# Bacterial Toxins: An Overview on Bacterial Proteases and their Action as Virulence Factors

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**Abstract:** Bacterial pathogenicity is a result of a combination of factors, including resistance to environmental threats and to the host's defenses, growth capability, localization in the host, tissue specificity, resource obtaining mechanisms and the bacterium's own defenses to aggression. A variety of bacterial components, often specific to each strain, are involved in the microorganism's survival, adhesion and growth in the host. Many of them are harmful and, therefore, are called virulence factors. The effects caused by the virulence factors determine the degree of aggressivity of the strain. In many cases the virulence factors are secreted proteins or enzymes, sometimes performing very specific functions. The enzymatic activity is directed to specific proteins from cell membranes, synaptic vesicle fusion proteins, among other important targets. One of the most toxic bacterial proteins is secreted by *Clostridium botulinum*, targeted to synaptic vesicle fusion proteins, cleaving them with a zinc-metalloprotease activity, which results in severe neurotoxic effects with a lethal dose as low as eight nanograms per kilogram of body weight. The tetanus neurotoxin acts in a similar way but is less active and *Bacillus anthracis* also presents a potent metalloprotease activity. In this work we describe a selection of these specially interesting and important bacterial proteins and proteases, stressing their relevance in the pathological process and in medical studies

**Key Words:** Proteases, virulence factors, bacteria, clostridium, *Bordetella pertussis*

## 1. INTRODUCTION

In any given ecosystem, individuals of every species are in a constant struggle to survive. In this struggle they establish a niche that can be defined as how an organism or population responds to, as well as it alters, the distribution of resources and competitors, from the same or different species, with which it interacts. In his theory of evolution through natural selection, Charles Darwin defines the relationship between species as an important selection factor that can modify a species' existing traits or originate new ones, thus providing itself advantages and increased fitness in the relationship with the other species [1].

The relationship between an individual of a given species with an individual or group from another species can be classified in one of 5 major classes:

Amensalism: A species restricts or prevents the growth of another species without any benefit for itself [2].

Commensalism: The relationship benefits one of the species without any harm or benefit to the other species [3].

Mutualism: Both species benefit from the relationship [4].

Competition: The fight for limited resources or a niche [5].

Predation: an organism feeds on another one, debilitating or killing it.

Parasitism, a special case of predation, involves the forceful acquisition of gathered resources by one species, the parasite, from another one, the host, which is much bigger. The parasite benefits from a prolonged and close association with the host, from which it obtains its needed resources, frequently causing harm. The parasite shows a high degree of specialization and reproduces more quickly and in greater numbers than its host. Pathogens fit in this scheme [6-9], establishing with their hosts a relationship that culminates in disease [10].

## 2. DEVELOPING VIRULENCE

Pathology is the culmination of the response of the host to the pathogen presence or a direct effect of pathogen derived structures, in which case these are named virulence factors [8]. But virulence factors did not evolve with such a purpose. Virulence is a side effect of these factors' main functions, which are to adapt the environment to the parasite and to provide resources or protection.

The nature of the structures associated with virulence varies as much as their functions [9], though they are intimately related to the pathological process [9]. In the *Bordetella* genus, several species can cause respiratory tract diseases in mammals, but the whooping cough characteristic of the human infection is caused only by strains capable of producing the pertussis toxin, which has an affinity for the tracheal ciliated cells [11]. Virulence is thus not only

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responsible for determining the characteristics of a pathology but it also determines the specificity of pathogens. Some *Escherichia coli* strains, for example, possess fimbria capable of mediating the adhesion to gut epithelial cells. This adhesion results from the interaction between fimbria and structures present on the cell surface and are very species specific [12].

### 2.1. Proteases as Virulence Factors: General Features

A number of virulence factors are proteases, and their actual physiological function is to provide peptidic nutrients to the microorganism through the hydrolysis of polypeptidic substrates [13]. However, in doing so, these proteases contribute to pathogenesis and, therefore, are considered virulence factors. Some authors even consider them as the main virulence factors among all other extracellular factors [14].

Proteases are a group of enzymes capable of catalyzing a hydrolytic cleavage of peptide bonds. They are produced by every organism, and play an important role in the regulation of metabolism as well as in infectious diseases [15]. They can be classified according to the location where the cleavage occurs in the targeted protein: if it occurs near or at the carboxi or amino terminal portions, they are called exoproteases; if it happens as far as five residues from these portions, the protease is called an endoprotease [16].

An additional classification is based on the functional group present at the catalytic site of the enzyme. According to this classification, there are four distinct groups of proteases: serinoproteases, aspartic proteases, cysteine-proteases and metalloprotease, each named after de residues of serine, aspartic acid, cystein or divalent metallic ion present respectively at the catalytic site. Some proteases, though, do not fit in this classification as is the case of the ADP-dependent proteases [17].

Proteases can be employed beyond the physiological processes, as there are many biotechnological uses for them [17-19]. The persistent study of protease mechanisms of action leads to a better understanding of the physiological and pathological process in which they are involved. This opens innumerable development possibilities for industrial uses, drug development and biotechnological modification.

Secreted bacterial proteases are involved in many different processes. Some act as direct defense mechanisms, cleaving proteins involved in the host immune defense, as is the case of ZapA from *Proteus mirabilis* [20]. Others function as maturation factors for other virulence factors, such as the secreted metalloprotease from *Listeria monocytogenes* which is responsible for the activation of two phospholipases important in the pathological process [21]. But bacterial proteases are mainly involved in the obtention of peptide nutrients for the microorganism, through the hydrolysis of large polypeptide substrates into smaller molecules which can subsequently be absorbed by the bacteria [13]. Consequently bacterial proteases often contribute to the pathogenesis of infections, in which case they can be considered as virulence factors. In fact, some authors regard proteases as the main virulence factors among all the bacterial extracellular factors [14]. Direct evidence on the molecular mechanisms through which bacterial proteases participate in the development of the pathology is often still lacking. But in some cases there is no doubt on the role of the proteolytic enzymes in the intoxication process, like, for instance, fragment A from diphteriae and pertussis toxins and the metalloproteases present in tetanus and botulinum neurotoxins [22].

Generally speaking, secreted bacterial proteins can be classified in three different groups, according to the site of action in the host: a) those acting in the extracellular space, by binding to specific membrane proteins or receptors or by degrading tissues and/or cellular components; b) those that cause changes to membrane permeability or interfering with transmembrane signaling pathways, and c) those that act in the intracellular space where they enzymatically modify specific cytosolic targets. The later normally consist of 2 main disulfide linked domains: domain A (presenting enzymatic activity) and domain B (binding and penetration domain) [23].

The mechanism of cell intoxication by this last group of toxins can be divided in four steps: 1) toxin binding through domain B to cellular protein or lipid receptors; 2) internalization through endocytosis of the toxin-receptor complex; 3) membrane translocation of at least domain A to the cytosol, and 4) domain A enzymatically modifies a cytosolic target, such as through the hydrolysis of membrane fusion proteins from neurotransmitter synaptic vesicles.

### 2.2. Clostridial Neurotoxins: Proteolytic Action and Specificity

Botulinum neurotoxins types A and E (BoNT/A and E) are members of the family of the botulinum neurotoxins (bontoxilysins as denominated by Barret *et al.* [24], EC 3.4.24.69), which contains seven structurally similar, but antigenically different, proteins (serotypes A to G). These toxins are produced by several strains of the anaerobic bacillus *Clostridium botulinum* and act on the peripheral nervous system of humans and animals, causing botulism, a frequently fatal disease characterized by a flaccid paralysis of skeletal muscles [25, 26]. Tetanus neurotoxin (TeNT) classified as EC 3.4.24.68 in the tentoxilysin family [24]is a protein produced by the anaerobic bacillus *Clostridium tetani*, which causes tetanic lethal spastic paralysis by blocking the release of inhibitory neurotransmitter at central synapses, and is a member of the clostridial neurotoxin family [22].

These clostridial neurotoxins are secreted as 150 kDa single chains, which are cleaved at an exposed loop, situated at approximately one third of the molecule's length from the N-terminal. This cleavage generates the active form of the toxin, which is composed of 2 fragments, of 50 and 100 kDa, linked by a disulfide bond and non-covalent interactions. The light chain, as is called the 50 kDa fragment, has a metalloprotease activity, while the heavy chain is important for the light chain translocation through the membrane into the host's neuronal cytosol, consequently playing a critical role in pore formation [27, 28].

The end protease activities of the light subunits [22, 28-31] were shown to cleave selectively, at a single characteristic site, one of the three neuronal proteins associated with exocytosis: synaptobrevin, also called vesicle-associated-membrane protein (VAMP), SNAP-25 (synaptosomal-NSF-

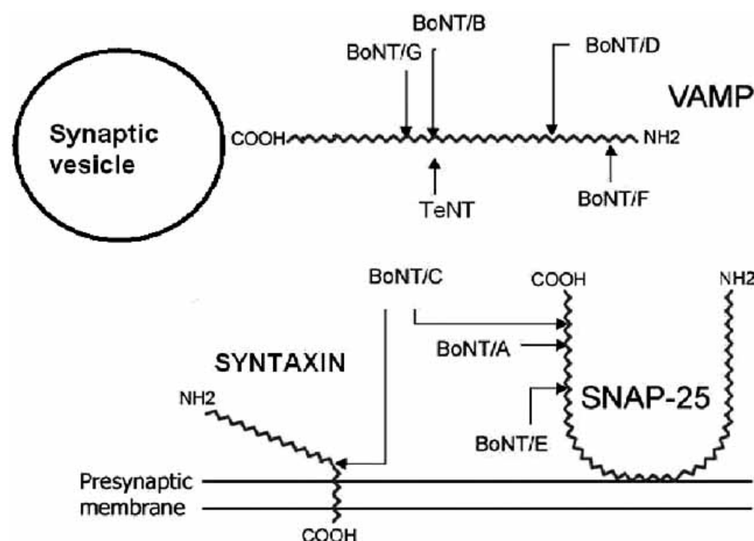


**Fig. (1).** Mechanism of action of clostridial neurotoxins (TeNT and BoNT).

attachment protein) or syntaxin (Table **1**). These proteins were demonstrated to form a complex that triggers the exocytosis process [32]. Among the neurotoxins, BoNT/A cleaves SNAP-25 between $Q_{197}$ and $R_{198}$, BoNT/E cleaves between $R_{180}$ and $I_{181}$, and TeNT claves the synaptobrevin between $Q_{76}$ and $F_{77}$ [30]. At present, there is no effective therapy against botulism, which remains a serious health problem. One potential solution to reduce BoNT toxicity would be to inhibit the proteolytic activity of its light chain (LC). Selective and potent inhibitors of BoNT could be obtained by a rational approach based on the knowledge of the mechanism of action of other zinc metallopeptidases [31, 33] and/or by screening libraries of various chemical compounds. Similarly, TeNT metalloprotease activity has been known for some time now, especially regarding its affinity for the vesicle membrane associated protein synaptobrevin, involved in the exocytotic machinery. However, further characterization of its affinity for other substrates, limitations regarding substrate size and preferential amino acid sequence at the P and P' positions is still needed.

### 2.3. Gram-Negative Virulence Factors Related to Proteases and their Multiple Targets and Actions

#### *Diphtheric Toxin*

The purified and inactivated diphtheric toxin, used in the anti-diphteric vaccine, has already been crystallized and partially characterized. It is an acid, globular protein with a molecular weight between 59 and 60 kDa. Though not as potent

**Table 1. Main Proteolytic Activity Found in Bacteria and Related to Virulence Components and their Pathogenicity**

| ENZYME | ACTIVITY | BACTERIA |
|---|---|---|
| ADP-Ribosilating toxins | Transferase and NADase | |
| Phospholipases | Citolytic phospholipases | *C. perfringens*, *C. sordellii*, *C. novyi*, *P. aeruginosa*, *P. aureofasciens*, *A. Hydrophila*, *B. cereus*, *Rickettsia prowazekii*, *S. aureus*, *Corynebacterium ovis, Yersinia pestis* |
| Adenilate cyclase | Adenilate cyclisation | *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *Bacillus anthracis*, *Pseudomonas aeruginosa* |
| Metalloproteases | Tetanus an botulinun A,B,C,D,E,F,G (light chain) and zinc-dependent toxins | *C. tetani*, *C. botulinum*, *Bacillus anthracis*, *Bacterioides fragilis* |
| RNA N-glicosidases | RNA N-glicosilation | *S. dysenteriae*, *E. colio*, *Aeromonas hydrophila*, *Enterobacter cloacae* |
| Glucosyl transferases | Glucose transferring | *C. difficile*, *C. sordellii*, *C. novyi* |
| Deamidase activity | Deamidating | *E. coli* |
| Protease activity | Proteolytic | *S. aureus*, *S. hyicus*, *E. coli*, *S. pyogenes* |
| Deoxyribonuclease activity | Ribonuclease deoxidating | Many gram-negative bacterias |

Adapted from Alouf J.E. & Popoff, M.R. (Comprehensive Sourcebook of Bacterial Protein Toxins, p. 5, **2006**, Academic Press.

**Table 2. Target and cleavage of peptide bond by BoNT subtypes and TeNT Cleaved Peptide Bond[a]**

| BoNT (Serotypes) | Target | $P_3$ | $P_2$ | $P_1$ | ↓ | $P_{1'}$ | $P_{2'}$ | $P_{3'}$ |
|---|---|---|---|---|---|---|---|---|
| A | SNAP-25 | $Ala_{195}$ | $Asn_{196}$ | $Gln_{197}$ | ↓ | $Arg_{198}$ | $Ala_{199}$ | $Thr_{200}$ |
| B | VAMP | $Ala_{74}$ | $Ser_{75}$ | $Gln_{76}$ | ↓ | $Phe_{77}$ | $Glu_{78}$ | $Thr_{79}$ |
| C | Syntaxin | $Thr_{251}$ | $Lys_{252}$ | $Lys_{253}$ | ↓ | $Ala_{254}$ | $Val_{255}$ | $Lys_{256}$ |
| C | SNAP-25 | $Asn_{196}$ | $Gln_{197}$ | $Arg_{198}$ | ↓ | $Ala_{199}$ | $Thr_{200}$ | $Lys_{201}$ |
| D | VAMP | $Asp_{57}$ | $Gln_{58}$ | $Lys_{59}$ | ↓ | $Leu_{60}$ | $Ser_{61}$ | $Glu_{62}$ |
| E | SNAP-25 | $Ile_{178}$ | $Asp_{179}$ | $Arg_{180}$ | ↓ | $Ile_{181}$ | $Met_{182}$ | $Gly_{183}$ |
| F | VAMP | $Arg_{56}$ | $Asp_{57}$ | $Gln_{58}$ | ↓ | $Lys_{59}$ | $Leu_{60}$ | $Ser_{61}$ |
| G | VAMP | $Thr_{79}$ | $Ser_{80}$ | $Ala_{81}$ | ↓ | $Ala_{82}$ | $Lys_{83}$ | $Leu_{84}$ |
| TeNT | VAMP | $Ala_{74}$ | $Ser_{75}$ | $Gln_{76}$ | ↓ | $Phe_{77}$ | $Glu_{78}$ | $Thr_{79}$ |

(a) The cleavages shown are in VAMP-2, SNAP-25 e syntaxin IA from rat (Rattus novergicus) (table adapted from *Handbook of Proteolytic Enzymes*, **1998**).

as other bacterial toxins, it still presents considerable toxicity [34].

The diphtheric bacterium secretes the toxin as a single polypeptidic chain containing two disulfide bonds, which is toxic for animals, but not enzymatically active. The enzymatic activity can be induced through protease treatment, such as with tripsin, followed by the reduction of the disulfide bonds, breaking the toxin into fragments A and B. This cleavage is indistinguishable from the natural process performed by bacterial proteases [35]. After cleavage and reduction of the polypeptide, fragments A and B remain bound by non-covalent forces.

Fragment A presents a high elongation factor 2 (Ef-2) ADP ribosilation activity, although different activities can be detected in certain experiments with non-pure toxin preparations. However, fragment A's main characteristic is stability. It can be heated to 100 $^{o}$C at neutral pH, or exposed to pHs between 2 and 12 at ambient temperature for short periods of time, with no loss of activity. Fragment B, on the other hand, has apparently no enzymatic activity, it denatures and precipitates as the toxin's fragments dissociate after cleavage and can't be found in crude toxin preparations, which is the reason why it has been much less studied than fragment A.

### *Bordetella Toxins*

*Bordetella* are Gram negative bacteria that cause respiratory tract infections in humans and animals. While at least five different species of *Bordetella* are known to exist, attention has been primarily given to *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* species. In their virulent phase, all of these bacteria produce a nearly identical set of virulence factors that have been identified and characterized at the molecular level and of which the molecular mechanisms are now well understood. The production of most virulence factors is coordinately regulated by a two-component signal transduction system composed of the regulator BvgA and the sensor protein BvgS, which mediates the transition between at least three identifiable phases: a virulent (Bvg+) phase, an avirulent (Bvg-) phase and an intermediate (Bvg(i)) phase, in response to specific environmental signals. These virulent factors can be grouped into two major categories: ADHESINS such as filamentous hemagglutinin (FHA), fimbriae and pertactin, and TOXINS such as a bifunctional adenylate cyclase/hemolysin, dermonecrotic toxin, tracheal cytotoxin, pertussis toxin specific to *B. pertussis* and type III secreted proteins specific to *B. bronchiseptica*.

*Bordetella* colonize the ciliated respiratory mucosa, a surface designed to eliminate foreign particles, thereby making the adherence and persistence mechanisms of these bacteria crucial. The adhesins and toxins act in concert to establish infection. Some adhesins exert their effects synergistically or redundantly, functioning only in the absence of other adhesins, illustrating the importance of the adhesion in infection. Most virulence factors are secreted into the culture supernatant or exposed at the surface of the bacterial cell. A notable exception is the dermonecrotic toxin, which remains in the cytoplasmic compartment of bacterial cells. Most *Bordetella* virulence factors are produced by all the three main *Bordetella* species: *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica.* However, some, such as the pertussis toxin and the tracheal colonization factor, are only produced by *B. pertussis*. The development of suitable animal models for *B. bronchiseptica* has enabled the study of *Bordetella* pathogenesis in the context of natural host-pathogen interactions. Evolutionary studies among the various *Bordetella* species and detailed analysis of differential regulation of Bvg-activated/repressed genes has greatly improved our understanding of the mechanisms of *Bordetella* pathogenesis [36]. In addition, our understanding of *Bordetella* virulence at the molecular level has led to the development of new acellular vaccines against whooping cough, and to the use of genetically attenuated *B. pertussis* strains as recombinant live bacteria vaccine vectors for homologous and heterologous protection.

### *Pertussis TOXIN (PTx)*

*Bordetella pertussis* causes whooping cough in humans. Infection is established through the respiratory route and

**Table 3. Virulence Factors and Protective Antigens of *B. pertussis***

| Virulence Factor | Putative Role in Pathogenicity |
|---|---|
| Pertussis toxin | Adesion, invasion, interference on immune effector cells |
| Filamentous haemagglutinin | Adesion, invasion |
| Fimbrial antigen (aglutinogens 2&3) | Adesion, invasion |
| Pertactin | Adesion, invasion |
| Adenylate cyclase toxin | Interference in immune effector cells |
| Heat labile toxin (dermonecrotic) | Local inflammatory effects |
| Tracheal Cytotoxin | Cilliar paralysis |
| BrkA | Adesion, invasion, serum resistence for bacteria |
| Tracheal colonizing factor | Adesion, invasion |

remains generally localized in the upper respiratory tract, causing respiratory disease [37, 38]. After aerosol inoculation, the bacteria adhere to the nasopharyngeal mucosa mainly *via* the filamentous hemagglutinin protein and fimbriae, and then proliferate and spread to the respiratory tract. *B. pertussis* produces a complex mixture of adhesins, agresins and toxins, important for the colonization of the human host [39] and causing the loss of cilia from the bronchial columnar epithelium and cell damages. The expression of these virulence factors is finely controlled through a series of highly sophisticated mechanisms, such as reversible mutations, in response to inputs from the ambient. Because of this complexity, and in spite of the growing knowledge on the biology of *B. pertussis*, the pathologic mechanisms of the disease are not yet clear. Some studies found that PTx efficiently ADP ribosylates protein G of airway macrophages both *in vitro* and *in vivo* after intranasal administration of PTx in mice. These observations indicate that PTx targets airway macrophages promoting early infection of the respiratory tract by *B. pertussis*.

In order to determine the role of each virulence factor, experiments have been performed both *in vitro* and *in vivo*, particularly in mice. Recent studies revealed a degree of heterogeneity between the different *B. pertussis* strains that might translate into antigenic variation. These findings on the pathogenicity of *B. pertussis* may have important implications on the development of the pertussis vaccine, and several of many recently identified virulence factors are in different testing stages as candidates for new acellular pertussis vaccines.

The main *B. pertussis* virulence factor is the pertussis toxin. The native pertussis toxin is a protein with a wide range of *in vivo* biological activities, including the induction of leucocytosis, histamine sensitization, increased insulin production with consequent hypoglycaemia, potentiation of anaphylaxis and lethality in mice [40]. PTx is also believed to be a major contributor to the reactogenicity of pertussis vaccines including, controversially, neuropathology [41]. More recently, new evidences indicate that PTx is able to induce IL-1β production in the mouse brain. IL-1β can affect neuroendocrine functions and modulate the release of neurotransmitters, and has been claimed to play a role in neurological reactions observed in children immunized with pertussis vaccines of high PTx content [42]. Although the biological effects of PTx have been under extensive research, the mechanism(s) of its toxicity is still unclear. As a consequence of these potent activities, the pertussis toxin probably has a central role in the pathogenicity of *B. pertussis*, causing the typical symptoms of the disease.

PTx has the A–B type structure typical of many bacterial toxins, with an enzymatically active A-protomer, the S-1 subunit and a binding B-oligomer consisting of subunits S-2 through S-5 [43, 44]. The holotoxin's molecular weight is estimated to be between 90 and 107 kDa. The intact B-subunit is required for the binding of the holotoxin to receptor sites on the target cell surface and entry of the A-protomer into the cells [48]. The A-protomer catalyses the ADP-ribosylation of eukaryotic GTP-binding regulatory proteins, preventing hormonal inhibition of adenylate cyclase and resulting in an increase in intracellular levels of cAMP [45, 46]. The B-oligomer also presents biological activity, e.g. as a mitogen and haemagglutinin. However, these effects require much higher concentrations (>1μg) of PTx or B-subunit than those dependent on the enzymatically active site [47]. Furthermore, the toxic activities of PTx *in vivo*, including histamine sensitization, are all abolished when the enzyme active site is inactivated by site-directed mutagenesis, as in a genetically detoxified PTx (g-PTx) [48, 49]. This is the basis for the hypothesis that the ADP-ribosylation enzyme activity is directly responsible for the toxicity reflected *in vivo* by the histamine-sensitization test (HIST).

### *Tracheal Cytotoxin (TCT)*

Tracheal cytotoxin (TCT) is a low molecular weight glycopeptide released during the logarithmic growth-phase of *Bordetella pertussis*. It is the only *B.pertussis* product that reproduces the respiratory cytopathology observed during pertussis (whooping cough) [50]. This pathology includes

ciliostasis and specific extrusion of ciliated cells from the respiratory epithelium. In the absence of ciliary activity, coughing becomes the only way to clear the airways of accumulating mucus, bacteria, and inflammatory debris. Thus, TCT-mediated destruction of ciliated cells may trigger the violent coughing episodes symptomatic of pertussis. In addition, the absence of a ciliary clearance mechanism predisposes patients to secondary pulmonary infections, the primary cause of pertussis mortality [51]. TCT has been purified and its structure was determined by fast atom bombardment (FAB)-MS as GlcNAc-1,6-anhydro-MurNAc-L-Ala-y-D-Glu-meso-A2pm-D-Ala [52], where MurNAc is N-acetylmuramic acid and A2pm is diaminopimelic acid. TCT belongs to a family of compounds known as muramyl peptides, which have many biological activities including adjuvanticity, somnogenicity, and pyrogenicity [53-55]. Naturally occurring muramyl peptides are of bacterial origin; they are fragments of peptidoglycans, the polymeric components that confer structural rigidity to the cell wall. Muramyl peptides are released during the growth of *Bordetella* Spp. [56, 57]

### *Dermonecrotic Toxin (DNT)*

DNT was one of the first virulence factors to be described from *Bordetella pertussis*, by Bordet and Gengou in 1909[66], although they misidentified it as an endotoxin [58]. DNT is so called because it produces a characteristic skin lesion when injected in rabbits, mice, and guinea pigs [57-60].

It is inactivated by heat above 56°C [38] and has been shown to be cytoplasmic rather than secreted by the bacteria [60-61]. The most recent reports suggest that DNT is composed of a single polypeptide chain with a molecular mass of 140 kDa [62, 63], and a minimal dose of 0.40 pg is required for a skin reaction [63]. The role of DNT in the pathogenesis of whooping cough is uncertain since mutants lacking DNT appear to be as virulent as the wild type in a mouse model [64]. Some observations suggest that DNT is an important virulence factor in the swine disease atrophic rhinitis [65], which can be caused by *B. bronchiseptica* [65, 66], an organism which can also cause kennel cough in dogs [65]. *B. parapertussis*, both a human pathogen and a sheep pathogen [67], and *B. avium*, a pathogen of domestic fowl [68, 69], also produce DNT, but, as in *B. pertussis* infections, there are no data regarding the role of DNT in the pathologies caused by them.

The DNTs from these species have been compared at the genetic level. *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* DNTs present a remarkable similarity, though they differ both genetically and biologically from *B. avium* DNT. Certain strains of *Pasteurella multocida* are implicated in atrophic rhinitis [65], and these strains also produce a toxin, PMT, which causes dermonecrosis in test animals [61, 70, 71].

### *Adenylate Cyclase Toxin (CyaA)*

CyaA consists of a single large polypeptide chain. The N-terminal part of the protein contains the catalytic domain, while the C-terminal part mediates its binding to the target cells. CyaA enters eukaryotic cells both in a receptor-independent manner [72, 73] and through high affinity binding to the $\alpha_M\beta_2$ integrin, CD11b/CD18, which is present on macrophages, neutrophils, dendritic cells and natural killer cells [74]. After membrane translocation, the CyaA catalytic domain remains attached to the cytosolic surface of the plasma membrane where, following activation by $Ca^{2+}$/calmodulin, it rapidly converts cellular ATP into cyclic AMP (cAMP), thus generating large quantities of cAMP [72, 75]. CyaA can also form cation-selective pores in cell membranes independently of translocation, thereby disrupting ion homeostasis [76]. Moreover, Fiser *et al*. [77] have recently reported a third activity of CyaA, which involves sustained elevation of intracellular $Ca^{2+}$ promoted by membrane translocation of the adelnylate cyclase domain and appears to be independent from both the adenylate cyclase activity and the pore-forming activity of the toxin. The use of *B. pertussis* strains lacking specific virulence factors in mice has provided proof that CyaA actively participates in the pathogenesis by favoring colonization of the respiratory epithelium and infection of the host [78]. Crucial to the successful establishment and spreading of the infection is the capacity of this toxin to attenuate the immune defenses of the host at the site of colonization and to delay the development of a systemic immune response. Indeed, CyaA inhibits a variety of innate immune effector functions, including phagocytosis, oxidative burst and production of pro-inflammatory cytokines [75, 76, 79]. CyaA has been recently reported to also interfere with the initiation of adaptive immune responses by driving monocyte-derived dendritic cell differentiation to a semi-mature state characterized by increased expression of MHCII and the co-stimulatory molecules CD80, CD83 and CD86 [80]. This state has been associated with decreased pro-inflammatory cytokine production and increased expression of the suppressive cytokine IL-10, which promotes the expansion of regulatory T cells [56]. Furthermore, CyaA promotes macrophage apoptosis, which not only impairs bacterial killing but also prevents antigen presentation [76].

The immunosuppressive activities of CyaA on phagocytes and dendritic cells have been largely attributed to its capacity to increase intracellular cAMP levels. cAMP has indeed been demonstrated to act as a potent immunosuppressant by promoting activation of protein kinase A (PKA) [81]. Furthermore, the Rap specific guanine nucleotide exchange factor EPAC1 has been recently identified as a primary target of cAMP [57]. Inhibition of monocyte and macrophage activation by cAMP results from disruption of PKA dependent intracellular signaling through the inhibition of LPS-induced activation [82,83]. On the other hand, the inhibitory activity of cAMP on phagocytosis by both alveolar and monocyte-derived macrophages has been ascribed to EPAC1 activation [84,85]. The cAMP has long been known to suppress T lymphocyte activation and chemotaxis. The immunosuppressive effects of cAMP on T cells involve perturbation by PKA and EPAC1 of a number of intracellular pathways which participate in T cell activation and migration [81, 86]. Among these, of paramount importance are MAP kinase cascades, which can be modulated at multiple steps of their activation by cAMP [87]. Here we have addressed the effects of *B. pertussis* CyaA on T lymphocytes. A comparative analysis of the effects of CyaA on T cells and macrophages identifies MAP kinase modules as common targets of

the toxin's PKA modulating activity in the signaling pathways triggered by receptors as diverse as antigen receptors, chemokine receptors and Toll-like receptors [88].

## 3. CONCLUDING REMARKS

Proteases are probably the most effective of all bacterial compounds in the establishment of an infection, thus conferring an adaptative advantage over bacteria lacking such proteases. As enzymes, proteases present a large turnover, processing enormous amounts of substrate in little time (high $k_{cat}$ value), while proteins and other molecules that act by binding to targeted receptors or tissues, even with high affinity, are limited to single or few events. Additionally, proteases can open their own ways to get into the host tissues and cells, thus representing a huge and valuable tool for colonization and spreading of the bacteria. Most secreted bacterial endoproteases are highly specific against their substrates in the host and, as opposed to exoproteases, they cleave at very specific sites within the target molecule, which confers a characteristic symptom, typical of the disease caused by the pathogen secreting it. In many cases, they also constitute a defense mechanism against the host's immune system.

Although many bacterial and viral proteic virulence factors were not mentioned in this review, most of them share a great number of the characteristics presented here which strongly reinforce the idea that to have a good knowledge on bacterial biology and pathogenesis it is important to characterize their secreted proteolytic activities and specificities. It is also interesting to note that newly described strains often result from modifications or over expression of virulence factors, including the proteolytic factors, from previously characterized strains. As inhibition mechanisms are elucidated, targets may be defined for the search and development of inhibitors of eventual pharmaceutical use.

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