advantage of being widely used; the drawback, however, is that high doses in the 100-nM range are usually necessary. Doses strongly depend on the cell type, presumably reflecting the distribution of an unknown α-toxin receptor. By contrast, aerolysin has not yet been widely used as a tool in cell-permeabilization experiments, but it has the advantage of being effective on any mammalian cell at picomolar concentrations. This is because aerolysin binds to glycosylphosphatidylinositol (GPI)-anchored proteins, a ubiquitous class of membrane-associated proteins, and binding occurs through the GLYCAN core[9] that is present in all the lipid anchors of this protein family.

LIPID RAFTS
Dynamic assemblies of cholesterol and sphingolipids on the cell surface, which can change their composition in response to intracellular and extracellular stimuli.

Large pores, which allow the passage of proteins, can be formed by members of the cholesterol-dependent toxin family, which includes streptolysin O (SLO)[5,10] (FIG. 2). Members of this large toxin family require cholesterol for pore formation, and they generate channels of variable size, up to 35 nm in diameter, corresponding to about 50 subunits. During polymerization, each subunit contributes 4 β-strands to the pore-forming β-barrel, generating a transmembrane structure composed of ~200 β-strands[11]. Walev *et al.*[10] recently reported that pore formation can be reversible under appropriate experimental conditions. Indeed, short exposure to SLO, followed by incubation of the cells in a toxin-free buffer in the presence of 1–2-mM calcium, resulted in repair of the membrane lesion.

We must, however, keep in mind that pore-forming toxins induce a wide spectrum of cellular events as a result of the permeabilization process. These include activation of G proteins[12], production of cytokines[13] or vacuolation of the endoplasmic reticulum (ER)[14]. Some of these effects are probably triggered by ion fluxes, calcium entry or depletion of cytoplasmic components, whereas others are still unexplained. These events must be taken into account when interpreting the data from experiments with pore-forming toxins involving plasma-membrane permeabilization of living cells.

### Tagging specific molecules

Toxins are generally active even at very low concentrations. This high efficiency is due to the fact that most toxins act catalytically, resulting in an amplification of cell responses, and also because essential surface molecules on target cells are opportunistically used by the toxins as receptors. This high affinity and specificity for their surface receptors make toxins excellent probes to follow the molecules with which they interact. Two examples will be highlighted here: cholera toxin and lysenin, which bind to the ganglioside GM1 (REF. 15) and sphingomyelin[16], respectively.

The receptor-binding unit of cholera toxin (CT-B) is a homopentamer, in which each subunit can interact with ganglioside GM1, a ubiquitous monosialylated glycosphingolipid found in eukaryotic cells (FIG. 2). This pentavalent binding unit has a higher affinity for clustered GM1 (REF. 15), which can be found in cholesterol–glycosphingolipid-rich microdomains of the plasma membrane called LIPID RAFTS[17]. Lipid rafts are the subject of intensive research because of their role in various fundamental cellular processes such as membrane sorting, signalling and cholesterol homeostasis, and markers for this membrane compartment are scarce. Therefore, CT-B (labelled with peroxidase, colloidal gold or fluorophores) has been an extremely useful tool as it allows the detection of GM1 not only on dot blots, but also on cells by either electron or fluorescence microscopy[18].

Lysenin, a newly characterized toxin, could be similarly useful to study lipid rafts, as it binds specifically to the raft component sphingomyelin[16]. Little is known about the intracellular distribution of sphingomyelin — not only under normal conditions, but also under pathological conditions, such as, for example, lipid-storage diseases[19]. With appropriate labelling, lysenin should allow detection of sphingomyelin on blots as well as on cells (FIG. 2) and permit the study of the distribution and transport of this lipid. So far, lysenin has been successfully used to select mutant cells that are defective in sphingolipid biosynthesis[20].

### Removing lipids or lipid-anchored molecules

Several bacterial species produce enzymes that affect lipids[1], and are therefore extremely useful in cell biology. These include soluble phosphatidylinositol-specific phospholipase Cs (PI-PLCs), the prototype of which is the enzyme purified from *Bacillus cereus*. PI-PLCs recognize and cleave both phosphatidylinositol and its glucosyl derivatives, and are therefore exploited to release GPI-anchored proteins from the cell surface (FIG. 2). This treatment has been the method of choice to determine whether or not a protein is GPI-anchored[21].

A second class of enzymes of great interest are sphingomyelinases[1] (FIG. 2), which cleave sphingomyelin to generate ceramide, a second messenger involved in cell

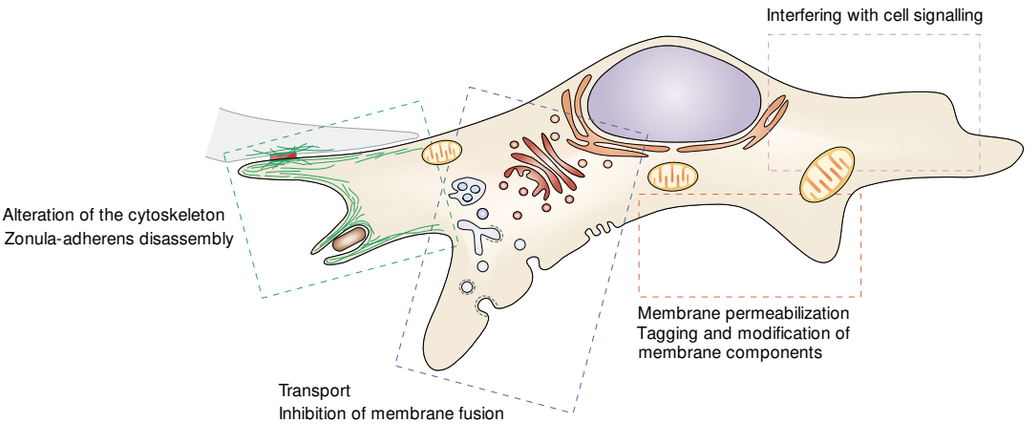


Figure 1 | **Cellular processes targeted by bacterial toxins.** These have been subdivided into four classes, each represented by examples in FIGS 2 to 5.

growth, differentiation, apoptosis and membrane transport[22,23]. The coupling of ceramide signalling to specific signal-transduction cascades is specific to both the stimulus and the cell type, and seems to be determined by the subcellular topology of its production[23]. Interestingly, transfection with *Bacillus cereus* sphingomyelinase expressed under the control of an inducible promoter led to the identification of a signalling pool of sphingomyelin that is distinct from the pool accessible to exogenous sphingomyelinase[24].

### Probing intracellular routes

With the notable exception of pore-forming toxins mentioned above, most bacterial toxins have an enzymatic

Table 1 | **The toxin toolkit**

| Aim | Toxins used | Producing organism | Activity |
|---|---|---|---|
| ***Plasma membrane permeabilization*** | | | |
| Small pores | α-toxin | *Staphylococcus aureus* | Pore formation |
| | Aerolysin | *Aeromonas* spp. | Pore formation |
| Large pores | Streptolysin O | *Streptococcus pyogenes* | Pore formation |
| Reversible permeabilization | Streptolysin O | *Streptococcus pyogenes* | Pore formation |
| ***Labelling of specific membrane components*** | | | |
| Sphingomyelin | Lysenin | *Eisenia foetida* (earthworm) | Binding |
| GM1 | Cholera toxin B-subunit | *Vibrio cholera* | Binding |
| ***Removing specific molecules*** | | | |
| GPI-anchored proteins | PI–PLC | *Bacillus cereus* and others | Phospholipase |
| Sphingomyelin | Sphingomyelinase | *Bacillus cereus* and others | Phospholipase |
| ***Inhibition of specific molecules*** | | | |
| Heterotrimeric G proteins | Pertussis toxin | *Bordetella pertussis* | ADP ribosylation |
| RhoA*, RhoB*, RhoC* | Exoenzyme C3 | *Clostridium botulinum* | ADP ribosylation |
| | C3-like toxins | *Clostridium limosum* and others | ADP ribosylation |
| | A and B toxins (LCT) | *Clostridium difficile* | Monoglucosylation |
| | α-toxin (LCT) | *Clostridium novyi* | Monoglucosylation |
| | EDIN | *Staphylococcus aureus* | ADP ribosylation |
| RhoA*, RhoB*, RhoC*, RhoE* | C3stau2 | *Staphylococcus aureus* | ADP ribosylation |
| Rac* | A and B toxins (LCT) | *Clostridium difficile* | Monoglucosylation |
| | Lethal toxin (LCT) | *Clostridium sordellii* | Monoglucosylation |
| | α-toxin (LCT) | *Clostridium novyi* | Monoglucosylation |
| Cdc42* | A and B toxins (LCT) | *Clostridium difficile* | Monoglucosylation |
| | Lethal toxin (LCT) | *Clostridium sordellii* | Monoglucosylation |
| | α-toxin (LCT) | *Clostridium novyi* | Monoglucosylation |
| Ral*, Ras*, Rap* | Lethal toxin (LCT) | *Clostridium sordellii* | Monoglucosylation |
| G-actin (not α-actin isoforms) | C2 | *Clostridium botulinum* | ADP ribosylation |
| | CTDA and CTDB | *Clostridium difficile* | ADP ribosylation |
| G-actin (also α-actin isoforms) | Iota toxin | *Clostridium perfringens* | ADP ribosylation |
| | Iota-like toxin | *Clostridium spiroforme* | ADP ribosylation |
| Actin | Vegetative insecticidal protein | *Bacillus cereus* | ADP ribosylation |
| VAMP‡ | Tetanus toxin | *Clostridium tetani* | Proteolysis |
| | *Botulinum* neurotoxin B,D,F, G | *Clostridium botulinum* | Proteolysis |
| SNAP-25‡ | *Botulinum* neurotoxin A, E, C | *Clostridium botulinum* | Proteolysis |
| Syntaxin‡ | *Botulinum* neurotoxin C | *Clostridium botulinum* | Proteolysis |
| DNA | Cytolethal distending toxins | *Haemophilus ducreyi* | DNAse |
| ***Activation of specific molecules*** | | | |
| Rho GTPases | Cytotoxic necrotic factor 1 and 2 | *Escherichia coli* | Deamidase |
| | Dermonecrotic toxin | *Bordetella* spp. | Deamidase/transglutaminase |
| Heterotrimeric G proteins | Cholera toxin | *Vibrio cholerae* | ADP ribosylation |
| | Heat-labile enterotoxin (Etx) | *Escherichia coli* | ADP ribosylation |
| ***Affecting cell polarity*** | | | |
| E-cadherin | *B. fragilis* toxin | *Escherichia coli* | Proteolysis |
| Claudins 3 and 4 | Enterotoxin | *Clostridium perfringens* | Proteolysis |
| ***Transport pathways*** | | | |
| Entry through caveolae | Cholera toxin | *Vibrio cholerae* | |
| Non-caveolae–non-clathrin entry pathway | Ricin | *Ricinus communis* (Plant) | |
| Retrograde pathway | Cholera toxin | *Vibrio cholerae* | |
| | Shiga toxin | *Shigella dysenteriae* | |
| | Ricin | *Ricinus communis* (Plant) | |
| Retrograde transport in neurons | Tetanus toxin | *Clostridium tetani* | |
| Transcytosis in intestinal cells | Botulinum toxins | *Clostridium botulinum* | |
| Transcytosis in neurons | Tetanus toxin | *Clostridium tetani* | |
| ***Vehicles for cytosolic delivery*** | | | |
| | Adenylyl cyclase toxin | *Bordetella pertussis* | |
| | Protective antigen | *Bacillus anthracis* | |
| | Heat-labile enterotoxin | *Escherichia coli* | |
| | Shiga toxin | *Shigella dysenteriae* | |
| | Diphtheria toxin | *Corynebacterium diphtheriae* | |

*Small GTPases. ‡Membrane docking and fusion apparatus.
GPI, glycosylphosphatidylinositol; LCT, large clostridial cytotoxin; PI-PLC, phosphatidylinositol-specific phospholipase C; SNAP-25, synaptosomal-associated protein-25; VAMP, vesicle-associated membrane protein.

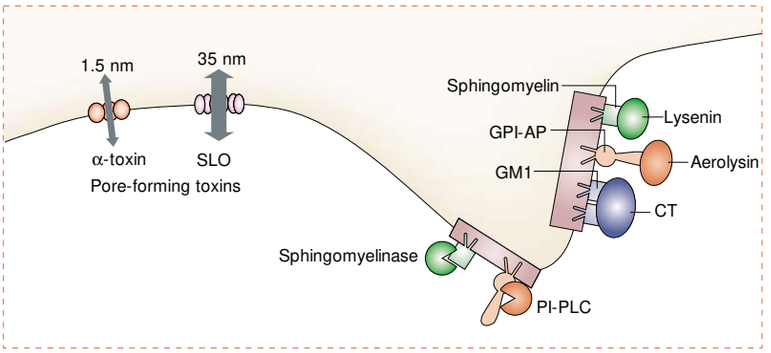


Figure 2 | **Targeting the plasma membrane.** Cells can be permeabilized with pore-forming toxins such as streptolysin O (SLO) or *Staphylococcus aureus* α-toxin. GPI-anchored proteins (GPI-APs) can either be tagged using aerolysin or removed using phosphatidylinositol-specific phospholipase C (PI-PLC). Sphingomyelin can be tagged using lysenin or cleaved using sphingomyelinases. Ganglioside GM1 can be detected using cholera toxin (CT) or its pentameric binding subunit (CT-B).

activity towards specific cytoplasmic targets. This implies that the toxin must cross the lipid bilayer and penetrate the cell. But this event rarely occurs at the plasma membrane. Toxins generally undergo endocytosis followed by transport to specific intracellular organelles before they translocate into the cytosol.

Distinct portions or subunits are involved in the different steps at which these toxins act. The B (binding) subunit is involved in receptor binding and translocation into the cytoplasm, whereas the A (active) subunit bears the enzymatic activity. On this functional basis, toxins with intracellular targets have been defined as A–B toxins. Studies of the routes used by these molecules turned out to be extremely interesting to cell biologists as they allowed the characterization of certain transport steps, and sometimes even revealed new intracellular pathways[25].

Toxins have hijacked all known entry points into a cell (FIG. 3). Some enter through CLATHRIN-COATED VESICLES (diphtheria toxin[26], *Pseudomonas* exotoxin A); others through the putative CAVEOLAR pathway (cholera toxin[15], heat-labile enterotoxin from *Escherichia coli*). Interestingly, the plant toxin ricin is internalized by all pathways including a putative clathrin and caveolae-independent route[27], probably as a consequence of its binding to several receptors.

The contribution of toxins to the field of membrane transport has, however, gone far beyond the initial internalization step. Studies of Shiga toxin were the first to show that a molecule can be transported from the cell surface to the ER[25,27]. More specifically, Shiga toxin binds to the cell surface by interacting with globotriaosylceramide (Gb3) and is then endocytosed through clathrin-dependent and clathrin-independent mechanisms and routed to the ER through the Golgi apparatus. Whether Shiga toxin remains bound to Gb3 is still not known.

The exact mechanisms that operate in this retrograde route seem to be new, and have not yet been fully characterized[28]. More than one retrograde transport pathway could exist, however, between the Golgi and the ER, as *Pseudomonas* exotoxin A and cholera toxin are also transported to the ER through the Golgi, but using a distinct mechanism[29]. Therefore, these bacterial toxins will continue to be useful tools to dissect retrograde transport pathways. In addition, they have been (and will be) helpful in studies on the still poorly understood communication routes between the endocytic and the biosynthetic pathways such as early endosomes to Golgi[29] or plasma membrane to Golgi[30].

Whereas cholera toxin can be used on almost any cell type owing to the ubiquitous expression of its receptor, some toxins have a remarkable cell type specificity. This is the case with Shiga toxin, which will affect only Gb3-positive cells such as those of the immune system. Members of the clostridial neurotoxin family, which is composed of tetanus and botulinum neurotoxins, bind at the neuromuscular junction and undergo differential transport in mammalian motor neurons[31]. Botulinum neurotoxins remain at the synapse, allowing the study of site-specific endocytosis,

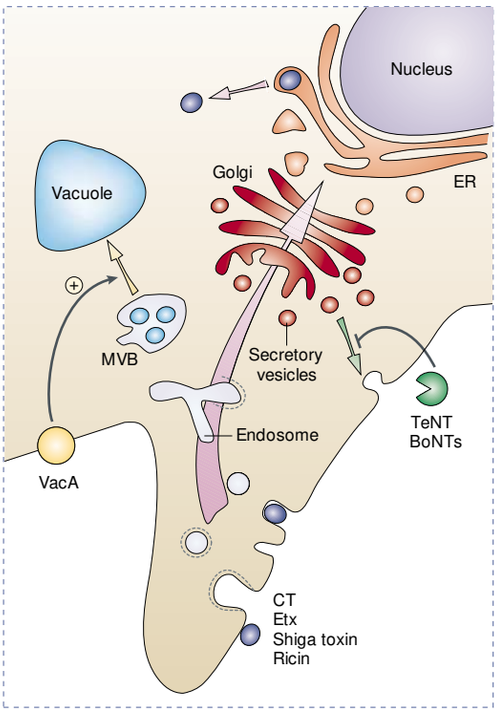


Figure 3 | **Studying intracellular membrane transport.** Various toxins are internalized and transported to intracellular organelles from where they are translocated into the cytoplasm. Different endocytic pathways can be studied using, for example, cholera toxin (CT) and heat-labile enterotoxin (Etx) from *Escherichia coli* (caveolar pathway), Shiga toxin (clathrin-dependent and clathrin-independent pathways), and ricin (several pathways including clathrin- and caveolae-independent). Clathrin coating is shown by a grey dashed line. Molecular mechanisms of vesicle fusion can be addressed using tetanus neurotoxin (TeNT) and botulinum neurotoxins (BoNTs), which cleave specific SNARE proteins. Also shown is *Helicobacter pylori* vacuolating toxin (VacA), which triggers vacuolation of late endocytic compartments and has recently been suggested to translocate to mitochondria (BOX 1). ER, endoplasmic reticulum; MVB, multivesicular body.

CLATHRIN-COATED VESICLES
Coated vesicles implicated in protein transport. Clathrin heavy and light chains form a triskelion, the main building element of these clathrin coats.

CAVEOLAE
Flask-shaped, cholesterol-rich invagination of the plasma membrane that might mediate the uptake of some extracellular materials, and are proabably involved in cell signalling.

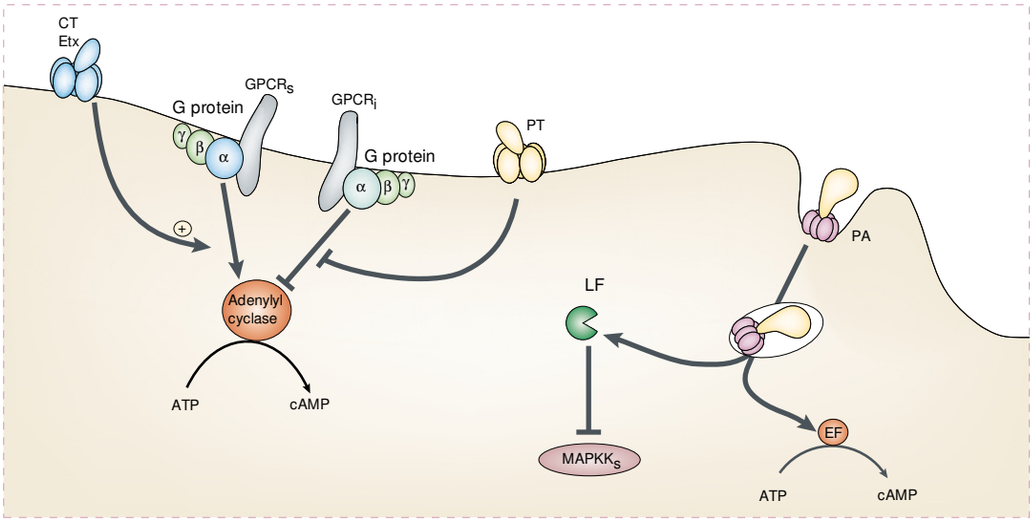


Figure 4 | **Interfering with cell signalling.** Cholera toxin (CT) and *Escherichia coli* enterotoxin (Etx) activate the α-subunit of heterotrimeric $G_s$ proteins downstream to stimulatory G-protein-coupled receptors ($GPCR_s$), leading to the constitutive activation of adenylyl cyclase and rapid elevation of cellular levels of cyclic AMP. Pertussis toxin (PT) instead inactivates the α-subunits of G proteins coupled to inhibitory GPCR ($GPCR_i$). This modification causes the silencing of the inhibitory input and induces the indirect activation of downstream effectors. Lethal factor (LF) requires the anthrax protective antigen (PA) to reach the cell cytoplasm, where it cleaves mitogen-activated protein kinase kinases (MAPKKs). PA is also essential for the binding and translocation of the oedema factor (EF), a powerful adenylyl cyclase, which elevates the cytoplasmic concentration of cAMP.

whereas tetanus toxin is retrogradely transported to the cell body and then transcytosed to inhibitory interneurons, making it a marker of choice to study retrograde transport in neurons (G. Lalli and G.S., unpublished data). In addition, botulinum neurotoxins, which are synthesized as large, multisubunit protein complexes, can be used to follow transcytosis in binding-competent epithelial cells such as colon cells, making use of the fact that these toxins can gain entry into an organism through the intestinal tract[32].

## Delivery of proteins into cells

As mentioned above, many toxins can translocate their catalytic moiety across a membrane into the target cell cytoplasm. The binding and/or translocation domains of toxins can therefore be used as vehicles for intracellular delivery of peptides and proteins. A molecule of choice for such an application is the adenylyl cyclase toxin from *Bordetella pertussis*[33]. The mode of entry of this toxin is unique because the catalytic domain, harbouring the adenylyl cyclase activity, is directly translocated across the plasma membrane of the target cell, independently of any endocytic step.

When making fusion proteins between the selected peptide or protein and an inactive variant of the adenylyl cyclase toxin, efficient translocation of the cargo into the cytoplasm can be monitored with a concomitant, sought biological response. This approach has been successfully used to present peptides to the cytosolic pathway for major histocompatibility (MHC) class-I-restricted antigen presentation[34]. In this pathway, peptides that reach the cytoplasm are processed by the proteasome, translocated into the ER and loaded onto newly synthesized MHC class I complexes, which are then transported to the cell surface. Similarly, fusion proteins harbouring the binding subunits of Shiga toxin allowed the MHC-class-I-restricted presentation of tumour peptides in dendritic cells[35].

The adenylyl cyclase and Shiga toxins are not only useful for studying antigen presentation but they are also attractive vectors for vaccine development as they elicit a response from cytotoxic T lymphocytes (CTLs) *in vivo*. CTLs are thought to have a key role in the control of virus-infected cells and tumour growth, but immunization with peptides generally fails to trigger this response.

Cytoplasmic delivery of heterologous proteins and peptides has also been successful with anthrax toxin[36], the binding subunits of *Clostridium botulinum* C2 binary toxin[37], the *E. coli* heat-labile enterotoxin[38], and diphtheria toxin[39]. Most of these systems take advantage of a relatively low selectivity for the target cells — a property that reflects the ubiquitous nature of their respective receptors. These are the ganglioside GD1 (REF. 40) for the enterotoxin and the heparin-binding epidermal-growth-factor-like growth factor precursor[41,42] for diphtheria toxin, whereas the receptor for anthrax toxin is unknown. Specificity can, however, be generated in some cases by the use of chimaeras. For example, neuronal delivery can be obtained by fusing the protein of interest with the non-toxic C fragment of tetanus toxin, which is involved in binding, as demonstrated by the targeting of CuZn superoxide dismutase to spinal cord motor neurons[43].

## Interfering with cell signalling

The enzymatic activity provided by A–B toxins can also be very useful for altering specific signal-transduction

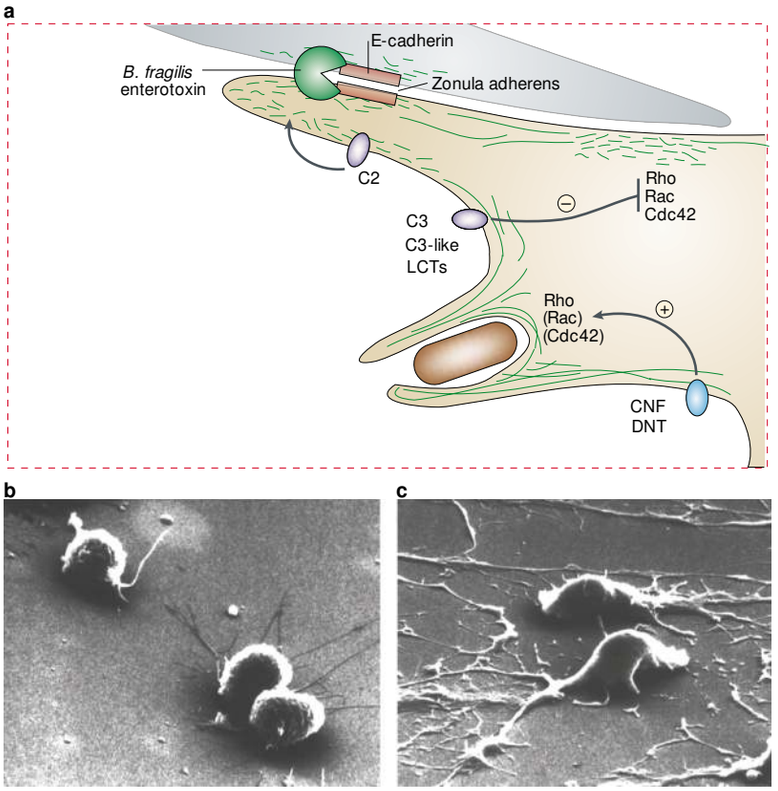


Figure 5 | **Affecting the cytoskeleton or cell polarization. a** | Cell treatment with large clostridial toxins (LCTs) or clostridial binary toxins, such as *Clostridium botulinum* C2, lead to disassembly of the actin cytoskeleton caused by the modification of actin or the inhibition of small GTPases. Rho inactivation can also be triggered by the smaller exoenzyme C3 and C3-like toxins. By contrast, *Escherichia coli* cytotoxic necrotic factor (CNF) and *Bordetella* dermonecrotic toxin (DNT) activate Rho, which can in turn promote the appearance of stress fibres and Rho-dependent macropinocytosis. *Bacteroides fragilis* enterotoxin cleaves E-cadherin, causing the disassembly of zonula adherens and indirectly the redistribution of actin cytoskeleton. **b**,**c** | Cytopathic effects induced by Rho-modifying LCTs in epithelial cells (**b**) and fibroblasts (**c**). (Courtesy of E.C. Olarte, E. Freer and M. Thelestam.)

pathways within the cell. In particular, pertussis toxin was instrumental in the discovery of inhibitory heterotrimeric G proteins, and it is extensively used in the definition of the initial steps in signalling cascades in plant and animal cells. This toxin ADP-ribosylates the α-subunits of several G proteins, including $G_i$, $G_o$ and $G_{gust}$[44], uncoupling them from their cognate receptors. In the case of $G_i$, this modification causes the silencing of the inhibitory input of the signalling cascade, which induces the indirect activation of downstream effectors, such as adenylyl cyclase, phosphodiesterase and ion channels[4] (FIG. 4). In contrast to pertussis toxin, cholera toxin and *E. coli* enterotoxin ADP-ribosylate activatory G proteins ($G_s$), including $G_t$ and $G_{olf}$[44], by blocking their GTPase activity. This leads to the constitutive activation of adenylyl cyclase and the rapid elevation of cyclic AMP levels in the cell (FIG. 4).

The 'lethal factor' produced by *Bacillus anthracis* (LF) can also affect signalling, as this zinc-endopeptidase specifically cleaves several members of the mitogen-activated protein kinase kinase (MAPKK) family[45,46] (FIG. 4), with the notable exception[47] of MAPKK5. Lethal factor elicits the hyper-stimulation of macrophage inflammatory responses and can be used to induce cytokine expression and oxidative burst in these cells[48]. Similarly, the oedema factor produced by the same bacterium transiently increases intracellular cAMP by means of its adenylyl cyclase activity, and modulates the inflammatory immune response[48] (FIG. 4).

### Inhibition of membrane fusion

In addition to their use in studying intracellular transport routes, botulinum and tetanus neurotoxins have been crucial in understanding membrane fusion, in particular during neurotransmitter release. These clostridial neurotoxins are characterized by extensive sequence similarity and are typical examples of A–B toxins. Their catalytic subunits contain a zinc-endopeptidase activity that is specific for synaptic members of the SNARE (soluble NSF attachment protein receptor, where NSF stands for *N*-ethylmaleimide-sensitive fusion protein) superfamily: VAMP (vesicle-associated membrane protein), syntaxin and SNAP-25 (TABLE 1)[31,49]. SNAREs are not only involved in the fusion of synaptic vesicles with the presynaptic plasma membrane, but they are also implicated at all stages of vesicular transport, during which they mediate targeting fidelity and the fusion of lipid bilayers[50]. The action of these toxins is restricted to neurons and neuronally differentiated cells, but cell permeabilization by means of pore-forming toxins or transfection with the active domains of tetanus and botulinum neurotoxins allow the inactivation of specific SNAREs within any cell type[51]. In addition, targeted expression of the active subunit of clostridial neurotoxins can be used to ablate specific SNARE proteins in selected tissues or even in entire organisms[52,53].

### Affecting the cytoskeleton

Clostridial species not only produce the above-mentioned SNARE-specific endopeptidases, but also several cytotoxins that affect the cytoskeleton (FIG. 5). A great deal of information on the cytoskeleton and its ability to undergo dynamic changes — the basis of numerous cellular processes, such as cell migration, polarization and cytokinesis — has been gathered by studying the effects of these toxins (TABLE 1). Their targets include actin itself as well as several regulatory components of the actin cytoskeleton[1,54,55].

The large family of clostridial binary toxins (CBTs) comprises several bi-chain toxins (*C. botulinum* C2, iota and iota-like toxins), produced by different species of *Clostridium* sp., but all belonging to a class of ADP-ribosylating enzymes that act on monomeric G actin (TABLE 1). ADP-ribosylation of actin inhibits its polymerization and leads to the dissociation of actin microfilaments (FIG. 5). By contrast, most other bacterial toxins that affect the actin cytoskeleton modify small GTP-binding proteins of the Rho subtype, which seem to be the most important regulators of actin microfilament dynamics[54,55]. Large clostridial cytotoxins (LCTs) form the biggest family in this toxin subgroup and are

Box 1 | **Novel toxins**

***Cell-cycle arrest.*** **Whereas most bacterial toxins that act inside host cells either destroy or modify host cell proteins, cytolethal distending toxins (CDTs) have a DNase-I-like activity. CDTs cleave DNA during replication, thereby activating host cellular checkpoint mechanisms, which result in cell-cycle arrest[67,68]. Although they have not yet been used for such purposes, these toxins may be useful in studies on the mitotic checkpoint and also in membrane transport. For their cytotoxic action, CDTs must indeed undergo endocytosis, be transported through the Golgi and possibly through the endoplasmic reticulum[68,69]. However, in contrast to other toxins that undergo retrograde transport and are then translocated into the cytoplasm, CDTs must reach the nucleus. This could occur either through a cytoplasmic intermediate, or directly from the ER through an as-yet-unknown pathway.**

***Apoptosis.*** **The list of toxins and other bacterial virulence factors that induce apoptosis is increasing exponentially. Recent findings indicate that three bacterial proteins might translocate to host cell mitochondria and cause programmed cell death: *Neisseria gonorrhoeae* Por B[70], enteropathogenic *Escherichia coli* Orf19 (REF. 71) and *Helicobacter pylori* vacuolating toxin (VacA)[72]. VacA is also responsible for the well-established alteration of the endocytic pathway resulting in the selective swelling of late endosomes/pre-lysosomal structures[73] (FIG. 3).**

characterized by extensive sequence homology, similar structural organization and identical glycosyl-transferase activity. Target specificity varies among members of the LCT family. It can be restricted to Rho, Rac and Cdc42, as in the case of the A and B toxins from *Clostridium difficile*[54,55]. Or it can cover a wider spectrum, including Ras, Rap and Ral, but with the notable exception of Rho, as, for example, the lethal toxin from *Clostridium sordellii*[56] (TABLE 1).

Finally, selective inactivation of Rho GTPases can be achieved by treatment of cells with members of a heterogeneous group of single-chain, low-molecular-weight ADP-ribosylating toxins of common ancestral origin. These comprise *C. botulinum* C3 toxin, staphylococcal epidermal cell differentiation inhibitor (EDIN), staphylococcal C3stau2 (which also modifies RhoE)[57] and other, similar, C3-like toxins produced by various bacteria including *Clostridium limosum* and *Bacillus cereus*. The general effect of cell intoxication with these negative regulators of small GTP-binding proteins is a depolymerization of actin filaments (F-actin) with a consequent collapse of the actin cytoskeleton[56].

By contrast, the cytotoxic necrotizing factor (CNF) from *E. coli* and the dermonecrotic toxin (DNT) from different *Bordetella* species activate Rho GTPases by specific deamidation[58–60] (or transglutamination[61,62]) of an essential glutamine residue, therefore increasing the formation of actin filaments. This process leads to the appearance of stress fibres and promotes macropinocytosis[54,55] (FIG. 5).

To bypass the various cell-type specificities of these toxins, which are imposed by the presence of selective and largely unidentified cell-surface receptors[56], the catalytic moiety can be directly delivered into the cytoplasm by permeabilizing cells with SLO[10]. Alternatively, the catalytic portion can be fused to the binding and translocation domains of a toxin with ubiquitously distributed receptors. This strategy has been successful for efficient delivery of C3 toxin coupled with the binding fragment of *C. botulinum* C2 binary toxin[37].

By contrast, the enterotoxin of *Bacteroides fragilis* does not require entry into the cytoplasm to trigger the reorganization of the actin cytoskeleton. This novel toxin is a metalloprotease, which cleaves E-cadherin on the basolateral side of polarized cells[63]. The specific proteolysis of this essential component of the ZONULA ADHERENS causes the disruption of intercellular junctions, with consequent loss of cell polarization and changes in cell barrier permeability (FIG. 5).

ZONULA ADHERENS
A cell–cell adherens junction that forms a circumferential belt around the apical pole of epithelial cells.

## Bacterial two-hybrid screening

The ability of two complementary fragments of the adenylyl cyclase toxin from *B. pertussis* to reassociate into a fully active enzyme is at the basis of a recently established two-hybrid system for the screening of protein–protein interactions in bacteria[64,65]. Re-association of the adenylyl cyclase toxin fragments requires either calmodulin or the genetic fusion of each of the two fragments to two interacting proteins, X and Y. Following the interaction of X and Y, the two toxin fragments are brought into close proximity, resulting in functional complementation. This is followed by production of cAMP, which then triggers transcriptional activation of catabolic operons, yielding a characteristic phenotype used for the selection. The association between the hybrid proteins, which occurs in the cytoplasm, can therefore be spatially separated from the nuclear transcriptional activation readout, offering a more flexible experimental tool to monitor protein–protein interactions occurring in the cytosol[65].

## Concluding remarks

In their never-ending evolutionary search for new ecological niches, bacteria have provided life scientists with invaluable tools to inhibit specifically or modulate key cellular events. This review has presented a subset of bacterial protein toxins that are useful to cell biologists, but several others have been studied (some are mentioned in BOX 1 and TABLE 1). No doubt hitherto-unknown toxins will emerge as many bacterial species remain to be discovered, and others may become pathogenic in the future[66]. Moreover, entire genomes of bacterial pathogens have been or will be sequenced. This should allow the rapid identification of new virulence factors, the mode of action of which will surely be unravelled, thanks to the rising interest in cellular microbiology.

## Links

**DATABASE LINKS** **PI-PLCs | MAPKK family | MAPKK5 | VAMP |syntaxin | SNAP-25 | Cdc42 | Rap | Ral**
**FURTHER INFORMATION** **Schiavo lab | Van der Goot lab**
**ENCYCLOPEDIA OF LIFE SCIENCES** **Toxin action: molecular mechanisms | Pore-forming toxins**

1. Alouf, J. E. & Freer, J. H. *The Comprehensive Sourcebook of Bacterial Protein Toxins* (Academic, London, 1999). **The latest dictionary on bacterial toxins, with detailed chapters.**
2. Cossart, P., Boquet, P., Normark, S. & Rappuoli, R. Cellular microbiology emerging. *Science* **271**, 315–316 (1996).
3. Schiavo, G. *et al.* Tetanus and botulinum B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* **359**, 832–835 (1992).
4. Ui, M. in *Pathogenesis and Immunity in* Pertussis (eds Wardlaw, A. C. & Parton, R.) 121–145 (John Wiley, Chichester, 1988).
5. Bhakdi, S. *et al.* Staphylococcal α-toxin, streptolysin-O, and *Escherichia coli* hemolysin: prototypes of pore-forming bacterial cytolysins. *Arch. Microbiol.* **165**, 73–79 (1996).
6. Van der Goot, F. G. (ed.) *Pore Forming Toxins* in *Current Topics in Microbiology and Immunology* (Springer Verlag, Berlin Heidelberg, 2001). **Up-to-date reviews on the best-characterized classes of pore-forming toxins, covering all aspects of the mode of action.**
7. Song, L. *et al.* Structure of *Staphylococcal* a-hemolysin, a heptameric transmembrane pore. *Science* **274**, 1859–1866 (1996).
8. Abrami, L., Fivaz, M. & van Der Goot, F. G. Adventures of a pore-forming toxin at the target cell surface. *Trends Microbiol.* **8**, 168–172 (2000).
9. Diep, D. B., Nelson, K. L., Raja, S. M., Pleshak, E. N. & Buckley, J. T. Glycosylphosphatidylinositol anchors of membrane glycoproteins are binding determinants for the channel-forming toxin aerolysin. *J. Biol. Chem.* **273**, 2355–2360 (1998).
10. Walev, I. *et al.* Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-O. *Proc. Natl Acad. Sci. USA* **98**, 3185–3190 (2001).
11. Shatursky, O. *et al.* The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins. *Cell* **99**, 293–299 (1999).
12. Krause, K. H., Fivaz, M., Monod, A. & van der Goot, F. G. Aerolysin induces G-protein activation and $Ca^{2+}$ release from intracellular stores in human granulocytes. *J. Biol. Chem.* **273**, 18122–18129 (1998).
13. Dragneva, Y. *et al.* Subcytocidal attack by staphylococcal α-toxin activates NF-κB and induces interleukin-8 production. *Infect. Immun.* **69**, 2630–2635 (2001).
14. Abrami, L., Fivaz, M., Glauser, P.-E., Parton, R. G. & van der Goot, F. G. A pore-forming toxin interact with a GPI–anchored protein and causes vacuolation of the endoplasmic reticulum. *J. Cell Biol.* **140**, 525–540 (1998).
15. Lencer, W. I., Hirst, T. R. & Holmes, R. K. Membrane traffic and the cellular uptake of cholera toxin. *Biochim. Biophys. Acta* **1450**, 177–190 (1999).
16. Yamaji, A. *et al.* Lysenin, a novel sphingomyelin-specific binding protein. *J. Biol. Chem.* **273**, 5300–5306 (1998).
17. Simons, K. & Toomre, D. Lipid rafts and signal transduction. *Nature Rev. Mol. Cell Biol.* **1**, 31–39 (2000).
18. Parton, R. G. Ultrastructural localization of gangliosides; GM1 is concentrated in caveolae. *J. Histochem. Cytochem.* **42**, 155–166 (1994).
19. Puri, V. *et al.* Cholesterol modulates membrane traffic along the endocytic pathway in sphingolipid-storage diseases. *Nature Cell Biol.* **1**, 386–388 (1999).
20. Hanada, K. & Nishijima, M. Selection of mammalian cell mutants in sphingolipid biosynthesis. *Methods Enzymol.* **312**, 304–317 (2000).
21. Higgins, J. A., Hitchin, B. W. & Low, M. G. Phosphatidylinositol-specific phospholipase C of *Bacillus thuringiensis* as a probe for the distribution of phosphatidylinositol in hepatocyte membranes. *Biochem. J.* **259**, 913–916 (1989).
22. Holthuis, J. Sphingoid base signalling controls yeast endocytosis. (2000). www.the-elso-gazette.org/magazines/issue2/mreviews/mreviews2.asp
23. Kolesnick, R. N. & Kronke, M. Regulation of ceramide production and apoptosis. *Annu. Rev. Physiol.* **60**, 643–665 (1998).
24. Zhang, P., Liu, B., Jenkins, G. M., Hannun, Y. A. & Obeid, L. M. Expression of neutral sphingomyelinase identifies a distinct pool of sphingomyelin involved in apoptosis. *J. Biol. Chem.* **272**, 9609–9612 (1997).
25. Sandvig, K. *et al.* Retrograde transport of endocytosed Shiga toxin to the endoplasmic reticulum. *Nature* **358**, 510–511 (1992).
26. Falnes, P. O. & Sandvig, K. Penetration of protein toxins into cells. *Curr. Opin. Cell Biol.* **12**, 407–413 (2000). **Broad review on the different intracellular routes taken by toxins with cytoplasmic targets.**
27. Sandvig, K. & van Deurs, B. Entry of ricin and Shiga toxin into cells: molecular mechanisms and medical perspectives. *EMBO J.* **19**, 5943–5950 (2000).
28. White, J. *et al.* Rab6 coordinates a novel Golgi to ER retrograde transport pathway in live cells. *J. Cell Biol.* **147**, 743–760 (1999).
29. Johannes, L. & Goud, B. Facing inward from compartment shores: how many pathways were we looking for? *Traffic* **1**, 119–123 (2000).
30. Nichols, B. J. *et al.* Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* **153**, 529–542 (2001).
31. Schiavo, G., Matteoli, M. & Montecucco, C. Neurotoxins affecting neuroexocytosis. *Physiol. Rev.* **80**, 717–766 (2000).
32. Maksymowych, A. B. & Simpson, L. L. Binding and transcytosis of botulinum neurotoxin by polarized human colon-carcinoma cells. *J. Biol. Chem.* **273**, 21950–21957 (1998).
33. Ladant, D. & Ullmann, A. *Bordetella pertussis* adenylate cyclase: a toxin with multiple talents. *Trends Microbiol.* **7**, 172–176 (1999). ***Bordetella pertussis* adenylyl cyclase toxin as a tool for epitope delivery, protein targeting and characterization of protein–protein interactions.**
34. Guermonprez, P., Ladant, D., Karimova, G., Ullmann, A. & Leclerc, C. Direct delivery of the *Bordetella pertussis* adenylate cyclase toxin to the MHC class I antigen presentation pathway. *J. Immunol.* **162**, 1910–1916 (1999).
35. Haicheur, N. *et al.* The B subunit of Shiga toxin fused to a tumor antigen elicits CTL and targets dendritic cells to allow MHC class I-restricted presentation of peptides derived from exogenous antigens. *J. Immunol.* **165**, 3301–3308 (2000).
36. Goletz, T. J. *et al.* Targeting HIV proteins to the major histocompatibility complex class I processing pathway with a novel gp120–anthrax toxin fusion protein. *Proc. Natl Acad. Sci. USA* **94**, 12059–12064 (1997).
37. Barth, H., Hofmann, F., Olenik, C., Just, I. & Aktories, K. The N-terminal part of the enzyme component (C2I) of the binary *Clostridium botulinum* C2 toxin interacts with the binding component C2II and functions as a carrier system for a Rho ADP-ribosylating C3-like fusion toxin. *Infect. Immun.* **66**, 1364–1369 (1998).
38. Loregian, A. *et al.* Intranuclear delivery of an antiviral peptide mediated by the B subunit of *Escherichia coli* heat-labile enterotoxin. *Proc. Natl Acad. Sci. USA* **96**, 5221–5226 (1999).
39. Liu, X. H., Castelli, J. C. & Youle, R. J. Receptor-mediated uptake of an extracellular Bcl–$x_L$ fusion protein inhibits apoptosis. *Proc. Natl Acad. Sci. USA* **96**, 9563–9567 (1999).
40. Wolf, A. A. *et al.* Ganglioside structure dictates signal transduction by cholera toxin and association with caveolae-like membrane domains in polarized epithelia. *J. Cell Biol.* **141**, 917–927 (1998).
41. Mitamura, T. *et al.* The 27-kD diphtheria toxin receptor-associated protein (DRAP27) from vero cells is the monkey homologue of human CD9 antigen: expression of DRAP27 elevates the number of diphtheria toxin receptors on toxin-sensitive cells. *J. Cell Biol.* **118**, 1389–1399 (1992).
42. Naglich, J. G., Metherall, J. E., Russell, D. W. & Eidels, L. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* **69**, 1051–1061 (1992).
43. Figueiredo, D. M. *et al.* Delivery of recombinant tetanus-superoxide dismutase proteins to central nervous system neurons by retrograde axonal transport. *Exp. Neurol.* **145**, 546–554 (1997).
44. Hepler, J. R. & Gilman, A. G. G proteins. *Trends Biochem. Sci.* **17**, 383–387 (1992).
45. Duesbery, N. S. *et al.* Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. *Science* **280**, 734–737 (1998).
46. Vitale, G. *et al.* Anthrax lethal factor cleaves the N-terminus of MAPKKs and induces tyrosine/threonine phosphorylation of MAPKs in cultured macrophages. *Biochem. Biophys. Res. Commun.* **248**, 706–711 (1998).
47. Vitale, G., Bernardi, L., Napolitani, G., Mock, M. & Montecucco, C. Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor. *Biochem. J.* **352** Pt 3, 739–745 (2000).
48. Leppla, S. A. in *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds Alouf, J. E. & Freer, J. H.) 243–263 (Academic Press, London, 1999).
49. Schiavo, G. & Montecucco, C. in *Bacterial Toxins. Tools in Cell Biology and Pharmacology* (ed. Aktories, K.) 169–186 (Chapman & Hall, London, 1997).
50. Chen, Y. A. & Scheller, R. H. Snare-mediated membrane fusion. *Nature Rev. Mol. Cell Biol.* **2**, 98–106 (2001).
51. Lang, J., Regazzi, R. & Wollheim, C. B. in *Bacterial toxins. Tools in Cell Biology and Pharmacology* (ed. Aktories, K.) 217–237 (Chapman & Hall, London, 1997).
52. Sweeney, S. T., Broadie, K., Keane, J., Niemann, H. & O'Kane, C. J. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* **14**, 341–351 (1995).
53. Baines, R. A., Robinson, S. G., Fujioka, M., Jaynes, J. B. & Bate, M. Postsynaptic expression of tetanus toxin light chain blocks synaptogenesis in *Drosophila*. *Curr. Biol.* **9**, 1267–1270 (1999).
54. Boquet, P., Munro, P., Fiorentini, C. & Just, I. Toxins from anaerobic bacteria: specificity and molecular mechanisms of action. *Curr. Opin. Microbiol.* **1**, 66–74 (1998). **Anaerobic bacteria as a goldmine of protein toxins targeting several physiological pathways.**
55. Busch, C. & Aktories, K. Microbial toxins and the glycosylation of Rho family GTPases. *Curr. Opin. Struct. Biol.* **10**, 528–535 (2000). **Insights into the mechanism of action of large clostridial cytotoxins and the role of their physiological targets in regulation of the actin cytoskeleton.**
56. Thelestam, M., Chaves–Olarte, E., Moos, M. & von Eichel–Streiber, C. in *The Comprehensive Sourcebook of Bacterial Protein Toxins* (eds Alouf, J. E. & Freer, J. H.) (Academic, London, 1999).
57. Wilde, C., Chhatwal , G. S., Schmalzing, G., Aktories, K. & Just, I. A novel C3-like ADP-ribosyltransferase from *Staphylococcus aureus* modifying RhoE and Rnd3. *J. Biol. Chem.* **276**, 9537–9542 (2001).
58. Flatau, G. *et al.* Toxin-induced activation of the G protein p21 Rho by deamidation of glutamine. *Nature* **387**, 729–733 (1997).
59. Schmidt, G. *et al.* Gln63 of Rho is deamidated by *Escherichia coli* cytotoxic necrotizing factor-1. *Nature* **387**, 725–729 (1997).
60. Horiguchi, Y. *et al. Bordetella bronchiseptica* dermonecrotizing toxin induces reorganization of actin stress fibers through deamidation of Gln63 of the GTP-binding protein Rho. *Proc. Natl Acad. Sci. USA* **94**, 11623–11626 (1997).
61. Schmidt, G. *et al.* Identification of the C-terminal part of *Bordetella* dermonecrotic toxin as a transglutaminase for rho GTPases. *J. Biol. Chem.* **274**, 31875–31881 (1999).
62. Masuda, M. *et al.* Activation of Rho through a cross-link with polyamines catalyzed by *Bordetella* dermonecrotizing toxin. *EMBO J.* **19**, 521–530 (2000).
63. Wu, S., Lim, K. C., Huang, J., Saidi, R. F. & Sears, C. L. *Bacteroides fragilis* enterotoxin cleaves the zonula adherens protein, E-cadherin. *Proc. Natl Acad. Sci. USA* **95**, 14979–14984 (1998).
64. Karimova, G., Pidoux, J., Ullmann, A. & Ladant, D. A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc. Natl Acad. Sci. USA* **95**, 5752–5756 (1998).
65. Karimova, G., Ullmann, A. & Ladant, D. A bacterial two-hybrid system that exploits a cAMP signaling cascade in *Escherichia coli*. *Methods Enzymol.* **328**, 59–73 (2000).
66. Hacker, J. & Kaper, J. B. Pathogenicity islands and the evolution of microbes. *Annu. Rev. Microbiol.* **54**, 641–679 (2000). **Comprehensive coverage of the origin, functions and evolutionary significance of pathogenicity islands and horizontal gene transfer.**
67. Elwell, C. A. & Dreyfus, L. A. DNase I homologous residues in CdtB are critical for cytolethal distending toxin-mediated cell cycle arrest. *Mol. Microbiol.* **37**, 952–663 (2000).
68. Lara-Tejero, M. & Galan, J. E. A bacterial toxin that controls cell cycle progression as a deoxyribonuclease I-like protein. *Science* **290**, 354–357 (2000).
69. Cortes-Bratti, X., Chaves–Olarte, E., Lagergard, T. & Thelestam, M. Cellular internalization of cytolethal distending toxin from *Haemophilus ducreyi*. *Infect. Immun.* **68**, 6903–6911 (2000).
70. Müller, A. *et al.* Targeting of the pro-apoptotic VDAC-like porin (PorB) of *Neisseria gonorrhoeae* to mitochondria of infected cells. *EMBO J.* **19**, 5332–5343 (2000).
71. Kenny, B. & Jepson, M. Targeting of an enteropathogenic *Escherichia coli* (EPEC) effector protein to host mitochondria. *Cell. Microbiol.* **2**, 579–590 (2000).
72. Galmiche, A. *et al.* The N-terminal 34 kDa fragment of *Helicobacter pylori* vacuolating cytotoxin targets mitochondria and induces cytochrome *c* release. *EMBO J.* **19**, 6361–6370 (2000).
73. Montecucco, C. & Rappuoli, R. Understanding *Helicobacter pylori* survival in the stomach. *Nature Rev. Mol. Cell Biol.* **2**, 457–466 (2001).



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