Toxinology

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P. Gopalakrishnakone *Editor-in-Chief*
Brad Stiles · Alberto Alape-Girón
J. Daniel Dubreuil · Manas Mandal *Editors*

Microbial Toxins

Springer

Toxinology

Editor-in-Chief
P. Gopalakrishnakone

In recent years, the field of toxinology has expanded substantially. On one hand it studies venomous animals, plants and microorganisms in detail to understand their mode of action on targets. While on the other, it explores the biochemical composition, genomics and proteomics of toxins and venoms to understand their interactions with life forms (especially humans), development of antidotes and exploring their pharmacological potential. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy and pharmacology. In addition, there is a fast-developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of toxins on the human body. Given the huge impact of toxin-based deaths globally, and the potential of venom and toxin in generation of drugs for so-far incurable diseases (for example, diabetes, chronic pain), the continued research and growth of the field is imminent. This has led to the growth of research in the area and the consequent scholarly output by way of publications in journals and books. Despite this ever-growing body of literature within biomedical sciences, there is still no all-inclusive reference work available that houses all of the important biochemical, biomedical and clinical insights relating to toxinology. Composed of 12 volumes, *Toxinology* provides comprehensive and authoritative coverage of the main areas in toxinology, from fundamental concepts to new developments and applications in the field. Each volume comprises a focused and carefully chosen collection of contributions from leading names in the subject.

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With 77 Figures and 16 Tables

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Series Preface

The term TOXIN is derived from the Greek word Toeikov and is defined as a substance derived from tissues of a plant, animal, or microorganism that has a deleterious effect on other living organisms. Studying their detailed structure, function, and mechanism of action as well as finding an antidote to these toxins is the field of TOXINOLOGY, and the scientists are called TOXINOLOGISTS.

In recent years, the field of toxinology has expanded substantially. On one hand, it studies venomous animals, plants, and microorganisms in detail to understand their habitat, distribution, identification, as well as mode of action on targets, while on the other, it explores the biochemical composition, genomics, and proteomics of toxins and venoms to understand their interaction with life forms (especially humans), the development of antidotes, and their pharmacological potential for drug discovery. Therefore, toxinology has deep linkages with biochemistry, molecular biology, anatomy, pharmacology and drug development. In addition, there is a fast developing applied subfield, clinical toxinology, which deals with understanding and managing medical effects of venoms and toxins on the human body following envenomations. Given the huge impact of envenomation-based deaths globally, and the potential of venom and toxin in the generation of drugs for debilitating diseases (e.g., diabetes, chronic pain, and cancer), the continued research and growth of the field is imminent.

Springer has taken the bold initiative of producing this series, which is not an easy task of generating 12 volumes, namely, biological toxins and bioterrorism, clinical toxinology, scorpion venoms, spider venoms, snake venoms, marine and freshwater toxins, toxins and drug discovery, venom genomics and proteomics, evolution of venomous animals and their toxins, plant toxins, and microbial toxins.

Singapore

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I would like to sincerely thank the section editors of this volume, Brad Stiles, Alberto Alape-Girón, J. Daniel Dubreuil and Manas Mandal for the invaluable contribution of their expertise and time and the authors who obliged with my request and provided a comprehensive review on the topics.

Springer provided substantial technical and administrative help by many individuals at varying levels, but special mention should go to Sarah Mathews, Sunali Mull, Meghna Singh, Mokshika Gaur, and Audrey Wong for their tireless effort in bringing these volumes to reality.

Singapore

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Volume Preface

This volume provides an overview of microbial toxins from diverse bacterial and fungal origins. These molecules, produced by various species and consisting of protein or small organic molecules, can play a pivotal role in the pathogenesis of plants, animals, and humans that in turn can lead to the survival/dissemination of the host microbe. Many of these microbes, due to their toxins, impact our society from a health and economic perspective. In particular, this volume addresses the diverse niches of these organisms focused upon their associated toxins. The structures, functions, and genetics of these toxins will be addressed. Besides the ill-effects elicited by these toxins, it must be noted that there is immense potential for turning some of these harmful molecules into useful tools such as specific receptor probes, novel drugs or drug-conjugates for the treatment of various diseases, and immunomodulating agents for targeted vaccine delivery. Recent progress in bacterial genome mapping and establishment of three-dimensional crystal structures of several bacterial toxins provides a deeper knowledge and greater understanding of structure-function relationships. Moreover, the emergence of some bacteria such as *Bacillus anthracis*, *Burkholderia pseudomallei*, and *Staphylococcus aureus*, and their toxins, as biological weapons also necessitates a thorough understanding of these agents, their pathophysiology, and development of countermeasures. This volume will also serve as a common resource for researchers interested in many other medically relevant microorganisms, and their toxins, that include *Clostridium botulinum*, *C. difficile*, *C. perfringens*, *C. tetani*, *Escherichia coli*, *Helicobacter pylori*, and *Listeria monocytogenes*.

This current handbook, consisting of 24 chapters, is a major reference work on microbial toxins written by a panel of international experts.

Contents

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Prof. Gopal has also got a new appointment in the newest University in Singapore, Singapore Institute of Technology (SIT), as a professor of anatomy in the Health and Social Sciences Cluster. Prof. Gopalakrishnakone is also a consultant to the Defence Science Organization in Singapore; adjunct senior research scientist at the Defence Medical Research Institute; and an honorary principal fellow at the Australian Venom Research Unit, University of Melbourne, Australia.

His research studies include structure function studies, toxin detection, biosensors, antitoxins and neutralization factors, toxinogenomics and expression studies, antimicrobial peptides from venoms and toxins, and PLA2 inhibitors as potential drug candidates for inflammatory diseases. The techniques he employ include quantum dots to toxinology, computational biology, microarrays, and protein chips.

Prof. Gopalakrishnakone has more than 160 international publications, 4 books, about 350 conference presentations, and 10 patent applications.

He has been an active member of the International Society on Toxinology (IST) for 30 years and was president from 2008 to 2012. He is also the founder president of its Asia Pacific Section, a council member, as well as an editorial board member of *Toxicon*, the society's official journal.

His research awards include the Outstanding University Researcher Award from the National University of Singapore (1998); Ministerial Citation, NSTB Year 2000

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His awards in teaching include Faculty Teaching Excellence Award 2003/4 and NUS Teaching Excellence Award 2003/4. Professor Gopalakrishnakone also received the Annual Teaching Excellence Award in 2010 at both university and faculty levels.

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Dr. Brad Stiles is a microbiologist by training with a keen appreciation for diverse topics in biology. His education in science was formally forged at Pennsylvania State University, Virginia Tech, and then the University of Texas. He spent over two decades in a Department of Defense laboratory studying protein toxins from snakes, marine cone snails, and bacteria. The goals were to further a basic understanding of how toxins affect cells, and subsequently generate methods for detection as well as neutralization via vaccines, therapeutic antibodies, and small molecular-weight inhibitors. Results from his laboratory led to numerous peer-reviewed publications with various national and international laboratories. National and international students have studied in his laboratory and now further the tradition of understanding science. Besides working in the United States, he has formally studied in France and Germany at various universities and public/private laboratories. Dr. Stiles now teaches microbiology at Wilson College in the United States.

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Dr. Manas Mandal received Bachelor's (Honors) and Master's degrees in Human Physiology from the University of Calcutta, India. He earned a Ph.D. degree from the Jadavpur University, Calcutta, in 1992, working on snake venom vaccine development project at the Calcutta School of Tropical Medicine and Indian

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His research interest involves the identification of bacterial toxin in vaccine delivery, drug-induced immune modulation, and salivary biomarker in orthodontic patients. Dr. Mandal has published in major journals in toxinology, immunology, and drug delivery. He serves as a scientific reviewer of journals, grants, and abstracts. He is professionally involved with American Association of Colleges of Pharmacy, US National Association of Boards of Pharmacy, and for the Accreditation Council for Pharmacy Education. Dr. Mandal gave invited talks and presentations and chaired scientific sessions in United States and abroad. He was awarded senior research fellowship by the Indian Council of Medical Research, Upjohn and Vahlteich award by the University of Michigan, and intramural research grant by the University of Southern Nevada.

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Part I

Bacillus Toxins

Bacillus anthracis Toxins: Efficient Biochemical Weapons for the Infectious Battle

1

Yannick Trescos, Clémence Rougeaux, and Jean-Nicolas Tournier

Abstract

Edema and lethal toxins represent two of the main virulence factors of *Bacillus anthracis*. They are formed by three components: protective antigen (PA, the binding component), edema factor (EF), and lethal factor (LF) that can associate to give lethal toxin (LT) and edema toxin (ET). EF and LF bear the activity, which are an adenylate cyclase and a metalloprotease, respectively. During the last two decades, numerous studies have improved our knowledge about the biochemical effects of these toxins.

The main biochemical effects of the toxins are presented first, describing how the toxins enter target cells through binding with receptors and are finally

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delivered to the cytosol. In a second section, the critical targets of the toxins, during the early and late stages of the infection, are discussed.

Keywords

Adenylate cyclase • Anthrax • Cardiomyocytes • Cell death • Cytoskeleton • Hepatocytes • Immune system • Metalloproteinase • Toxi-infection

Contents

Introduction

Bacillus anthracis, the agent of anthrax, is a veterinary disease affecting livestock as well as a major agent of biological warfare and is thus of interest for biodefense (Mock and Fouet 2001). The attacks in 2001, related to letters contaminated with anthrax spores in the United States, have confirmed its potential use as a weapon of bioterrorism and further justify the growing needs in understanding the pathophysiology of this dreadful pathogen. *B. anthracis* is a Gram-positive aerobic bacterium that can form, in poor environments, very resistant spores. Its virulence is linked to two main factors: the capsule formed by poly γ-D-glutamic acid, whose operon is encoded by the plasmid pXO2, and the two toxins (edema toxin (ET) and lethal toxin (LT)) encoded on the other virulence plasmid named pXO1 (Moayeri and Leppla 2009).

Anthrax is a toxi-infection: an association of toxemia and rapidly spreading infection progressing to septicemia. The pathogenicity of *B. anthracis* mainly depends on two plasmid-encoded virulence factors, toxins and a poly-gamma-*D*-glutamate capsule (PDGA), anchored to the cell wall which protects bacilli from the immune system, thus promoting systemic dissemination. A more complex picture for capsule function has emerged beyond the simplistic view of a rampart against degradation. The pathogen indeed sheds capsule degradation products through capsule depolymerase CapD, which are associated with virulence (Makino et al. 2002). Thus, the capsule is not neutral to immune cells, as it has suppressive effects on human monocyte-derived dendritic cell (DC) functions (Jelacic et al. 2014).

There are three classical types of human infection: cutaneous, gastrointestinal, and inhalational. A recent fourth form named "injectional anthrax" has been described after an outbreak in Northern Europe caused by tainted heroin batches (Hicks et al. 2012). By the pulmonary route, *B. anthracis* induces within a few days a toxemia associated with sepsis and respiratory distress, rapidly progressing to death in the absence of treatment (Guarner et al. 2003). Anthrax toxins play a central role in the pathogenesis of the disease at two critical stages of the infection. In the early time of infection, the toxins target specifically the innate and adaptive immune cells to paralyze the immune response and take over the host response. During the late stage of the infection, when high levels of toxins circulate in the blood, toxins are responsible for high lethality by targeting specific organs (Tournier et al. 2009; Liu et al. 2014).

In this review, the biochemical properties of LT and ET will be first described, followed by their general effects at the early and late stages of infection.

Anthrax Toxins: A Presentation

Toxin Entry

Anthrax toxins are A/B type, formed by the association of three components. Components A bear the enzymatic activity. Edema factor (EF, 89 kDa) is a calmodulin-dependent adenylate cyclase (Moayeri and Leppla 2009; Tang and Guo 2009), while lethal factor (LF, 90 kDa) is a zinc-dependant metalloprotease (Moayeri and Leppla 2009; Tonello and Montecucco 2009). The component B, involved in binding to the cell receptor, is common for both toxins: it is named protective antigen (PA, 83 kDa -PA_{83}-) because of its immunogenic properties (review in Young and Collier (2007)). Overall, the association of EF and PA and LF and PA forms ET and LT, respectively. PA plays a major role in toxin entry. The mechanisms of cell penetration of toxins have been largely explored over the past decade and can be separated into three major steps: (i) receptor binding, (ii) internalization, and (iii) membrane translocation (reviewed in Young and Collier (2007)).

PA_{83} binds to at least two specific cell receptors: ANTXR1 or TEM-8 for *tumor endothelial marker*-8 and ANTXR2 or CMG-2 for *capillary morphogenesis protein*-2 (Bradley et al. 2001). TEM-8 (ANTXR1) and CMG2 (ANTXR2) are transmembrane proteins that anchor the actin cytoskeleton to several extracellular matrix proteins such as collagen IV, laminin, and fibronectin, but their exact functions are still largely unknown. Interestingly, ANTXR1 and ANTXR2 contain both an extracellular von Willebrand factor A (vWA) domain responsible for binding to PA domain 4.

Another coreceptor named LDL receptor protein (LRP)-6 has been proposed (Wei et al. 2006), while its effective role has been challenged (Young and Collier 2007). Anthrax toxin receptors can form a complex with LRP6 to control Wnt

signaling (Abrami et al. 2008). Integrin β1, which also contains a vWA domain, has also been suspected to play a role as receptor, although based upon limited evidence (Martchenko et al. 2010). The controversy about the potential alternative toxin cell receptors has vanished after the publication using ANTXR1 and ANTXR2 genetically-inactivated mice and showing that in vivo, ANTXR2 is the major receptor playing any physiological role (Liu et al. 2009, 2013).

The interaction of PA_{83} with its receptor allows its cleavage by a furin-like protein in two subunits of 63 (PA_{63}) and 20 kDa (PA_{20}), the latter being released. The cleavage of PA_{83} into PA_{63} can also occur in the plasma by unidentified proteases (Moayeri et al. 2007), while PA_{63} oligomers can assemble and associate with LF and EF before binding to its cell receptors (Liu et al. 2014). The respective role of circulating complexes and oligomers formed after cleavage on the cell surface is still unknown. PA_{63} subunits associate into oligomers, mainly heptamers or octamers, to form a prepore redistributed to the cell surface in lipid rafts (Kintzer et al. 2009). This allows the binding of EF or LF components in a stoichiometric ratio of 3 LF/EF to 7 or 8 PA molecules. The toxin-receptor complexes are then internalized by clathrin-dependent endocytosis through lipid rafts after activation by *src*-like kinase (Abrami et al. 2003, 2010a, b). Palmitoylation and ubiquitination of anthrax receptors may also participate in the process through unconventional adaptors (Young and Collier 2007; Abrami et al. 2010b). The pH decrease of early endosomes results in the translocation of these factors in multivesicular bodies (MVB), finally merging with the intracellular membrane of late endosomes (Abrami et al. 2013). The pH drop during the journey is responsible for the conversion of a prepore structure to a heat- and SDS-stable structure (Young and Collier 2007; Miller et al. 1999). The structural aspects of the unfolded EF and LF translocating through the pore have been thoroughly studied (Feld et al. 2012), and final demonstration has been afforded by cryo-electron microscopy that support the Brownian ratchet model and the Φ-clamp (Jiang et al. 2015). Final translocation occurs through back fusion of intraluminal MVBs filled with LF and EF (Abrami et al. 2004). This last step is responsible for the translocation of LF into the cytoplasm, while EF stays associated to the late endosome membrane (Guichard et al. 2012).

Lethal Toxin Cell Targets

LF specific activity is a zinc-dependent metalloproteinase that cleaves specifically the N-terminus of mitogen-activated protein kinase kinases (MAPKK or MEK) (Vitale et al. 1998). This cleavage results in a disruption of MEK signaling pathways of ERK 1/2 (extracellular signal-regulated kinases), JNK/SAPK (c-Jun N-terminal kinase/stress-activated protein kinase), and p38, signaling cascades essential in multiple cell function such as cell proliferation, cell cycle regulation, and immune function (review in Moayeri and Leppla (2009), Tournier et al. (2009)).

It has been known for a long time that macrophages from certain mouse (BALB/c) and rat (Fisher) strains are susceptible to a rapid cell death induced by LT (Friedlander et al. 1993). The cell death susceptibility of certain rodent strains was thought to be

related to MEK cleavage (Park et al. 2002), before genetic studies demonstrated a link to the *Nlrp1b* locus in mice (Boyden and Dietrich 2006). NLRP1 encodes the nucleotide oligomerization domain (NOD)-like receptor (NLR) protein 1, a component of inflammasomes, an intracellular sensor of microbial molecular patterns that triggers activation of caspase-1. Further studies finally demonstrated that LF cleaves NLRP1b and NLRP1, respectively, in susceptible mouse and rat strains at a site near their N-termini, resulting in their constitutional activation and cell death by pyroptosis (Liu et al. 2014).

The discovery of LF effects on NLRP1 could finally provide an explanation to the old paradox that susceptible mouse strains have macrophages susceptible to LF-induced cell death, but were naturally more resistant to the infection and vice versa (Friedlander 1986; Welkos and Friedlander 1988). Recent studies have shown that resistance to infection is mediated specifically by the *NLRP1b* locus (Terra et al. 2010). Cleavage of NLRP1b/NLRP1 in sensitive mouse/rat strains to LF-induced cell death induces caspase-1 activation and interleukin (IL)-1β and IL-18 release by targeted cells. These strong pro-inflammatory molecules induce a potent innate immune response, local recruitment of neutrophils, and control of the infection (Moayeri et al. 2010). It is interesting to note that human macrophages are not sensitive to LF-induced cell death, and this particular feature may partly explain the difference in sensitivity to anthrax infection between species.

Edema Toxin Cell Targets

EF biochemical activity is a calmodulin-dependent adenylate cyclase, producing a rise in intracellular cAMP, a classical second messenger of eukaryotic cells (Leppla 1982). After back fusion of MVBs to the late endosome membrane, EF stays associated to the membrane and generates intracellular cAMP gradients from the cell nucleus to the periphery (Guichard et al. 2012). EF is a very efficient adenylyl cyclase (Tang and Guo 2009) that induces cAMP that in turn activates at least two main cytosolic targets: protein kinase A (PKA) and the exchange protein activated by cAMP (Epac) (Liu et al. 2014).

ET disrupts endothelial homeostasis by playing upon multiple factors, although its role in edema is still discussed (Liu et al. 2013; Guichard et al. 2012). Interestingly, ET affects directly the cytoskeleton of endothelial cells by inducing transendothelial macroaperture tunnels (Maddugoda et al. 2011). The cytoskeleton can sense curvature induced by transendothelial macroaperture through an I-BAR domain protein missing in metastasis (MIM). A delicate balance exists between the ET-induced macroaperture and resealing by the cytoskeleton through recruitment of Arp2/3 actin polymerization, which induces actin waves that close the macroaperture (Maddugoda et al. 2011). ET also disrupts Rab11/Sec15 traffic at the exocyst, inducing reduced cadherin expression at the tight junctions (Guichard et al. 2010). ET induces cytoskeletal changes and inhibits chemotaxis through the action of downstream cAMP effectors EPAC and EPAC-related activators Rap-1 and EPAC and M-Ras-regulated guanine nucleotide exchange factor (MR-GEF)/

rapGEF5 (Hong et al. 2007). Moreover, using the macrophage micropatterning shows that ET induces a strong disruption of the actin cytoskeleton (Trescos et al. 2015), making the actin cytoskeleton an important emerging target of anthrax toxins.

Anthrax Toxin Effects During Infection: Critical Virulence Factors

Anthrax Toxin Effects at the Early Stage: Immune System Paralysis

The early stage of anthrax, also called the prodromal phase, is an asymptomatic stage in which LT and ET dismantle the innate and adaptive immune defenses of the host by acting on neutrophils, macrophages, monocytes, dendritic cells (DCs), and T and B cells (Tournier et al. 2009; Klezovich-Benard et al. 2012). It allows bacterial survival in the host and facilitates dissemination.

An extensive germination occurs as early as 15 min after inoculation into the ear cutaneous tissue (Corre et al. 2013) and in the absence of eukaryotic cell contact. It is accompanied by a rapid toxin production: *pagA* mRNA is detected as soon as 15 min after germination triggering (Cote et al. 2005). It was also reported that detectable LF levels were found in the blood of 50% of cutaneous anthrax cases at an early stage when the infectious process remained localized at the initial lesion site (Boyer et al. 2011). This suggests that *B. anthracis* toxins diffuse and act rapidly, locally, and at a distance.

Genetic inactivation of CMG2 anthrax receptor on myeloid cells, specifically macrophages and neutrophils, enables resistance of mice to anthrax infection (Liu et al. 2010). This study highlights the importance of both myeloid cells as targets of *B. anthracis*, and it shows that CMG2 is the main receptor for anthrax toxin in vivo. Both toxins impair myeloid cells in order to evade the scavenging functions of neutrophils and successfully establish the infection. Neutrophils, considered the first responders to acute infection, contribute importantly to control of *B. anthracis* infection (Liu et al. 2010). They kill *B. anthracis* spores (Mayer-Scholl et al. 2005), playing an important role in bacterial clearance and delaying time to death. LT, and to a lesser extent ET, disables the innate immune functions of the myeloid cells (Liu et al. 2010). LT diminishes neutrophil's ability to kill vegetative bacilli in vitro, prevents neutrophilic migration, and inhibits their accumulation at sites of inflammation in vivo. ET inhibits neutrophilic phagocytosis in vitro and decreases IL-8-driven transendothelial migration of neutrophils through human microvascular endothelial cells of the lung. Thus, the toxins affect neutrophil priming, chemotaxis, chemokine production, and superoxide responses (Tournier et al. 2009; Liu et al. 2014; Guichard et al. 2012; Moayeri et al. 2015).

During inhalational anthrax, alveolar macrophages are the first cells to phagocytose alveolar spores within 10 min, while lung DCs capture spores within 30 min and carry them rapidly to the lymph nodes (Cleret et al. 2007). LT and ET have disruptive

effects on murine DC cytokine secretion (Tournier et al. 2009), and both toxins induce a decrease of chemokines and cytokines by human DCs in vitro (Cleret-Buhot et al. 2012). Only LT inhibits neutrophil attraction (Cleret-Buhot et al. 2012). Interestingly, susceptibility of DCs to LT is dependent upon the maturation stage, suggesting that LT can kill immature DCs in peripheral tissues, while mature DCs in lymph nodes are protected (Tournier et al. 2009). ET treatment results in DC maturation and increases migration toward a specific lymph node-homing chemokine called macrophage inflammatory protein, thus favoring the diffusion of the pathogen (Tournier et al. 2009). In the local draining lymph nodes, exposure to LT kills macrophages and lymphocytes, while ET alters their activation, their migration, and the production of cytokines (Moayeri and Leppla 2009; Tournier et al. 2009). ET induces reorganization of F-actin in macrophages, affecting the cell size and nucleus (Trescos et al. 2015). LT also inhibits the sporicidal and bactericidal activities of macrophages (Tournier et al. 2009).

ET and LT impair the recruitment of natural killer (NK) cells and macrophages into the mouse draining lymph nodes, induced by *B. anthracis* infection (Klezovich-Benard et al. 2012). Both toxins inhibit the natural cytotoxicity function of NK cells in vitro and in vivo.

Anthrax toxins also prevent the development of specific immunity to *B. anthracis* by acting on lymphocytes. In a mouse model, injection of sublethal doses of LT or ET into mice directly inhibits the activation and proliferation of T lymphocytes (T-cells) (Tournier et al. 2009). LT also inhibits cytokine secretion. Both toxins inhibit chemotaxis of T-cells in vitro (Tournier et al. 2009). In human cell assays, LT hinders T-cell proliferation and IL-2 production (Tournier et al. 2009). In vitro and in vivo, LT decreases B-cell capacity to proliferate and produce immunoglobulin (Gnade et al. 2010). ET decreases both basal migration and chemokine-directed migration of B cells, perturbs cytokine production, and slightly impairs MHCII molecule expression which could have effects on the interaction between T-cell and B-cell conjugates (Gnade et al. 2010).

To sum up, ET and LT set up a complex immune evasion strategy by targeting key players in the orchestration of the immune defenses of the host. These protein toxins enable *B. anthracis* survival, germination, and dissemination.

Anthrax Toxin Effect at the Late Stage: Host Killer

The late stage of anthrax, generally considered as fulminant, is often characterized by toxic shock-like symptoms, represented by severe respiratory dysfunction and hypotension followed by cardiac failure.

Bacterial multiplication and dissemination via the bloodstream to multiple highly vascularized target tissues and organs are observed, as there is disruption of organ function and vascular integrity by both LT and ET (Guichard et al. 2012). Histopathological observations in human anthrax cases and in various animal models reveal edema and hemorrhage in a wide array of organs, whatever the route of

infection (Duong et al. 2006; Vasconcelos et al. 2003; Savransky et al. 2013; Abramova et al. 1993): the spleen, heart, liver, lungs, lymph nodes, meninges, kidney, and gastrointestinal tract.

For a long time, LT was considered the major toxin of *Bacillus anthracis*, but it has been demonstrated in mice that ET is lethal at lower doses (20–30 μg) and more rapidly than LT (40–100 μg) (Moayeri and Leppla 2009; Firoved et al. 2005). So, both toxins appear important to induce host death. Injection of mice with LT results in bone marrow necrosis, peritoneal fluid accumulation, pleural edema without visible lesions in lungs, as well as liver failure resulting from hypoxia (Moayeri et al. 2003). A recent study shows that LT treatment decreases the survival of hepatocytes by acting on the cellular responses to hypoxia (Ouyang et al. 2014). In another murine study, LT induces coagulation deficiency in the liver that could contribute to liver damage (Sun et al. 2015). In a murine model of infection, LT blocks intestinal crypt cell proliferation and induces villus tip apoptosis with subsequent mucosal erosion, ulceration, and bleeding of intestinal epithelium (Sun et al. 2012). In rats, LT damages cardiomyocytes, producing acute heart failure (Kuo et al. 2008). In murine heart, electron microscopy analyses show that LT affects myocytes and endothelium that leads to heart failure and important release of cardiac biomarkers (Moayeri et al. 2009).

Each toxin provokes particular lesions in the murine spleen and, when produced together, induces spleen lesions in a specific temporal pattern during the time course of infection (Dumetz et al. 2011). LT-induced lesions are observed before ET-induced lesions. Other combinatorial effects leading to worsening cardiac failure are observed in a rat model: ET increases heart rate, while LT decreases it, and ET produces greater as well as earlier reductions in blood pressure than LT (Cui et al. 2007).

Intravenously administration of ET in mice induces various effects that include extensive fluid accumulation into the gastrointestinal lumen, as well as necrotic lesions in the heart (cardiomyocytes degeneration), adrenal glands, bone, kidney, reproductive organs, and gastrointestinal mucosa (Firoved et al. 2005). ET effects on the heart appear as secondary effects due to general loss of circulatory fluids in many tissues. Effects on the adrenal gland induce endocrine perturbation known to alter LT sensitivity in murine models (Moayeri et al. 2005), which may explain sensitization (Moayeri and Leppla 2009).

Eventually, LT and ET affect many tissues and organs, but an elegant study has finally determined that *B. anthracis* toxins mainly target two vital organs. The generation of cell-type-specific CMG2-null mice and the corresponding cell-type-specific CMG2-expressing mice shows, after toxin challenge, that LT primarily targets cardiomyocytes and vascular smooth cells, while hepatocytes are the major targets of ET (Liu et al. 2013). Interestingly, this study also demonstrates that both toxins do not critically target endothelial cells in their model, although other studies involve both toxins during disruption of the endothelial barrier (Maddugoda et al. 2011; Guichard et al. 2010; Ghosh et al. 2012). Hence, LT induces reorganization of the cytoskeleton, redistribution of junction proteins as VE-cadherin,

and barrier dysfunction in cultured human endothelial monolayers (Warfel et al. 2005) while destroying the barrier function of human mucociliary lung epithelium (Lehmann et al. 2009). In a model of rats challenged with toxins, ET damages vascular endothelium (Kuo et al. 2008), probably explaining vascular hemorrhage observed in lung tissues. According to an infection model, LT and ET breach the vascular barrier between the bloodstream and tissues and change pressure that leads to hemorrhage, which then contributes to bacterial dissemination into target tissues.

Conclusion and Future Directions

Anthrax toxins have broad effects on the immune system and numerous vital systems such as endothelial vascular integrity, as well as heart and liver functions, as discovered recently.

Over the last decades, many important gaps have been filled, but numerous questions remain unanswered.

Future works should try to establish the level of circulating toxins and what is the level of toxins locally produced during an infection. There are some data for LF or EF concentrations in tissue, but mainly during late stages of the infection. This question is important for developing novel therapeutics that would target toxin in the proper organ and at an efficient concentration.

Another side question that remains unanswered is the LT/ET ratio and whether the association of both toxins generates a synergistic effect. Very few studies have evaluated the effects of both toxins at the same time in vivo.

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Mode of Action of Cry Toxins from *Bacillus thuringiensis* and Resistance Mechanisms

2

Mario Soberón, Rose Monnerat, and Alejandra Bravo

Abstract

Bacillus thuringiensis (Bt) insecticidal Cry toxins have been shown to be effective in controlling insect pests either in spray products or expressed in transgenic crops. All Cry toxins are expressed as protoxins that undergo proteolytic processing in the insect gut releasing the activated toxin. It has been shown that activated toxin binds to different insect protein molecules in gut cells leading to oligomerization, membrane insertion, and pore formation. However, it was recently shown that not only the activated toxin is able to specifically interact with receptors, since Cry1A protoxins bind gut receptor molecules leading also to oligomerization, membrane insertion, and pore formation. The final pores induced by protoxin or by activated toxin have different characteristics, suggesting dual mode of action of Cry proteins. In addition it was shown that different Cry1A resistant populations from different insect species are significantly more susceptible to Cry1A protoxins than to Cry1A activated toxins, supporting that Cry1A proteins may undergo two toxic pathways involving protoxin binding to receptors and binding of activated Cry toxins to gut receptor molecules. Here the authors will revise this dual mode of action of Cry proteins and discuss implications of the dual mode of action of Cry proteins for insect pest management in transgenic plants.

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Contents

Introduction

Bacillus thuringiensis is a gram-positive bacterium that produces different insecticidal proteins with different insect specificities named Vip, Cyt, or Cry toxins (Pardo-López et al. 2013). Cry and Cyt proteins are delta-endotoxins formed as crystal inclusion during the sporulation phase of growth, while Vip proteins are synthesized and secreted during vegetative phase of growth (Pardo-López et al. 2013; Chakroun et al. 2016). Among these toxins, the three-domain Cry toxins (3d-Cry), the biggest Cry toxin family, have been shown to be highly effective for agricultural pest control and also for the control of vectors of human diseases such as malaria and dengue (Pardo-López et al. 2013). Some 3d-Cry toxins, such as Cry1Ab, Cry1Ac, and Cry1Fa, have been expressed in transgenic crops, such as maize, soybean, or cotton, reducing the use of chemical insecticides and increasing yields in some cases (Qaim and Zilberman 2003; Sanahuja et al. 2011). In 20 years (1996–2015), a total of two billion hectares were planted with Bt crops (James 2015). However, evolution of resistance to Cry toxins threatens the sustained use of Bt crops.

Defining the mode of action of 3d-Cry toxins will set the basis for a rational and sustained efficacy against insect pests. All 3d-Cry toxins are produced as protoxins that are activated by larval gut proteases to yield a protease-resistant core named as activated toxin and formed by three domains (Pardo-López et al. 2013). Two types of 3d-Cry protoxins have been identified, short protoxins of around 70 kDa and long protoxins of 130 kDa (de Maagd et al. 2001). In the case of the short protoxins, approximately 40 amino acids of the N-terminal end are removed during activation with proteases, while in the case of long protoxins, besides N-terminal processing, around 500 amino acids from the C-terminal end are processed to yield a 60 kDa activated toxin (de Maagd et al. 2001). It is generally accepted that 3d-Cry toxins exert toxicity by recognizing different larval gut cell proteins leading to membrane insertion forming nonselective pores that lyse cells by osmotic shock (Soberón et al. 2009).

Recently the three-dimensional structure of the complete Cry1Ac protoxin was solved, showing that the C-terminal region of the protoxin is structured in four different domains. Two of them resemble carbohydrate-binding modules (Evdokimov et al. 2014). Despite that there are multiple studies about the mechanism of action of Cry proteins, little attention has been paid to the putative role of these other domains present in Cry1A protoxin in the insecticidal activity of these important proteins.

Recent data suggest that the mode of action of Cry1A protoxins may differ from that of activated toxins since protoxins are also able to bind to insect receptor molecules resulting in pore formation activity that has several differences with the pore formation activity induced by activated toxin. Based on these data, a dual mode of action of Cry proteins was proposed (Gómez et al. 2014), suggesting that the two pre-pore structures may have differential roles in toxicity against selected targets, increasing their range of action. Also that insect may harbor different receptors, but also different midgut proteases that would influence the rate of protoxin/toxin activation. In support to the dual mode of action of Cry proteins, it was reported that several insect populations that have developed resistance to Cry proteins showed significantly lower resistance ratios to Cry1A protoxins compared to the activated toxins (Tabashnik et al. 2015). Here the authors will revise the mode of action of 3d-Cry1A activated toxins compared to what is known for Cry1A protoxins and discuss the implications of this dual mode of action for resistance pest management.

Three-Dimensional Structure of 3d-Cry Toxins and Protoxins

Cry toxins are classified based on their amino acid sequence identity (Crickmore et al. 2016). At least five different Cry families not related by sequence or structure have been characterized. The 3d-Cry toxin family is the biggest group of Cry toxins, and this group is the best characterized structurally since the three-dimensional structure of eight 3d-Cry activated toxins and two protoxins has been solved (Bravo et al. 2015; Crickmore et al. 2016; PDB numbers 1CIY for Cry1Aa, 4ARX for Cry1Ac, 1DLC for Cry3Aa, 1JI6 for Cry3Bb, 2C9K for Cry4Aa, 1 W99 for Cry4Ba, 4D8M for Cry5B, and 3 EB7 for Cry8Ea-http://www.pdb.org).

Regarding the activated toxins, the structures of Cry1Aa, Cry1Ac, Cry3Aa, Cry3Bb, Cry4Aa, Cry4Ba, Cry5Aa, and Cry8Ea have shown that, although most of these toxins share low amino acid sequence identity, they share a similar fold consisting of three domains. Figure 1a shows the three-dimensional structures of Cry1Aa, Cry1Ac, Cry3Aa, Cry3Bb, Cry4Aa, and Cry8Ea. The domain I of these toxins is a bundle composed of seven alpha helices that in several toxins has been shown to be involved in toxin oligomerization, membrane insertion, and pore formation. Domain II is a beta prism with exposed loop regions that corresponded to the less conserved structures, and in several toxins it was shown that these loops mediate interaction with different insect gut proteins. Finally, domain III is a beta sandwich; this domain is also involved in recognition of insect gut proteins (Pardo-López et al. 2013). In the case of Cry4Ba and Cry5B toxins, the crystal structure

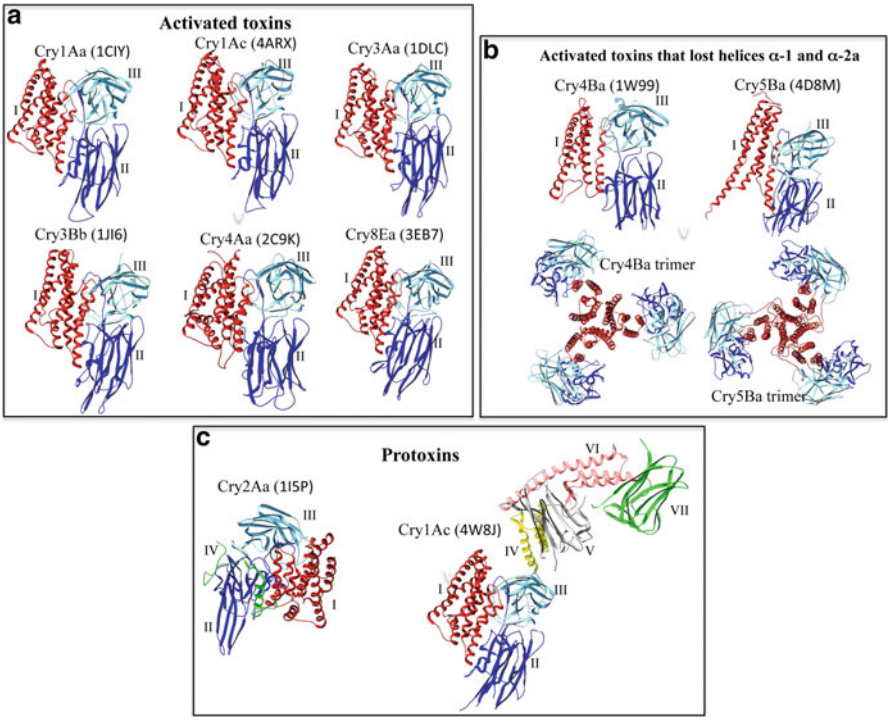

Fig. 1 Three-dimensional structure of Cry proteins. (Panel **a**) Three-dimensional structures of Cry1Aa, Cry1Ac, Cry3Aa, Cry3Bb, Cry4Aa, and Cry8Ea activated toxins. (Panel **b**) Crystal structures of Cry4Ba and Cry5B activated toxins where alpha helices 1 and 2a were cleaved resulting in a trimer array. (Panel **c**) Three-dimensional structures of Cry2A and Cry1Ac protoxins.

revealed that alpha helices 1 and 2a were removed during the crystallization process, resulting in a conformation change where truncated helix 2b and the loop connecting with helix 3 rearranged resulting in an extended helix 3 and a trimer array (Fig. 1b) (Boonserm et al. 2005; Hui et al. 2012).

In the case of protoxins, the three-dimensional structure of a short protoxin, Cry2Aa, and a long protoxin, Cry1Ac (Fig. 1c), was solved (PDB numbers 1I5P for Cry2Aa, 4W8J for Cry1Ac). The Cry2Aa protoxin showed an additional amino terminal end of 49 amino acid residues that are cleaved upon protease activation. This N-terminal region is structured in two helices. It was proposed that removal of this region is necessary for exposing a putative domain II region involved in receptor binding (Morse et al. 2001). Thus, activation of Cry2Aa is necessary for toxin binding to insect protein molecules (Morse et al. 2001). In the case of Cry1Ac protoxin, the structure corresponds to a Cry1Ac protoxin mutant lacking fourteen cysteine residues located in the protoxin domain. These mutations were introduced to avoid toxin aggregation (Evdokimov et al. 2014). The first 31 amino acids of the amino terminal end of Cry1Ac protoxin were not solved as they appeared disordered

(Evdokimov et al. 2014). Besides the 3d-Cry core structure of Cry1Ac, the C-terminal protoxin region was shown to be composed of four discrete domains (domains IV–VII), where domains IV and VI are composed of alpha helices and they resembled structural/interaction domains such as spectrin or fibrinogen-binding complement inhibitor. Domain V and VII are mainly composed of beta-strands and resemble carbohydrate-binding domains such as in sugar hydrolases (Fig. 1c; Evdokimov et al. 2014).

Mode of Action of 3d-Cry Toxins and Protoxins

It is generally accepted that 3d-Cry toxins exert toxicity due to their pore formation capacity (Soberón et al. 2009). However, an alternative mechanism involving activation of a signal transduction pathway by receptor binding was proposed (Zhang et al. 2006). It was proposed that interaction of 3d-Cry toxin with cadherin receptor induces activation of an intracellular death signal involving activation of protein G, adenylate cyclase, and protein kinase PKA. This model has been only studied in an insect cell line transfected with the cadherin gene from *Manduca sexta*. Mutants affected in cadherin binding would be affected in triggering this response (Zhang et al. 2006). However in the literature, there are multiple mutant toxins that still are able to bind to cadherin with similar affinity but lose completely the insecticidal activity indicating that further actions of the toxin besides cadherin binding may be important for in vivo toxicity (Pardo-López et al. 2013).

Nevertheless, both proposed mechanisms (pore formation or signal transduction) agreed that in order to exert toxicity, the 3d-Cry protoxins have to be activated by midgut proteases to yield the active toxin core of approximately 60 kDa in size, formed by the three-domain structure that binds toxin receptor molecules leading to midgut cell death (de Maagd et al. 2001; Pardo-López et al. 2013).

Different Cry-binding proteins have been characterized in different insect species (Pigott and Ellar 2007). Among these, aminopeptidase N (APN), alkaline phosphatases (ALP), cadherin, and ATP binding cassette Subfamily C member 2 (ABCC2) transporters have been shown to be involved in toxicity (Pigott and Ellar 2007; Pardo-López et al. 2013). Their participation as putative receptors is based on multiple data such as the isolation of Cry mutants affected in binding to some of these receptors that are severely affected in toxicity, the expression of these proteins in insect cell lines that renders cells sensitive to specific Cry toxins, or mutations in some of these proteins that resulted in insect resistant to Cry protein (Pigott and Ellar 2007; Pardo-López et al. 2013; Heckel 2015). The most complete model of the mode of action proposes that Cry toxins undergo a sequential binding mechanism with different insect gut proteins leading to toxin oligomerization, membrane insertion, and pore formation (Bravo et al. 2004). The first interaction of activated Cry1A toxins is binding to glycosylphosphatidylinositol (GPI)-anchored proteins such as APN or ALP. This first binding event is a low-affinity binding with the highly abundant APN or ALP molecules that approaches toxins to the microvilli membrane (Pacheco et al. 2009). Then Cry1A toxins may bind with high affinity to a less abundant cadherin protein. Binding to cadherin triggers toxin

oligomerization by facilitating removal of the N-terminal, helix 1, and part of helix 2a, forming a pre-pore oligomeric structure necessary for membrane insertion (Gómez et al. 2002). The pre-pore oligomer binds ALP or APN with higher affinity leading to membrane insertion and pore formation (Bravo et al. 2004; Gómez et al. 2006). In favor of this model, it was shown that modified Cry1A toxins (Cry1AMod) engineered to lack the N-terminal end up to domain I helix 1 formed oligomers in solution after protoxin activation with trypsin without cadherin binding and were toxic to a *Pectinophora gossypiella* Cry1Ac-resistant population whose resistance was shown to be linked to different cadherin mutant alleles (Soberón et al. 2007).

Other supporting data are the isolation of point mutations affecting Cry1Ab or Cry11Aa oligomerization that severely impact toxicity to lepidopteran or dipteran insects, respectively (Jiménez-Juárez et al. 2007; Muñoz-Garay et al. 2009), despite that the binding to cadherin was not affected (Jiménez-Juárez et al. 2007). Point mutations that affect pore formation activity without affecting binding to brush border membrane vesicles (BBMV) or to cadherin also support that pore formation activity of Cry toxin is important and binding of these toxins to receptors is not enough to trigger mortality in insects (Vachon et al. 2002; Girard et al. 2008; Rodríguez-Almazán et al. 2009).

The sequential interaction with different receptors is supported by the fact that reduced expression or loss of only one receptor molecule in insect populations resulted in development of resistance to Cry toxin action. For example, mutations in the cadherin gene have been liked to resistance in *Heliothis virescens*, *P. gossypiella*, and *Helicoverpa armigera* (Gahan et al. 2001; Morin et al. 2003; Xu et al. 2005; Fabrick et al. 2011). Similarly, mutations affecting the expression of receptors also are linked to a resistant phenotype such as the case of reduced expression of cadherin in *Diatraea saccharalis* (Yang et al. 2011) or reduced expression of APN1 and ALP in *Spodoptera exigua*, *H. armigera*, *H. virescens*, and *S. frugiperda* (Herrero et al. 2005; Zhang et al. 2009; Jurat-Fuentes et al. 2011). In addition, data from gene silencing have shown that RNAi of a single receptor molecule (cadherin, APN, or ALP) resulted in tolerance to Cry toxin intoxication (Gahan et al. 2001; Rajagopal et al. 2002; Morin et al. 2003; Xu et al. 2005; Herrero et al. 2005; Soberón et al. 2007; Zhang et al. 2009; Fabrick et al. 2011; Jurat-Fuentes et al. 2011; Yang et al. 2011; Flores-Escobar et al. 2013). All these data suggest that all receptors, not only cadherin, are important for Cry toxin action since the loss or reduced expression of a single receptor results in resistance or tolerance to Cry toxin action.

The fact that transfection of cell line *Trichoplusia ni* H5 with a single receptor cadherin induces susceptibility to Cry1Ab toxin was taken as evidence that only the interaction with this receptor is important to trigger insect death. However, some insect cell lines such as *T. ni* H5 and SF9 are naturally sensitive to other 3d-Cry toxins such as Cry1Ac that is toxic to *T. ni* H5 cells and Cry1C that is toxic to SF9 (Kwa et al. 1998; Liu et al. 2004), implying that participation of other toxin-binding molecules could not be excluded and they might be participating along with the transfected cadherin receptor to induce Cry1Ab susceptibility. In addition, the

transfection with cadherin did not always result in susceptibility to Cry toxins, for example, in the case of DmS2 cells transfected with the cadherin gene from *M. sexta*, only 12–14% of the successful-transfected cells were susceptible to Cry1A toxin action (Hua et al. 2004; Jurat-Fuentes and Adang 2006), indicating that other factors in these cells may be present to induce full toxicity of Cry toxins.

Finally, another evidence that supports the sequential model is that pore formation activity of activated Cry toxin is increased significantly in the presence of different receptors, such as APN that increased up to 250-fold the pore activity of Cry1A toxins in planar lipid bilayer (Schwartz et al. 1997) or a mixture of APN and ALP that increased 1000-fold the Cry1A-induced release of $^{86}Rb^{+}$ from the vesicles (Sangadala et al. 1994) or BBMV containing toxin receptors that improves 2–40-fold the pore formation activity and conductances of the Cry toxins (Lorence et al. 1995; Martin and Wolfersberger 1995; Peyronnet et al. 2001).

The hypothesis that Cry protoxins need to be processed by gut proteases in order to bind their receptors was challenged when it was shown that Cry1Ac protoxin binds cadherin from *P. gossypiella* (Fabrick and Tabashnik 2007). A follow-up study in *M. sexta* showed that Cry1Ab protoxin bound cadherin with similar affinity as the activated toxin (Gómez et al. 2014). Analysis of oligomerization and pore formation after Cry1Ab protoxin or toxin after binding to cadherin in the presence of midgut proteases revealed that two distinct pre-pores formed. Both oligomers are composed of 60 kDa monomers (Gómez et al. 2014), but important differences were observed; the pre-pore oligomer formed after protoxin binding to cadherin was more heat stable, was readily inserted into synthetic membrane vesicles, and formed pores with high open probability in black lipid bilayers. In contrast, the pre-pore oligomer formed from activated toxin was more heat sensitive, did not insert promptly into synthetic lipids, and formed unstable pores with different conductances (Gómez et al. 2014; Fig. 2). Both pre-pores inserted readily into *M. sexta* brush border membrane vesicles. These results suggested that both pre-pores could be involved in pore formation and toxicity (Gómez et al. 2014).

Evidence that both pre-pores are involved in toxicity came from the characterization of Cry1AbMod protein. As mentioned above, Cry1AbMod or Cry1AcMod could counter Cry1Ac resistance in *P. gossypiella* linked to cadherin mutations (Soberón et al. 2007). Also, Cry1AMod toxins could counter resistance linked to other resistance mechanisms as resistance linked to ABCC2 mutations in *Plutella xylostella*, *T. ni*, or *H. virescens* (Tabashnik et al. 2011). However, it was shown that in most lepidopteran species analyzed, the Cry1AbMod or Cry1AcMod showed lower potency to susceptible insects than native toxins and that the loss in potency is different among the insect species analyzed (Tabashnik et al. 2011). The only example where Cry1AbMod or Cry1AcMod showed higher potency than native toxins to susceptible populations was against *S. frugiperda* (Monnerat et al. 2015). Analysis of Cry1AbMod oligomerization in *M. sexta* BBMV showed that Cry1AbMod readily formed oligomers when protoxin was used, but it was much less efficient in oligomerization from activated toxin (Gómez et al. 2014). Interestingly, the toxicity to *M. sexta* larvae of Cry1AbMod activated toxin was eightfold lower compared to Cry1Ab activated toxin, but toxicity of Cry1Ab and Cry1AbMod

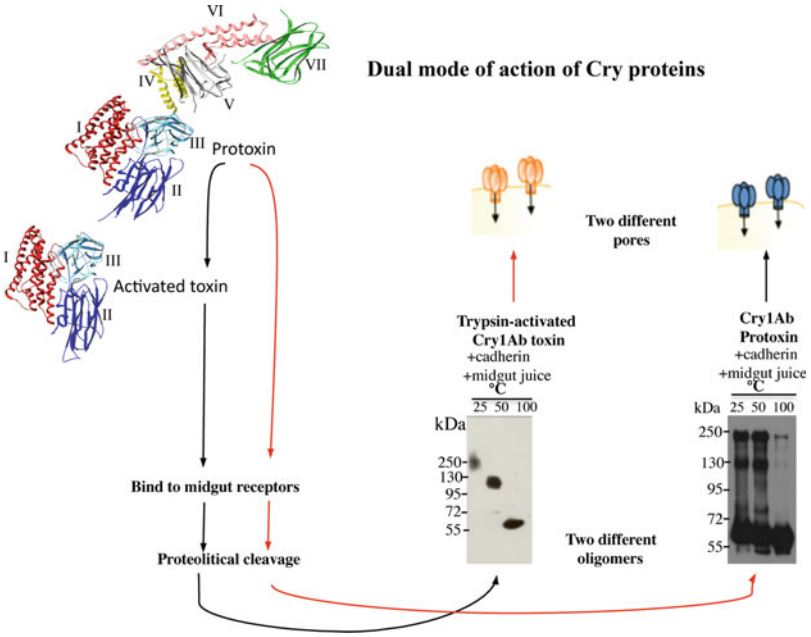

Fig. 2 Proposed model of the dual mode of action of Cry proteins. Protoxins (130 kDa) could be activated by proteases resulting in activated toxin; both molecules are able to bind to receptors located in midgut cells; after binding and extra cleavage by proteases, two different oligomeric structures are formed which showed differences in size, resistance to temperature, and capacity of insertion into synthetic membranes. The oligomeric proteins interact with receptors and insert into the membrane forming two different pores, which showed differences in open probability and conductance states (Gómez et al. 2014)

protoxins was similar (Gómez et al. 2014). These results show that both pre-pore oligomers are involved in toxicity. The differential role of both pathways in toxicity in different insect species could be related to the rate of Cry1A protoxin activation into toxin that is related to the protease composition of their midgut juice. Figure 2 shows the proposed model of the dual mode of action of Cry1A toxins.

In Vivo Evidence for the Dual Mode of Action of 3d-Cry Toxins

The major threat to the sustained use of Bt crops for pest control is evolution of insect resistance (Tabashnik et al. 2013). If Cry protoxins are inactive proteins that only become active after cleavage by proteases, it implies that in Cry1A-resistant insects, the resistance ratio to activated toxin would be similar or indeed twofold lower than resistant ratio to protoxins, since protoxins have twice the molecular weight compared to activated toxins. Comparison of the resistance ratios of seven Cry1Ac-resistant strains from three different insect species (*D. saccharalis*, *H. armigera*, and *H. zea*) showed that the resistance to activated Cry1Ab or

Cry1Ac was significantly higher to that observed with the same protoxins (Tabashnik et al. 2015). The average reduction in resistance ratio for protoxins of all comparisons was tenfold (Tabashnik et al. 2015). In addition, in *Ostrinia nubilalis* the resistance ratio for two different resistant populations was higher for activated Cry1Ab toxin compared with the resistance ratio to Cry1Ab protoxin (Siqueira et al. 2004). Overall the results show that in four different insect species, Cry1A protoxins are significantly more toxic to Cry1A-resistant populations supporting the dual model of mode of action of Cry toxins.

In the case of *H. armigera* population SCD-R1, Cry1Ac resistance is linked to a single cadherin gene allele showing 500-fold resistance to Cry1Ac activated toxin but only 30-fold resistance to Cry1Ac protoxin (Tabashnik et al. 2015). SCD-R1 strain was isolated by backcrossing individual moths that were collected in Bt cotton fields and selection for Cry1Ac resistance in the laboratory (Yang et al. 2009). Bt cotton planted in the region where moths were collected expresses *cry1Ac* gene corresponding to the activated toxin, not the full-length *cry1Ac* protoxin gene. The fact that SCD-R1 resistance to Cry1Ac protoxin is still 30-fold suggests that Cry1Ac activated toxin and Cry1Ac protoxin partially share cadherin as a functional receptor but that Cry1Ac protoxin could bind to a different receptor molecule to exert toxicity. Interestingly, a different *H. armigera* Cry1Ac-resistant population (LF60) selected using Cry1Ac protoxin under laboratory conditions is linked to a mutant gene allele of a different Cry1Ac receptor, ABCC2 (Xiao et al. 2014). Interestingly the resistance ratio of LF60 for activated Cry1Ac or protoxin was similar (1100–1400-fold, respectively) (Xiao et al. 2014). These results suggest that Cry1Ac activated toxin or protoxins rely equally on ABCC2 for toxicity and that cadherin and ABCC2 have a differential role on toxicity for activated toxin or Cry1Ac protoxin.

However, this is not a trend in all species since, for instance, the two *O. nubilalis* Cry1Ab-resistant strains were selected with Cry1Ab protoxin, but the resistance levels were substantially higher with activated toxin than with protoxin (Siqueira et al. 2004). Additional evidence characterizing more resistant colonies with different resistant mechanisms is necessary to identify the insect molecules involved in Cry1A protoxin and activated toxin mode of action.

Implications of the Dual Mode of Action for Sustained Use of Bt Crops

The evolution of two distinct modes of action by 3d-Cry toxins is likely to delay evolution of resistance in susceptible insects. Most Bt plants were engineered to express activated Cry toxins in order to avoid potential resistant mechanisms related to inefficient conversion of protoxins to activated toxins. However, this strategy avoids the mechanism of protoxins that could be important for delaying resistance. In *H. zea* it was shown that resistance to Cry1Ac evolved more rapidly to activated toxin than to protoxin (Anilkumar et al. 2008). Although there are different cultivars that express either activated toxins or protoxins, there are few examples of direct comparison of plants expressing both forms of toxins. In one such comparison,

Cry1Ac full-length protoxin or activated toxin was expressed in tomato plants, and expression of protein and toxicity against *H. armigera* was evaluated in the transgenic plants (Koul et al. 2015). The results show that plants expressing the Cry1Ac full-length protoxin were more effective in controlling larvae compared to plants expressing the truncated Cry1Ac gene corresponding to the activated form of the protein, although the expression levels of the Cry1Ac protoxin were less than those found in plants expressing the truncated Cry1Ac gene (Koul et al. 2015). This suggests that plant proteases could hamper the effective expression of full-length Cry protoxins. This could be overcome by expressing full-length toxin genes by an inducible promoter that will express the gene upon damage by insect feeding. Transgenic crops expressing short Cry protoxins have also been planted commercially like cotton plants expressing Cry2Ab or corn plants expressing Cry3Aa or Cry3Bb variants (James 2015). Whether short protoxins have also two pathways for toxicity still remains to be analyzed to determine if expression of protoxins vs. toxins could give advantages for delaying resistance or for increasing toxicity. *H. armigera* Cry2Ab-resistant or *Diabrotica* sp.-resistant Cry3Bb populations have been isolated and characterized (Tay et al. 2015; Gassman et al. 2013). It will be important to determine if there are any differences in susceptibility of these populations to protoxins compared to activated toxins.

Conclusion and Future Directions

Bt 3d-Cry toxins are produced as protoxins that are activated by insect gut proteases to yield an active three-domain toxin. Either Cry1A activated toxin or protoxin binds to cadherin receptor forming distinct oligomers that insert into the membrane forming lytic pores. The consequence of this dual mode of action is the reduction of potential evolution of resistance and possibly to broaden the spectrum of insect targets. For the sustained use of Bt crops, it is likely that achieving a stable expression of Cry full-length proteins will have the same consequences, delaying resistance and protection from a wider number of insect pests.

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Part II

Burkholderia Toxins

Burkholderia pseudomallei Toxins and Clinical Implications

3

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Abstract

Burkholderia pseudomallei is the causal agent of melioidosis. In spite of ongoing studies, the molecular mechanisms underlying toxin-induced pathogenesis of this bacterium are not clearly elucidated for this potential biological warfare pathogen. In this review, we highlight current information of *B. pseudomallei* toxins and their roles in pathophysiological effects in various experimental models. Several secretary proteins/lethal factors show lethal toxicity to cells in culture via filtrates of *B. pseudomallei* culture. These toxins are released in culture from

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strains isolated from soil, animals and humans. Toxins are also found in infected patients, which strongly correlate with severity of melioidosis. Melioidosis progression begins with an environmental reservoir and bacterial attachment in the host, invasion of epithelial/macrophage cells and subsequent intercellular spread. The molecular and cellular basis of pathogenesis in melioidosis will provide a better, rational understanding toward design and development of new drugs with novel mechanisms of action.

Keywords
Melioidosis • Soil pathogen • Lethal factors • Exotoxins

Contents

Introduction

The gram-negative bacterium *Burkholderia pseudomallei* causes melioidosis and has become a serious public health issue with potential bioterrorism implications worldwide (Gilad 2007). This is an emerging disease in Vietnam (Leelarasamee 2000), Northern Thailand (Waiwarawooth et al. 2008), Singapore, and Malaysia. In

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Northern Australia, 46% of severe cases become bacteremic, and 19% of these patients died (Currie et al. 2000). The first cases of pulmonary melioidosis have also been reported in Southern Cambodia (Overtoom et al. 2008), the Western Province of Papua New Guinea with most cases being in children (Warner et al. 2008), New Caledonia (Le Hello et al. 2005), as well as various Indian states (Whitmore and Krishnaswami 1912; Saravu et al. 2008). Recently, there were reported cases of acute and travel-related fatal melioidosis (Morosini et al. 2013). Not only *B. pseudomallei* but also other species such as *Burkholderia cepacia* are an important threat to patients in North America, as well as Europe (Hauser et al. 2001).

These bacteria are opportunistic pathogens in patients with cystic fibrosis or chronic granulomatous disease (Whitby et al. 2006). This bacterium is resistant to many antibiotics, and there is cross infection involving patient-to-patient transmission, especially nosocomially (Hunt et al. 2004). Almost 20% of colonized patients have acute necrotizing pneumonia and septicemia (Kitt et al. 2016). Pneumonia is one of the most frequent clinical presentations of melioidosis, especially acute, subacute, as well as chronic forms of pneumonia due to *B. pseudomallei* infection (Currie 2003). A recent clinical study also clearly shows that consolidated with cavitary lesions, hepatosplenomegaly and sputum analysis confirm the presence of *B. pseudomallei*. These melioidosis patients were also completely treated for primary pneumonia (Afroze et al. 2016). Other complications, such as coinfection of pulmonary tuberculosis (TB) in a diabetic condition, have also been reported (Sulaiman et al. 2013). However, approximately 60–80% of melioidosis patients possess high risk factors for this infection along with diabetes mellitus, chronic pulmonary infection, and heavy alcohol use (Wiersinga et al. 2012). Melioidosis and TB can be associated diseases, which have been reported in a neck abscess (Shenoy et al. 2009) and lung mass (Truong et al. 2015). Such complicated cases of neck melioidosis, defined as a parapharyngeal abscess, are treated by incision, drainage, and intravenous combination of antibiotics for 6 weeks along with oral antibiotic administration (Zulkiflee et al. 2008). In addition, *B. pseudomallei* has also been isolated from the patient's blood and pus of a left gluteal abscess in an imported case (Pelerito et al. 2016). Nearly 34 confirmed human, and 3 animal, cases have been reported from the USA/Puerto Rico during 2008–2013 that had histories of travel into areas endemic for melioidosis (Benoit et al. 2015).

Naturally, these bacteria can survive in the environment for more than a year. During nitrogen/amino acid starvation, these bacteria can use sigma factors such as RNA polymerase nitrogen-sigma factor (RpoS)/RNA and polymerase nitrogen-limitation (RpoN) to modulate gene expression for their adaptation and survival (Diep et al. 2015). In addition, this bacterium can survive in host cells by escaping reactive oxygen species (ROS) through the regulation of stress-responsive sigma factors (RpoS) (Chutoam et al. 2013). However, sigma factors E (σE) play an important role in regulating extra-cytoplasmic stress responses in various gram-negative bacteria (Daimon et al. 2015). Many bacterial toxins are proteins, encoded by the bacterial chromosomal genes, plasmids, or even phages (Lubran 1988). In addition, a variety of bacterial secretion systems have been characterized to date, and they are known as types I–VII (Kimelman et al. 2012). These toxins, when delivered

by these secretion systems, can locally damage the host at the site of bacterial infection (Henkel et al. 2010).

Various microbial or bacterial toxins are important virulence factors that induce pathological changes to hosts, causing severe diseases (Martin 2012). Bacterial toxins from all types of bacteria can cause sepsis-related mortality globally, for instance, in the USA alone there was an incidence rate of 240 per 100,000 people in 2013 (Martin 2012). However, *B. pseudomallei* also causes sepsis and leads to an uncontrolled inflammatory response by the host cells, which results in multiple organ failure and death (Morgan et al. 2016). Endotoxins are derived from the outer cell membrane of gram-negative bacteria (Michael and Silverman 1998). In particular, lipopolysaccharide (LPS) or endotoxin contains lipid A (core molecule) that induces diverse clinical-pathological effects in humans. These cell wall components are also responsible for severe cellular toxicity (Brown et al. 2015), and higher doses (6–25 ng/kg) of these toxins elicit fatal shock to humans, as well as other mammals. Generally, LPS produces high fever in the host via release of interleukin-1 (IL-1) and other mediators that cause a systemic inflammatory response, leading to cell death and organ dysfunction (Silverman and Ostro 1999).

Some pathogens secrete exotoxins containing enzymatic activity that is implicated in the necrosis of tissues (proteases, lipases, lecithinases, catalases, and hemolysins). These exotoxins can also be polypeptides devoid of enzymatic activity, such as those produced by *Staphylococcus aureus* that include toxic shock syndrome toxin-1, staphylococcal enterotoxins, and leukocidin (Martin et al. 2004). These protein toxins bind to specific receptors on cells and are also fatal to hosts, even in very small doses (Brown et al. 2015). Commonly, the membrane-perforating bacterial toxins are known as pore-forming toxins (Gurnev and Nestorovich 2014). However, small peptides such as alpha-hemolysin of *S. aureus* form channels that cause lesions on cellular membranes that simply punch holes in membranes. Currently, there are no enzymatic subunits that enter a cell (Yan et al. 2013). *Burkholderia pseudomallei* lethal factor 1 (BPSL1549) was the first discovered toxin from this bacterium, using X-ray crystallography studies of hypothetical proteins (Cruz-Migoni et al. 2011). Later, BPSL1549 was renamed as *Burkholderia* lethal factor 1 (BLF1). A recombinant version of BLF1 is toxic and kills mice (challenged intraperitoneally) and cultured macrophages (Hautbergue 2012). Further characterization of BLF1 has revealed its deadly molecular mechanism of action in human cells, identifying molecules that may prevent the modification of eukaryotic initiation factor 4A (Eif4A) by BLF1. The elevated levels of BLF1 promote or inhibit pathogenesis. However, BLF1 promotes deamidation of glutamine-339 (Gln339) of the translation initiation factor Eif4A, completely abolishing its helicase action and preventing translation. In addition, inactivation of BLF1 by various means might lead to an interesting vaccine candidate (Guillaume et al. 2009). *B. pseudomallei* has been isolated from various sources, yet bacteria isolated from melioidosis patients release higher levels of endotoxin in vitro compared to those isolated from other sources such as soil/animals (Chen et al. 2015). Collectively, these *B. pseudomallei* toxins contribute to poor clinical outcomes. The current review will discuss *B. pseudomallei* toxins and their clinical implications at a cellular, and molecular level of melioidosis.

Severity of Potential Soil Pathogens

The soil pathogen, *B. pseudomallei*, causes severe diseases such as melioidosis, which is endemic to Southeast Asia and other parts of the world. This pathogen is isolated from soil of agricultural lands; and also, the prevalence of *B. pseudomallei*-specific antibodies in humans increases significantly among residents affected by a typhoon/flood incident (Chen et al. 2015). *B. pseudomallei* has been isolated from seroma using sheep blood agar (Zong et al. 2012). Recent serology studies support the presence of melioidosis in Myanmar (Wuthiekanun et al. 2006), from where the original case of melioidosis was first recognized in 1911 (Whitmore and Krishnaswami 1912). All empyema aspirates of melioidosis patients test positive for arginine dihydrolase, and serological testing reveals a minimal 1:2560 antibody titer against *B. pseudomallei* (Tsang and Lai 2001). Following diagnosis, the patient's symptoms improve after treatment with high doses of ceftazidime (Lee et al. 2005). The bacterium is typically sensitive to co-trimoxazole, Augmentin, ceftazidime, cefoperazone, ciprofloxacin, chloramphenicol, and imipenem, while being resistant to aminoglycosides (Goldberg and Bishara 2012).

The most important routes for melioidosis infection are via aerosol, ingestion, or cutaneous inoculation via muddy water or wet soil. As a result, infection severely affects the main organs such as the liver, spleen, kidney, prostate, and brain, leading to bacteremia and septicemia, which elicits larger numbers of pro-inflammatory cytokines that trigger oxidative stress as well as cell death (Fig. 1). Thus, the bacterium invades cells via endocytic vesicles, as the bacterium attaches onto the host cell wall, disrupts the membrane, escapes into the cytosol, and finally travels to adjacent cells via cell-to-cell membrane contact. Bacteria can release endotoxin from the cell wall components (O-antigen/LPS), which promote secretion of interferon gamma that is linked to cellular signaling molecules of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway.

Clinical Implications of Bacterial Exotoxin

Until now, there are no vaccines available for the treatment of clinically relevant, multidrug-resistant (MDR) *B. pseudomallei*. Recent structural studies of a cytolethal exotoxin (CLT) demonstrate the molecular mechanism underlying the function of this *B. pseudomallei* protein. The toxin induces a more severe form of melioidosis (Haase et al. 1997). In particular, the exotoxin is one of the most important agents responsible for septicemic melioidosis in humans. The molecular weight of CLT is 10 kDa, as purified from the culture filtrates of *B. pseudomallei* grown in vitro. Bacterial isolates from soil, animals, and humans possess differential cytotoxic effects in in vitro systems. The toxic effects of culture filtrates include lethality and hemorrhagic dermonecrosis in mice (Haase et al. 1997). Additional studies show that two other *B. pseudomallei* proteins (31 and 35 kDa) possess proteolytic, as well as toxic, activities (Mohamed et al. 1989). Furthermore, BLF-1 (23 kDa) is responsible for inhibiting host protein synthesis during melioidosis (Ahmad et al. 2015), which is similar to that of the catalytic domain of *Escherichia coli* cytotoxic necrotizing factor

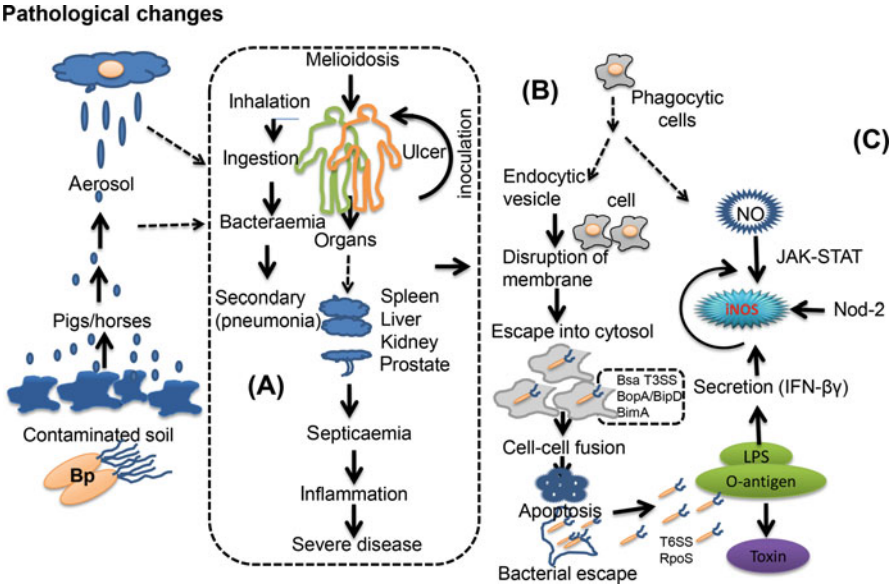

Fig. 1 (**a**–**c**) Muddy water and soil contaminated with *Burkholderia pseudomallei* in an endemic region transmit melioidosis by aerosol or inhalation, ingestion, inoculation, and human-to-human contact. This disease severely affects various organs such as the liver, spleen, kidney, prostate, and brain, leading to bacteremia/septicemia and increased pro-inflammatory cytokines that trigger oxidative stress as well as cell death. Bacteria enter phagocytes via endocytic vesicles, disrupt the membrane, and escape from the cytosol by cell-to-cell fusion. Bacteria also release toxin from their cell wall components (O-antigen/LPS), eliciting secretion of interferon gamma linked to cellular signaling molecules of JAK-STAT through Nod2

1 (CNF1-C). This further highlights that BLF-1 serves as a potent cytotoxin to not only eukaryotic cells but is also lethal to mice and promotes pathogenesis.

B. thailandensis (E264) also possesses contact-dependent growth inhibition (CDI) systems similar to the *B. pseudomallei* (strain 1026b), which modulates CDI-facilitated delivery of CdiA-CT toxins derived from other strains (Nikolakakis et al. 2012). This CDI system encodes CdiI immunity proteins that specifically bind to CdiA-CT, neutralize its toxin activity, and, as a result, protect CDI-positive cells from auto-inhibition. In addition, these variations in CDI toxin, as well as immunity proteins, reveal that these systems play an important role in bacterial self and nonself-recognition in microbial communities (Willett et al. 2015). The toxin-antitoxin (TA) system is commonly distributed in bacteria and linked to the formation of antibiotic-tolerant cells involved in chronic diseases (Daimon et al. 2015). A previous study shows that overexpression of *B. pseudomallei* HicA toxin (13 kDa) retards bacterial growth and generates cells tolerant to antibiotics such as ciprofloxacin/ceftazidime (Butt et al. 2014). These toxins are more pathogenic and responsible for severe disease.

Structure Activity Relationship of Bacterial Toxins

Structure analysis indicates that the HicA(a) toxin, HicA (H24A), is a mutant with histidine to alanine change at position 24. The protein consists of 95–135 amino acid (AA) residues. The N-/C-terminal AA residues and secondary structure reveal that the HicA (H24A) conserved side chains help form a hydrophobic core containing Val36, Val38, and Phe27, with charge distribution on the surface of HicA (H24A) (Fig. 2a, b). The active site consists of strongly conserved residues and includes cysteine, histidine, and multiple hydrogen bonds (Fig. 3a, b). However, the gene fragment containing amino acids 95–135 encodes a biologically active toxin that interacts with diverse cellular components like RNA, ribosomes, and DNA, which subsequently leads to killing of host cells (Yamaguchi et al. 2011). Earlier studies also show the toxin-antitoxin (TA) systems consisting of five different types (I–V), based on the gene products (Schuster and Bertram 2013). However, this antitoxin (RNA) or protein binds to the toxin and blocks its activity (Hayes 2003).

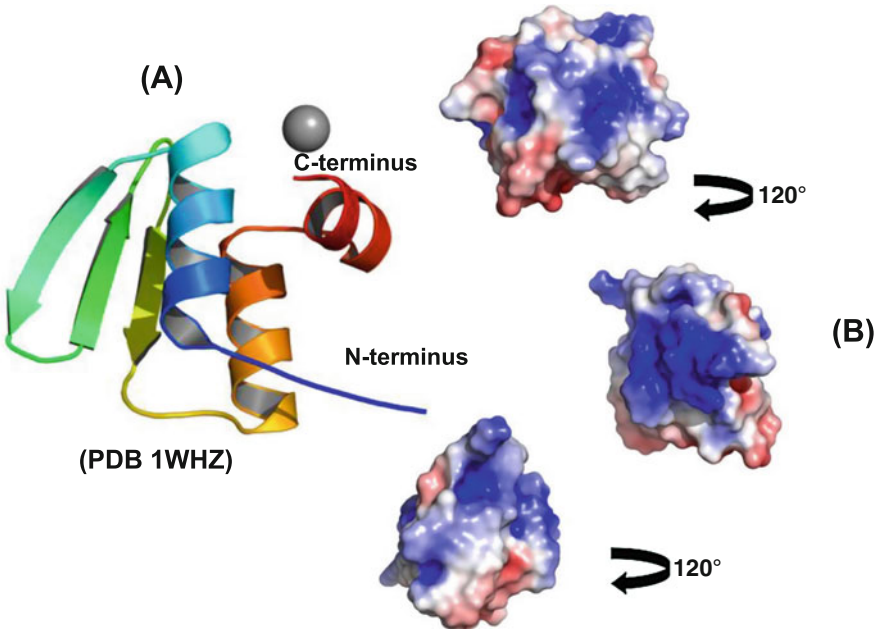

Fig. 2 Structure analysis of HicA(a) toxin. (**A**–**B**) The N-/C-terminal and secondary structure demonstrate that the HicA (H24A) conserved side chains contribute to the hydrophobic core containing Val36, Val38, and Phe27, with charge distribution on the surface of HicA (H24A) (PDB code 1 WHZ)

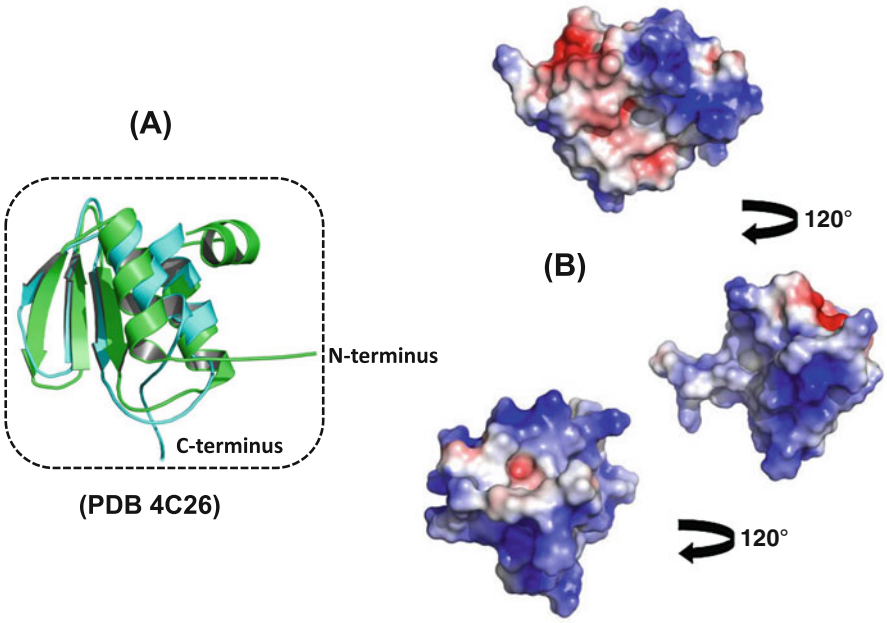

Fig. 3 (**A**) X-ray crystal studies clearly indicated that HicA bacterial toxin from *Burkholderia pseudomallei* plays an important role in persister cell formation and toxicity. (**B**) Crystal structure of the HicA toxin from *B. pseudomallei* responsible for persister cell formation (Butt et al. 2014)

Effect of Exotoxin (CLT) on Pathogenicity

Culture and Infection of Invasive Rodent Models

Toxic substances are produced by various bacteria that cause deleterious effects to targeted host cells as well as promote bacterial proliferation (Ashida et al. 2014). In the case of CLT, cell death is confirmed by formation of a pore in the endosomal membrane, which facilitates *B. pseudomallei* escape into the cytosol (Dubail et al. 2000). Murine melioidosis models of acute (BALB/c, Th2 phenotype) and chronic (C57BL/6, Th1 phenotype) infections mimic the disease stages in humans (Lazar Adler et al. 2009; Leakey et al. 1998). Infected C57BL/6 mice exhibit an early influx of neutrophils, followed by better activation and clearance of *B. pseudomallei* with moderate pro-inflammatory cytokine secretion contributing to chronic melioidosis (Ulett et al. 2000). Whereas, infected BALB/c mice lead excessive inflammation (Gan 2005) and exhibit increased levels of pro-inflammatory cytokines such as IL-6, IL-12, IL-15, IL-18, TNF-α, and IFN-γ at 24–48 h, with reduced macrophage/lymphocyte recruitment and activation, contributing to the development of acute disease (Leakey et al. 1998; Wiersinga et al. 2007). *B. pseudomallei* is an important biological warfare agent because it poses a threat to humans (Aquino and Wu 2011).

This species is the main causal agent of melioidosis, causing deadly sepsis in various areas (Williams et al. 2015). Currently, there is much mortality due to higher rates of relapse and severe inflammatory responses (Currie et al. 2000). Inflammation is an important biological response of the human body to harmful stimuli, such as bacterial pathogens or damaged cells. As a result of inflammation, a protective host response is initiated by various mediators such as immune cells, blood vessels, and specific cellular mediators. Inflammation also eliminates cell injury, necrotic cells, and damaged tissues from the injury site and initiates tissue repair mechanisms.

Several rodent models have been developed for the study of melioidosis. BALB/c mice are highly susceptible to *B. pseudomallei* by the aerosol route of infection in an acute model, with higher bacterial counts in the lungs/spleen (1×10^3–1×10^5 per gram of tissue versus other organs). Focal points of acute inflammation and severe necrosis are in the lungs, liver, and spleen (Lever et al. 2009). The murine aerosol model is well established and most suitable for not only *B. pseudomallei* infection but also other respiratory pathogen infections. Such murine models can also be useful for identifying biomarkers that may lead to effective treatments (Massey et al. 2014). Besides mice, marmosets can be used as a model for melioidosis. When the latter are challenged with different strains of *B. pseudomallei*, there ensues a severe acute disease with mild dissimilarity of the time of death and pathologic appearance versus mice. Fevers with bacteremia, bacterial dissemination, necrotizing hepatitis, splenitis, and pneumonia are common observations (Nelson et al. 2015). In our study, more foci were found in the lung of challenged animals versus the liver and spleen (unpublished work). Viable bacteria are found in the liver, spleen, and kidney that rapidly increase during 24 and 48 h postinfection, with the number of viable bacteria drastically increasing to a peak of 1×10^5 cfu/ml in 48 h (Fig. 4a–f).

Histologic analysis demonstrates acute necrotizing alveolitis and pneumonia in the lungs of mice, 24 h after exposure to *B. pseudomallei,* particularly in alveolar spaces and walls that are infiltrated by neutrophils/macrophages. Larger foci of consolidation exist, consisting of enlarged macrophages predominating in some bronchi lesions and thickening of alveoli walls, 48 h postinfection. Infected mouse lung sections show diffuse inflammatory infiltrates, including neutrophils and macrophages. Furthermore, lobes are filled with cellular debris, while proteinaceous fluid and inflammatory cells are found in airways/bronchial lumen on day 3 postinfection. In addition, there are small lesions containing infected macrophages and large lesions surrounded by noninfected neutrophils.

Several studies prove that human macrophages and neutrophils play a vital role in preventing infection by highly virulent strains of *B. pseudomallei* versus less virulent strains (Massey et al. 2014). Capsular polysaccharide influences the deposition of critical complement component C3, which essentially controls this bacterium through nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase stimulation by human neutrophils (Woodman et al. 2012). Polymorphonuclear neutrophils (PMNs) are also implicated in the pathogenesis of melioidosis, which includes formation of weblike structures called neutrophil extracellular traps (NETs); however, NETs killing of *B. pseudomallei* until now has been rather ambiguous (Riyapa

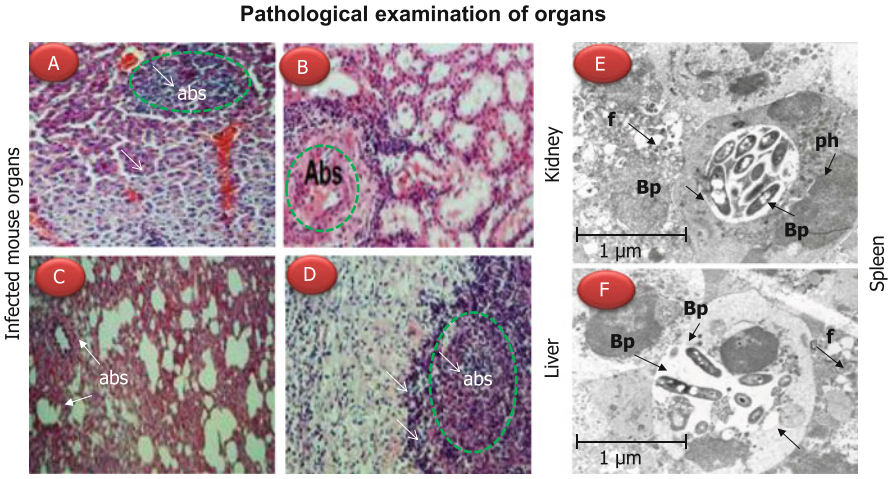

Fig. 4 Light micrograph showing *Burkholderia pseudomallei* infection in mouse spleen, liver, and kidney sections stained by hematoxylin and eosin, magnification 10 & ×20. (**A**–**D**). Large abscesses (*Abs*) with focal areas of necrosis, surrounded by a rim of meshed fibrous tissue, are evident after 2-week intraperitoneal (i.p.) challenge with 1.7×10^5 CFU/ml. The bacterial invasion is more pronounced in the spleen and liver than the kidney. (**E**–**F**) Transmission electron microscopic examination of BALB/c mouse spleen infected with *B. pseudomallei* 2 weeks following i.p. challenge with 1.7×10^5 CFU/ml. Large abscesses (*Bp*) with focal areas of cell, surrounded by a rim of meshed fibrous tissue, are evident. Abbreviations: *Bp B. pseudomallei, n* nucleus, *ph* phagocytosis, *abs* abscess, *f* fibrous tissue

et al. 2012). However, these NETs effectively trigger innate activation of plasmacytoid dendritic cells (pDCs) through Toll-like receptor-9 (TLR-9). The pDCs are potent producers of type I interferons (IFN), and a recent study has investigated whether the pDCs and type I IFN play a role during the early stages of *B. pseudomallei* infection (Williams et al. 2015). Virus-induced transient immunosuppressed C57BL/6 mice, infected with a low dose of biofilm-defective mutant (M10) of *B. pseudomallei*, relapse with severe inflammation (Panomket et al. 2016). Glycogen synthase kinase-3β (GSK3β) serves a vital role during an innate inflammatory response caused by bacterial pathogens. The effect of lithium chloride (LiCI), a GSK3β inhibitor, in an experimental murine model of acute melioidosis reveals improved survival of infected mice. These animals have elevated levels of anti-inflammatory cytokines such as IL-10/IL-1Ra in the sera as well as organs, while pro-inflammatory cytokines such as IL-1β, TNF-α, and IFN-γ are greatly reduced by LiCI (Tay et al. 2012). In this study, *B. pseudomallei*-infected peripheral blood mononuclear cells (PBMC), as well as infected diabetic/normal rats, generate raised levels of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-10, IL-12, and IL-18. This occurs via the NOD-like receptor nucleotide-binding domain, leucine-rich pyrin domain-containing-3 (NLRP3), or Nod-like receptor protein-3/pyroptosis through NLRC4 (Bast et al. 2014; Abderrazak et al. 2015) and phosphorylation of NF-kB in certain cells (Maniam et al. 2015). However, the blocking of dysregulated

GSK3β in PBMC from diabetic animals leads to inactivation of NF-kB and modulation of inflammatory cytokines.

Several gram-negative bacterial pathogens have secretion systems that inject structural proteins (flagellin) into the host cytosol, leading to caspase-1 activation/pyroptotic cell death (Abderrazak et al. 2015). A previous study shows that *B. pseudomallei* factors trigger caspase-1 activation, downstream signaling pathways, and effector mechanisms of caspase-1. Furthermore, the type 3 secretion system (T3SS3) modulated-NLRC4, caspase-1 activation, pyroptosis, and caspase-1-dependent/-independent cell death mechanisms contribute to the pathogenesis of *B. pseudomallei* infection of macrophages (Bast et al. 2014).

Effect of Exotoxin (CLT) on Virulence

The bacterial virulence factors playing an important role during an acute infection were determined using gene expression profiles in the spleen, lung, and liver of BALB/c (Th2 phenotype) and C57BL/6 (Th1 phenotype) mice via DNA microarrays. This analysis identified BPSS1521 (*bprD*), a predicted transcriptional regulator located in the type III secretion system (T3SS-3) operon, to be upregulated and specifically so in C57BL/6 mice. Whereas, BALB/c mice infected with a *bprD* mutant (a knockout) also resulted in death but in a shorter time and with more inflammation, as determined by histopathological analysis/enumeration of bacteria in the spleen. A large number of multinucleated giant cells (MNGCs), a hallmark of human melioidosis, were detected in animals infected with either wild-type or *bprD* mutants. One striking observation was the increased expression of BPSS1520 (*bprC*), located downstream of *bprD*, in the *bprD* mutant. BprC is a regulator of T6SS-1 that is required for the virulence of *B. pseudomallei* in murine infection models. Deletion of *bprD* led to the overexpression of *bprC* and a decreased time to death. The *bprD* expression was elevated in C57BL/6 mice, as compared to BALB/c, which suggests a role for BprD in the natural resistance of C57BL/6 mice to *B. pseudomallei*.

The negative regulation of *bprC* by BprD sheds further light on the complexity of regulation between T3SS-3 and T6SS-1, suggesting further investigation of suppression upon T6SS-1, as the latter is considered one of the most important *B. pseudomallei* virulence factors (Stevens et al. 2005). The actin-based motility of *B. pseudomallei* involves a distinctive mechanism of activation. For example, *bimA* homologs in *B. mallei* and *B. thailandensis* induce actin-based motility in J774.2 cells (Stevens et al. 2005). Another study also shows that T6SS-1 plays a vital role in actin-based motility in RAW 264.7 cells. Mutants (*tssE*) undergo vacuolar escape, and in the cytoplasm of host cells containing these bacteria, there are defects in actin polymerization, as evidenced by decreased intra-, as well as inter-, cellular spreading (Burtnick et al. 2010). Whereas, *B. pseudomallei*-induced actin polymerization is due to $bimA_{Bm}$ genes being highly expressed, as evidenced in 556 melioidosis cases (human) from Australia (Sarovich et al. 2014). In addition, a filamentous hemagglutinin gene, *fhaB3*, has been observed in positive blood cultures; however, it was

negatively correlated with localized skin lesions without sepsis (Sarovich et al. 2014).

Secretion of Bacterial Molecules Responsible for Virulence

Molecules that play key roles in *B. pseudomallei* virulence include capsular polysaccharide, lipopolysaccharide, adhesins, specialized secretion systems, actin-based motility, and various secreted factors (Stone et al. 2014). After internalization, bacteria escape from endocytic vacuoles into the cytoplasm of infected cells, subsequently forming membrane protrusions by inducing actin polymerization at one pole of the bacterium that helps to propel it out of the host cell. Survival within phagocytic cells, and cell-to-cell spread via actin protrusions, is required for full virulence. Previous studies reveal the role of a putative type III protein secretion apparatus (Bsa) during the interaction of *B. pseudomallei* with host cells. These murine-based findings indicate that the Bsa type III secretion system critically modulates the intracellular behavior of *B. pseudomallei* (Stevens et al. 2002). *B. pseudomallei* bipD mutants, lacking a component of the translocation apparatus, are significantly attenuated as determined by intraperitoneal or intranasal challenge studies in BALB/c mice. Furthermore, a bipD mutant is attenuated in C57BL/6 IL-12 p40 (−/−) mice, which are highly susceptible to *B. pseudomallei* infection. Mutation of bipD impairs bacterial replication in the liver and spleen of BALB/c mice during the early stages of infection (Stevens et al. 2004).

Deletion of Hcp from cluster 1 (Hcp1) strongly attenuates *B. pseudomallei*, suggesting a prominent role of the T6SS cluster 1 (T6SS1, BPSS1496 to BPSS1511) for virulence in mammalian hosts (Chirakul et al. 2014). The type VI secretion system (T6SSs) and their effectors play an important role in pathogenesis and inter-bacterial competition (Attar 2015). A previous study shows that the bacterial toxin-antitoxin system mediates this transition by controlling bacterial motility in response to extracellular stress (Hadjifrangiskou et al. 2011). However, the bacterial capsule and a type III protein secretion apparatus enable *B. pseudomallei* to survive intracellular killing and facilitate intercellular spread. Since one of the capsules produced by *B. pseudomallei* is important in virulence, the genes encoding the proteins responsible for its biosynthesis may be considered as potential targets to disable the bacterium and halt disease progression (Reckseidler-Zenteno et al. 2009).

Effect of Exotoxin (CLT) on Resistance and Drug-Efflux Mechanism

Currently, recommended antibiotics such as chloramphenicol, doxycycline, co-trimoxazole, and kanamycin are often ineffective in patients with severe melioidosis infections (Estes et al. 2010). The main resistance mechanisms affecting these antibiotics include enzymatic inactivation, target deletion, and efflux from the bacterium, which are all mediated by chromosomally encoded genes (Schweizer

2012). Overexpression of efflux pumps strongly correlates with clinically relevant drug resistance (Sun et al. 2014; Chan et al. 2004, 2007; Chan and Chua 2005). Excessive release of mutations in the class A PenA beta-lactamase causes ceftazidime and amoxicillin-clavulanic acid resistance. Removal of penicillin binding protein-3 (PBP-3) leads to ceftazidime resistance. Whereas, the BpeEF-OprC efflux pump expression causes trimethoprim and trimethoprim-sulfamethoxazole resistance (Schweizer 2012). Of clinical relevance, particularly in gram-negative bacteria, efflux pumps of the resistance nodulation cell division (RND) play a vital role. Diverse efflux pumps exist in *B. cenocepacia*, which confer resistance to many potent antibiotics such as chloramphenicol, tetracyclines, and aminoglycosides (Podnecky et al. 2015). BpeAB-OprB strains of KHW mediate the efflux of aminoglycosides, while macrolides play a major role in quorum sensing and virulence (Mima and Schweizer 2010).

Toxins Modulating the Mechanism of Action and Cellular Signaling

Mouse macrophages (RAW 264.7), treated with LPS from *B. pseudomallei* (BP-LPS), produce significantly less nitric oxide (NO) and TNF-α than those treated with *Escherichia coli* or *Salmonella typhi* LPS (Utaisincharoen et al. 2000). Interestingly, plasma levels of endotoxin are an important predictor of multiple organ failure and death in systemic infections (Brandtzaeg et al. 1989). Mammalian cells respond to LPS by activating protein kinase cascades, which leads to new gene expression. This includes mitogen-activated protein kinase (MAPK), targeted by endotoxin, and hyperosmolarity in mammalian cells (Han et al. 1994). However, the p38 MAPK signaling pathway is activated by various stress stimuli such as osmostress, which also regulates multiple biological processes that include the p38 signaling pathway (Ben Messaoud et al. 2015). In addition, MAPK-interacting protein kinases 1 and 2 (Mnk1/Mnk2) play a vital role in controlling signals essential for mRNA translation (Joshi and Platanias 2014). The extracellular regulated kinase (Erk)/p38 MAPK pathways play key roles in mediating various biological functions such as development, apoptosis, autophagy, and inflammation (Roux and Blenis 2004). Bacterial pathogens can release toxic proteins to outmaneuver the host's immune system. The NADPH oxidase enzyme family generates ROS that contributes to cell signaling, the development of immune responses, as well as promoting proliferation and transcription (Bokoch et al. 2009). On the other hand, these proteins target regulatory GTPases belonging to the RHO family that organize the host's actin cytoskeleton (Aktories 2011). Therefore, the molecular basis of actin-based motility of these bacterial pathogens will be useful to understand fully the novel insights of pathogenesis and host-cell pathways (Stevens et al. 2006).

Melioidosis is a severe form of infection endemic to Southeast Asia, Northern Australia, and other parts of the world. Present treatment of melioidosis is highly challenging and complicated because of resistance to many existing antibiotics. It is very difficult to treat this disease as it results in very high mortality and morbidity.

Currently, there is no vaccine, and the organism has become multidrug resistant, which often induces relapse. Bacterial cell wall-secreted proteins or toxins responsible for severe inflammation/tissue destruction, pathological changes, cell death, and organ failure lead to death of the host. These bacterial toxins play an important role in invasion, virulence, drug-efflux mechanisms, and cellular signaling. Melioidosis is manifested by an acute/chronic septicemia that can be asymptomatic, leading to septic shock. Pathology examination of human melioidosis, via solid organs, is usually performed in the lungs, liver, and spleen. Disease-attributed abnormalities can also be detected in the brain or kidney of mice infected with *B. pseudomallei.* Necrotizing lesions of melioidosis can be attributed to bacteria-released toxins that kill host cells. *B. pseudomallei* produce numerous exotoxins that lead to acute progression of the disease but are not found in aerosol-infected BALB/c mice (Lever et al. 2009). The bacterial burden is higher in the lung versus other organs in aerosol-infected mice. Experimental evidence clearly shows elevated levels of toxins secreted from in vitro cultured bacteria isolated from patients suffering from severe melioidosis. Several culture methods, as well as rodent models, are well established and useful for studying the pathophysiology of melioidosis. However, the lack of diagnostics presents a challenge not only in endemic regions but also globally.

Conclusion and Future Directions

In conclusion, further experimental studies are required to understand *B. pseudomallei* toxins, their clinical implications, and to subsequently design and develop new drug targets against melioidosis in the near future.

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Part III

Clostridium Toxins

Clostridium perfringens Epsilon Toxin: Structural and Mechanistic Insights

4

Michel R. Popoff, Bradley G. Stiles, and Bernard Poulain

Abstract

Epsilon toxin (ETX) is produced by strains of *Clostridium perfringens* classified as type B or D. ETX belongs to the heptameric β-pore-forming toxins including *Aeromonas* aerolysin and *Clostridium septicum* alpha toxin, which are characterized by the formation of a pore through the plasma membrane of eukaryotic cells and containing a β-barrel composed of 14 amphipathic β-strands. In contrast to aerolysin and *C. septicum* alpha toxin, ETX is a much more potent toxin, which is responsible for enterotoxemia in animals, mainly in sheep. ETX induces perivascular edema in various tissues and accumulates particularly in the kidneys and in the brain, where it causes edema and necrotic lesions. ETX is able to pass through the blood-brain barrier (BBB) and to stimulate the release of glutamate, which accounts for the nervous excitation symptoms observed in animal enterotoxemia. At the cellular level, ETX causes a rapid swelling followed by a cell death involving necrosis. Recently, ETX has been found to induce demyelination and could be involved in demyelinating diseases like multiple sclerosis. The precise mode of action of ETX remains undetermined.

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Contents

Introduction: *Clostridium perfringens*, Its Toxins, and Disease

Clostridium perfringens (also termed *C. welchii*) is a Gram-positive, rod-shaped, anaerobic, and sporulating bacterium which produces the largest number of different toxins compared to other bacteria. According to the main lethal toxins (alpha, beta, epsilon, and iota), *C. perfringens* is divided into five toxinotypes (A to E). Epsilon toxin (ETX) is synthesized by toxinotypes B and D. However, the high diversity of toxin combinations, which can be produced by *C. perfringens* strains, makes more complex the classification into five toxinotypes (Petit et al. 1999; Popoff and Bouvet 2009). *C. perfringens* is widely distributed throughout the environment (soil, dust, sediment, cadavers, etc) and can be found in the digestive tract of healthy humans and animals. Albeit *C. perfringens* is largely spread in the environment, some toxinotypes are preferentially associated with certain animal species such as type C in pigs, type D in sheep and goats, and type E in bovine.

Based on the toxins produced, *C. perfringens* is responsible for diverse pathologies in man and animals, resulting from a gastrointestinal or wound contamination and including food poisoning, enteritis, necrotic enteritis, enterotoxemia, gangrene, and puerperal septicemia. Epsilon toxin (ETX) contributes with beta toxin to the pathogenesis of toxinotype B, and it is the causative virulence factor of all symptoms

and lesions due to toxinotype D. Indeed, isogenic *C. perfringens* ETX – null mutants – induce neither symptoms nor lesions in experimental animals (Garcia et al. 2013). ETX is one of the most potent toxins known. Its lethal activity ranges just below the botulinum neurotoxins. Indeed, the lethal dose by intraperitoneal injection in mice is 1.2 ng/Kg for botulinum neurotoxin A and 70 ng/Kg for ETX (Gill 1987; Minami et al. 1997). For this reason, ETX is considered a potential biological weapon classified as a category B agent, although very few ETX-mediated natural diseases have been reported in humans (Mantis 2005).

Toxin Plasmids: Expression and Regulation

ETX Gene

C. perfringens strains contain a chromosome of about 3 Mbp and various size plasmids (Popoff and Bouvet 2013). The *plc* gene for alpha toxin (which is a phospholipase C) is localized at the same site on a variable region of the chromosome near the origin of replication, and therefore is produced by all *C. perfringens* strains (Tsutsui et al. 1995; Rood 1998; Justin et al. 2002). In contrast, the other toxin genes [*cpb* for *C. perfringens b*eta toxin, *cpb*2 for *b*eta*2*, *etx* for *e*psilon *t*ox*i*n, *ia* and *ib* for the two components of *i*ota toxin*, netB* for NetB (*n*ecrotic *e*nteritis *t*oxin *B*-like), and *tpeL* for TpeL (*t*oxin *C. perfringens l*arge cytotoxin)] are localized on plasmids of varying sizes. For instance, *etx* can be found in at least five different plasmids ranging from 48 to 110 kb (Sayeed et al. 2007) and can be associated with other toxin genes like *cpe* (for *C. perfringens e*nterotoxin) and *cpb2*. A single *C. perfringens* type D strain may possess an *etx* plasmid and additional plasmids containing *cpe* and *cpb2*, together or separately, or a unique plasmid type harboring the three toxin genes (Sayeed et al. 2007; Gurjar et al. 2010).

Expression of ETX Gene

ETX is synthesized and secreted during the exponential growth of *C. perfringens*. Culture medium and environmental factors greatly influence ETX production. For example, maximum yield of ETX production is achieved when *C. perfringens* growth is maintained at a neutral pH (Mollby et al. 1976). ETX synthesis is a highly regulated process including the quorum sensing system, which controls adaptive responses to cell density. In Gram-positive bacteria, quorum sensing is based on the secretion of a signaling molecule (autoinducer peptide, AIP, encoded by *agrD*), which is activated by the protease, AgrB. When activated AIP reaches a threshold level in the external medium, it is sensed by a two-component system (*agrA/agrC)* in *Staphylococcus aureus* which activates the transcription of a regulatory RNA. In *C. perfringens*, ETX synthesis is controlled by a two-component system including *agrB/agrD*, but *C. perfringens* lacks *agrA/agrC* ortholog genes, and the two-component system involved in the control of ETX-dependent quorum sensing

system remains unidentified (Chen and McClane 2012). In addition, recent data show that ETX production is linked to metabolism. The proposed model includes the NanI sialidase which releases free sialic acid from glycoproteins or glycolipids, uptake of sialic acid into *Clostridium* cytoplasm, generation of carbohydrates such as fructose 1,6 bisphosphate, and activation of CcpA (catabolite control protein A) and subsequently CodY which are global gene regulators. CcpA and CodY binding sites are located upstream of the *etx* gene (Li et al. 2015). Interestingly, ETX production is upregulated upon contact of *C. perfringens* with enterocytes like $CaCo_2$ cells, and this regulation pathway includes the quorum sensing *agrD/agrB* genes (Chen and McClane 2012). Thereby, *C. perfringens* is able to sense interaction with host cells and adapt to the new environment by producing specific molecules like toxins or virulence factors. Upregulation of ETX production in the digestive tract of animals is critical in the onset of enterotoxemia and enteritis.

Structure and Biochemistry of ETX

Production of ETX

ETX is synthesized as a single protein containing a signal peptide (32 N-terminal amino acids). The secreted protein (32.98 kDa) is poorly active and generically called a prototoxin (Hunter et al. 1992). The prototoxin is activated by proteases produced by the host and/or *C. perfringens*, such as trypsin, α-chymotrypsin, and λ-protease, which is produced by *C. perfringens*. Activation by λ-protease is comparable to that obtained with trypsin plus α-chymotrypsin. The λ-protease removes 11 N-terminal and 29 C-terminal residues whereas trypsin plus α-chymotrypsin cleaves 13 N-terminal residues and the same C-terminal amino acids. This results in a reduced size (28.6 kDa) and an important decrease in pI from 8.02 to 5.36, which is likely accompanied probably by a conformational change. The charged C-terminal residues might prevent protein interaction with its substrate or receptor (Minami et al. 1997).

Structure/Function of ETX

ETX retains an elongated form and contains three domains, which are mainly composed of β-sheets (Cole et al. 2004) (Fig. 1). Despite poor sequence identity (14%), the ETX overall structure is significantly related to that of the pore-forming toxin aerolysin produced by *Aeromonas* species (Parker et al. 1994), *C. perfringens* enterotoxin (CPE) (Briggs et al. 2011; Kitadokoro et al. 2011), and model of *Clostridium septicum* alpha toxin (ATX), an agent of gangrene (Melton et al. 2004). However, ETX is a much more potent toxin with a 100 times more mouse lethal activity than aerolysin and *C. septicum* alpha toxin (Minami et al. 1997; Tweten 2001). The main difference between both toxins is that the aerolysin domain I, which is involved in initial toxin interaction with cells, is missing in ETX. Domain 1 of

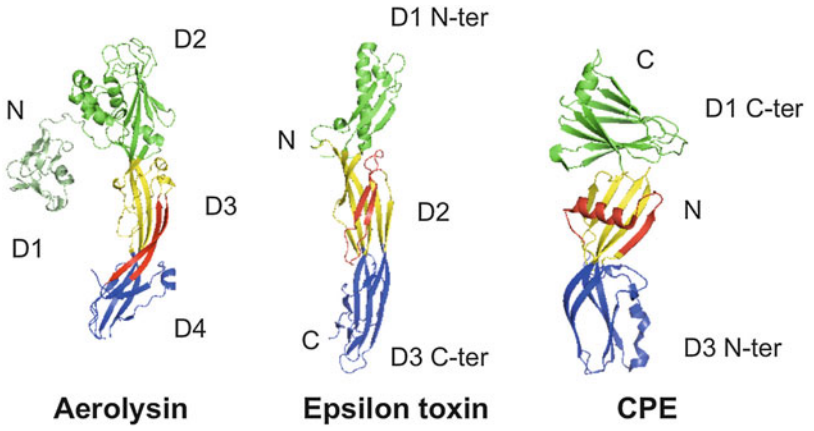

Fig. 1 Structure of *C. perfringens* epsilon toxin (ETX) and related β-PFTs. Aerolysin monomer (Protein Data Bank (pdb) record 1PRE), *C. perfringens* ETX monomer (pdb, 1UYJ), and *C. perfringens* enterotoxin (CPE) (pdb, 2XH6^ and 2QUO). The receptor binding domains are in *green*, the domains containing the pre-stem loop are in *yellow* with the pre-stem loop in *red*, and the domains containing the propeptide which are involved in controlling oligomerization are in *blue*. Figures were produced with the program MacPyMOL

ETX contains a large α-helix followed by a loop and three short α-helices, similar to domain 2 of aerolysin, which interacts with the glycosylphosphatidylinositol (GPI) anchors of proteins. This domain of ETX could have a similar function of binding to receptor. A cluster of aromatic residues (Tyr42, Tyr43, Tyr49, Tyr209, and Phe212) in ETX domain 1 could be involved in receptor binding (Cole et al. 2004). Domain 2 is a β-sandwich structurally related to domain 3 of aerolysin. This domain contains a two-stranded sheet with an amphipathic sequence predicted to be the channel-forming domain (see below). In contrast to the cholesterol-dependent cytolysins, only one amphipathic β-hairpin from each monomer is involved in the pore structure of ETX and other heptameric β-pore-forming toxins (β-PFTs) like aerolysin. Domain 3 is also a β-sandwich analogous to domain 4 of aerolysin, which contains the cleavage site for toxin activation. Domain 3, after removing the C-terminus, is likely involved in monomer-monomer interaction required for oligomerization (Cole et al. 2004; Knapp et al. 2010b).

The pore-forming domain of ETX has been identified in domain 2. His151-Ala181 contains alternate hydrophobic-hydrophilic residues, which are characteristic of membrane-spanning β-hairpins, forming two amphipathic β-strands. Site-directed mutagenesis confirms that this segment is involved in ETX channel activity in lipid bilayers (Knapp et al. 2009). Interestingly, the ETX pore-forming domain has higher sequence similarity to those of the binding components of clostridial binary toxins [Ib of Iota toxin, C2-II of C2 toxin, CDTb *of C. difficile* transferase (CDT), CSTb of *C. spiroforme* toxin (CST)]. There is some, yet less, sequence similarity of the ETX pore-forming domain with that of *B. anthracis* protective antigen (PA, the binding component of anthrax toxins) and aerolysin. However, ETX residues

(Lys162 to Glu169) exposed to the transmembrane side of the channel and forming the loop linking two β-strands of the transmembrane β-hairpin are unrelated at the amino acid sequence level to those of other β-PFTs. This ETX loop is flanked by two charged residues, Lys-162 and Glu-169, and contains a central proline like the corresponding aerolysin loop. Binding components share a similar structure organization with that of β-PFTs and notably contain an amphipathic flexible loop that forms a β-hairpin, playing a central role in pore formation (Geny and Popoff 2006; Schleberger et al. 2006). This suggests that binding components and β-PFTs have evolved from a common ancestor. However, β-PFTs have acquired a specific function consisting of translocating corresponding enzymatic components of binary toxins through the membrane of acidified endosomes. In contrast, β-PFTs such as ETX and aerolysin can form pores in plasma membrane at neutral pH, which are responsible for cytotoxicity.

Essential amino acids for the lethal activity of ETX have been identified by biochemistry and mutagenesis. Previous work with chemical modifications shows that His residues are required for the active site, and Trp and Tyr residues are necessary for binding to target cells (Sakurai 1995). ETX contains a unique Trp and two His. Amino acid substitutions reveal that His106 is important for the biological activity, whereas His149 and Trp190 are probably involved in maintaining the structure but not activity (Oyston et al. 1998).

ETX Mechanism of Action with an Overview of Animal and Cell Culture Studies

Interaction of ETX with Cell Membrane

The first step of ETX activity, as for the other PFTs of the aerolysin family, is the binding to cell surface receptor. Very few cell lines besides those renal from various species such as dog (Madin-Darby Canine Kidney, MDCK), mouse (murine renal mpkCCDcl4 collecting duct cells), and to a lesser extent human (human leiomyoblastoma G-402 and adenocarcinoma hypernephroma ACHN) are sensitive to ETX (Payne et al. 1994; Shortt et al. 2000). Surprisingly, kidney cell lines from ETX-susceptible animal species, like lamb and cattle, are ETX resistant and suggest that the receptor in primary cells is lost in cultured cell lines ((Payne et al. 1994) and unpublished).

ETX binds to MDCK cells, preferentially the apical surface, and recognizes a specific membrane receptor not present in insensitive cells. Binding of the toxin to its receptor generates large membrane complexes which are very stable at 37 °C. In contrast, the complexes formed at 4 °C are dissociated by SDS and heating (Petit et al. 1997). This suggests a maturation process like a prepore and then a functional pore formation (Robertson et al. 2011). Overall, the toxin remains associated to the outer cell membrane throughout the intoxication process, with no endocytosis and internalization of the toxin during the early steps of intoxication (Petit et al. 1997), albeit presence of ETX into early endosomes has been reported (Nagahama

et al. 2011). The ETX large membrane complex in MDCK cells and synaptosomes corresponds to the heptamerization of ETX monomers within the membrane and pore formation (Petit et al. 1997; Miyata et al. 2001, 2002). ETX prototoxin binds to sensitive cells but does not oligomerize, in contrast to activated ETX (Chassin et al. 2007). Thus, the 23 C-terminal residues of prototoxin control toxin activity by preventing the heptamerization. These amino acids are removed by proteolysis and not found in the active toxin molecule (Miyata et al. 2001).

ETX binding to susceptible cells or synaptosomes and subsequent complex formation are prevented by protease pretreatment of cells or synaptosomes. Phospholipase C, glycosidases, and neuraminidase have little or no effect upon ETX intoxication, thus indicating the protein nature of the ETX receptor (Nagahama and Sakurai 1992; Petit et al. 1997; Dorca-Arevalo et al. 2008). It is possible that ETX receptor could be related to a 34 or 46 kDa protein or glycoprotein sensitive to trypsin in MDCK cells (Petit et al. 1997; Manni et al. 2015) and to a 26 kDa sialoglycoprotein in rat brain (Nagahama and Sakurai 1992). Further work with cell membranes and giant unilamellar vesicles exclusively containing lipids emphasizes the fact that ETX requires a protein receptor on sensitive cells (Manni et al. 2015). The phosphatidylserine receptor *H*epatitis *A* *v*irus *c*ellular *r*eceptor *1* (HAVCR1, also known as KIM1 for *K*idney *I*njury *m*olecule *1*, or TIM1 for *T*-cell *i*mmunoglobulin (Ig) *m*ucin domain protein *1*) facilitates ETX cytotoxicity in MDCK cells and the human kidney cell line ACHN. ETX binds to HAVCR1 in vitro (Ivie et al. 2011). However, it is not yet clear whether HAVCR1 can serve as functional ETX receptor. Indeed, expression of HAVCR1 in cells resistant to ETX is not sufficient to confer the sensitivity to this toxin (Ivie et al. 2011). Caveolins 1 and 2, which are scaffolding proteins in cholesterol-enriched membrane microdomains forming invaginations (caveolae) involved in cell signaling and receptor-independent endocytosis, reportedly facilitate ETX oligomerization via an unknown mechanism (Fennessey et al. 2012b). More recently, the *m*yelin *a*nd *l*ymphocyte protein (MAL), which is a tetraspan membrane protein associated with lipid rafts in the apical membrane of certain epithelial cells, is evidently required for ETX cytotoxicity. MAL is expressed by most cells susceptible to ETX like renal cells, endothelial cells, oligodendrocytes, and Schwann cells but not neurons (Schaeren-Wiemers et al. 1995; Rumah et al. 2015), and it is required for both ETX binding and cytotoxicity (Linden et al. 2015; Rumah et al. 2015). Albeit, no direct interaction between ETX and MAL has been evidenced, ETX was totally unable to bind tissues from MAL knockout mice. MAL could be a specific receptor for ETX, or a scaffold protein involved in assembly of a multi-protein complex or assembly of lipid microdomain required for ETX interaction with membrane and subsequent activity (Rumah et al. 2015). It is noteworthy that MAL is expressed by oligodendrocytes which are target cells for ETX in the nervous tissue (see below).

Moreover, although ETX does not directly interact with a lipid, the lipid environment of ETX receptor is critical for the binding of ETX to cell, since detergent treatment prevents ETX binding to the cell surface (Petit et al. 1997). It is noteworthy that ETX can interact with artificial lipid bilayers and form functional channels. This binding is without the requirement of a specific receptor in contrast to cell

membrane. Lipid bilayers have a smooth, structure-less surface versus a cell membrane naturally decorated with surface-exposed carbohydrates and proteins. This means that the toxins can interact with the hydrocarbon core of the lipid bilayer and insert without a surface receptor, whereas in contrast receptors are required to promote such an interaction in the cell membrane (Petit et al. 1997; Manni et al. 2015). It is proposed that monomeric ETX binds to a protein receptor in non-lipid raft areas of plasma membrane, and then the ETX receptor complex is distributed in lipid raft microdomains where ETX forms oligomers (Chassin et al. 2007; Nagahama et al. 2011). Recently, it was shown that ETX exclusively binds to lipids forming the raft microdomains (phosphatidylserine, sulfatide, and phosphatidylinositol monophosphate), but weakly at best to non-raft-containing lipids. Removal of sulfate groups by sulfatase results in a reduced cytotoxic activity of ETX but does not prevent the oligomer formation. Sulfatide is present on the cell membranes of various tissues targeted by ETX, such as the gastrointestinal tract, kidney, and nervous system which could account for toxin binding to myelin. Interestingly, sulfatide also binds to MAL (Gil et al. 2015). However, the precise association of ETX with its cell membrane receptor and then with lipid rafts that enable oligomerization and pore formation remains to be clarified.

Toxin interaction with cell membranes has been further analyzed with ETX and ATX coupled to europium-doped oxide nanoparticles. Toxin monomers bound to their receptor are mobile on the cell surface but in confined areas corresponding to lipid rafts (Masson et al. 2009; Turkcan et al. 2012). Indeed, ETX and ATX receptors are localized into lipid rafts (Gordon et al. 1999; Miyata et al. 2002; Chassin et al. 2007). Raft confinement seems to be mainly due to the composition and spatial organization of the lipids around the proteins and subsequent molecular interactions (local electrostatic interactions, hydrophobic interactions, lipid-protein specific and/or nonspecific interactions). Thereby, membrane depletion of cholesterol by cholesterol oxidase or sphingolipids results in the release of confinement, and ETX and ATX bound to their receptors move in a wider area on the membrane surface. The actin and microtubule cytoskeleton is not directly involved in the ETX and ATX mobility (Turkcan et al. 2012, 2013b). However, albeit the toxin receptors are not directly linked to actin filaments, other lipid raft proteins are connected to the actin cytoskeleton which mediates lipid raft displacement throughout the membrane (Turkcan et al. 2013b). Mobility of toxin monomers bound to their receptors in confined areas leads to a concentration of toxin molecules and facilitates their interactions and subsequent oligomerization.

Pore-Forming Activity of ETX

Pore formation of β-PFTs has been solved at the structural level with aerolysin. An aerolysin heptamer adopts a mushroom shape similar to *Staphylococcus* α-hemolysin. However, in contrast to α-hemolysin, the aerolysin heptamer associates with the membrane in an inverse orientation. The mushroom cap of aerolysin faces the membrane and the stalk in the extracellular milieu, since domains 1 and

2 bind to the receptor located in the cap. The heptamer then undergoes a vertical collapse. Domains 3 and 4 rotate and completely flatten, and the β-hairpin from domain 3 moves through a cavity between the two monomers. The β-hairpins of the seven monomers refold in a β-barrel which lies in the opposite orientation to that of the prepore mushroom stalk, which inserts into the membrane (Degiacomi et al. 2013). Based on the structure similarity between ETX and aerolysin, a similar mechanism of pore formation is probably involved with ETX. The lipid composition and physical properties of the membrane influence ETX access to the receptor, assembly into an ETX heptamer, and insertion of the ETX pore into the membrane. Indeed, lipids such as diacylglycerol and phosphatidylethanolamine, which induce a negative membrane curvature, increase ETX pore formation in liposomes, whereas lipids having an opposite effect, like lysophosphatidylcholine, impair ETX activity (Nagahama et al. 2006). This is consistent with the model of an ETX prepore formation and subsequent insertion into the membrane to form a functional channel. The structure of an ETX pore has been defined as a cone shape (Nestorovich et al. 2010), and thus its insertion into a lipid bilayer might be favored by a specific lipid membrane organization.

ETX Effects in Cultured Renal Cells

In sensitive cultured cells, ETX induces a marked swelling in the first phase of intoxication, followed by mitochondria disappearance, blebbing, and membrane disruption. The cytotoxicity can be monitored by using an indicator of lysosomal integrity (neutral red) or mitochondrial integrity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT) (Payne et al. 1994; Lindsay et al. 1995; Petit et al. 1997; Shortt et al. 2000; Borrmann et al. 2001). At low concentration, cell vacuolation and ETX internalization into early endosomes have been reported (Nagahama et al. 2011).

Cytotoxicity is associated with a rapid loss of intracellular K^+, and an increase in Cl^- and Na^+, whereas the increase in Ca^{++} occurs later. In addition, loss of viability also correlates with the entry of propidium iodide, indicating that ETX forms large pores in the cell membrane. Pore formation is evident in artificial lipid bilayers. ETX induces water-filled channels permeable to hydrophilic solutes up to a molecular mass of 1 kDa, which represent general diffusion pores slightly selective for anions (Petit et al. 2001; Nestorovich et al. 2010). In polarized MDCK and mpkCCDc14 cells, ETX induces a rapid and dramatic increase in permeability. Pore formation in the cell membrane is likely responsible for the permeability change of cell monolayers. The actin cytoskeleton and organization of tight and adherens junctions are not altered, and the paracellular permeability to macromolecules is not significantly increased upon ETX treatment (Petit et al. 2003; Chassin et al. 2007). In addition, cells treated with ETX lose coenzymes critical for energy production, notably nicotinamide adenine dinucleotide (NAD^+ and NADH) and coenzyme A, with subsequent membrane depolarization and impaired activity of mitochondria (Fennessey et al. 2012a; Ferrarezi et al. 2013). ETX causes a rapid cell death by necrosis characterized by a

marked reduction in nucleus size without DNA fragmentation. Toxin-dependent cell signaling leading to cell necrosis is not yet fully understood and includes ATP depletion, AMP-activated protein kinase stimulation, mitochondrial membrane permeabilization, and mitochondrial-nuclear translocation of apoptosis-inducing factor, which is a potent caspase-independent cell death factor (Chassin et al. 2007). The early and rapid loss of intracellular K^+ induced by ETX or *C. septicum* alpha toxin seems to be the early event leading to cell necrosis (Knapp et al. 2010a). It is intriguing that ETX, which has a pore-forming activity related to that of aerolysin and *C. septicum* alpha toxin, is much more active. Does ETX induce a specific intracellular signal responsible for a rapid cell death? Methyl-beta-cyclodextrin (MβCD), which prevents ETX pore formation in lipid rafts, does not inhibit the sudden decrease in cellular ATP and cell necrosis (Chassin et al. 2007). A subset of ETX channels unaffected by MβCD might be sufficient to trigger an intracellular signal leading to cell necrosis, excluding the requirement of a large diffusion pore to induce the intracellular toxic program. Therefore, ETX is a very potent toxin that alters the permeability of cell monolayers such as epithelium and endothelium, causing edema and cell death. However, its precise mode of action remains unclear.

Evidence for ETX Neurotoxicity and Emerging Links with Multiple Sclerosis

Attack of the Nervous System by ETX

ETX-intoxicated animals with large amounts of toxin (goats, sheep, lambs, calves, and rodents), be it during the course of enterotoxemia or experimental intoxination, display severe neurological manifestations (i.e., convulsions) culminating with animal death. In that case, postmortem investigations reveal severe, generalized brain vasogenic edema and few microscopic lesions (Finnie 2004). Further, following intoxination with low amounts of ETX, neurotoxicity evolves in a more chronic manner, associated with altered animal behavior, during which tissue lesions develop in several sites of the central nervous system (CNS) (as in the cerebral cortex, hippocampus, internal capsule, thalamus, basal ganglia, cerebellum). Lesions are characterized by their relative symmetry (i.e., focal symmetrical encephalomalacia) (Finnie 2004) and includes dark perivascular edema, hemorrhagic foci, degeneration or distortion of the white matter, (Finnie 1984b) brain necrosis, and presence of altered neurons (Buxton and Morgan 1976; Finnie 1984a, b; Uzal et al. 1997; Uzal and Kelly 1997; Miyamoto et al. 1998; Finnie et al. 1999; Miyamoto et al. 2000; Fernandez Miyakawa and Uzal 2003; Finnie 2004; Uzal et al. 2004; Wioland et al. 2013). Several evidences indicate these lesions result from the action of ETX upon brain. Indeed, hematogenously disseminated ETX accumulates in the brain (Nagahama and Sakurai 1991), and binding of ETX has been detected in

hippocampus, thalamus, cerebral white matter and commissures, basal ganglia, and cerebellum (Dorca-Arevalo et al. 2008; Lonchamp et al. 2010; Wioland et al. 2013, 2015).

The way by which ETX gains access to brain tissue has been clarified. *B*lood-*b*rain *b*arrier (BBB) is formed by the tight junctions between endothelial cells of capillary vessels in the CNS and has a cut-off size between 0.5 and 8 kDa (Banks 2009). Circulating ETX binds to vascular endothelial cells, crosses the BBB (Finnie 1984a; Soler-Jover et al. 2007), and, via an undefined mechanism, then opens endothelial tight junctions (Zhu et al. 2001; Soler-Jover et al. 2007). All of this causes penetration of fluid and ETX into the cerebral parenchyma (Zhu et al. 2001; Soler-Jover et al. 2007). Interestingly ETX has been proposed to facilitate the transport of bleomycin, through the BBB for the treatment of experimental malignant brain tumors in mice (Hirschberg et al. 2009). However, keep in mind that ETX attacks various cell types in the CNS and that a non-active derivative retaining the ETX targeting properties would be required for the use of this toxin as a drug vehicle.

The numerous neurological disorders caused by ETX result from two distinct actions. First, there is mechanical damage of neural parenchyma caused by penetration of fluids through the BBB with ensuing edema in the brain (Buxton and Morgan 1976; Uzal et al. 1997). Second, ETX binds to and directly affects (described below) certain types of neurons and glial cells, like oligodendrocytes but not astrocytes (Dorca-Arevalo et al. 2008; Lonchamp et al. 2010; Wioland et al. 2013, 2015).

Oligodendrocytes and Neurons Are Targeted by ETX

At high concentrations (10^{-7}M), immunostaining of cerebellum slices has revealed ETX binding on neurons of mice but not rats (Lonchamp et al. 2010; Wioland et al. 2013, 2015). However, in primary culture of cerebellar neurons, ETX binds to granule cells from both mouse and rat (Lonchamp et al. 2010; Wioland et al. 2015) at 10^{-7}M, but not lower concentrations. By using ETX tagged with green fluorescent protein, no neuronal binding has been found (Dorca-Arevalo et al. 2008). In the mouse and rat cerebellum, ETX binding is not detected onto the cerebellar GABAergic neurons (Lonchamp et al. 2010; Wioland et al. 2015). Overall, these findings indicate that certain neurons express a low-affinity ETX receptor, differentially regulated during development, and among neuron subtypes and animal species. The identity of the neuronal receptor for ETX remains unknown. It is likely distinct from MAL, which is expressed in oligodendrocytes but not in neurons (Schaeren-Wiemers et al. 1995).

In the CNS, another very abundant cell type includes the glial cells. The ability of ETX to bind to astrocytes is unlikely (Dorca-Arevalo et al. 2008; Lonchamp et al. 2010; Wioland et al. 2013, 2015). No binding of ETX to microglia has been observed (Wioland et al. 2015). However, this latter issue deserves further investigation. In the CNS, the most important cell target for ETX is likely the oligodendrocyte possessing protrusions which wrap around axons and form the myelin

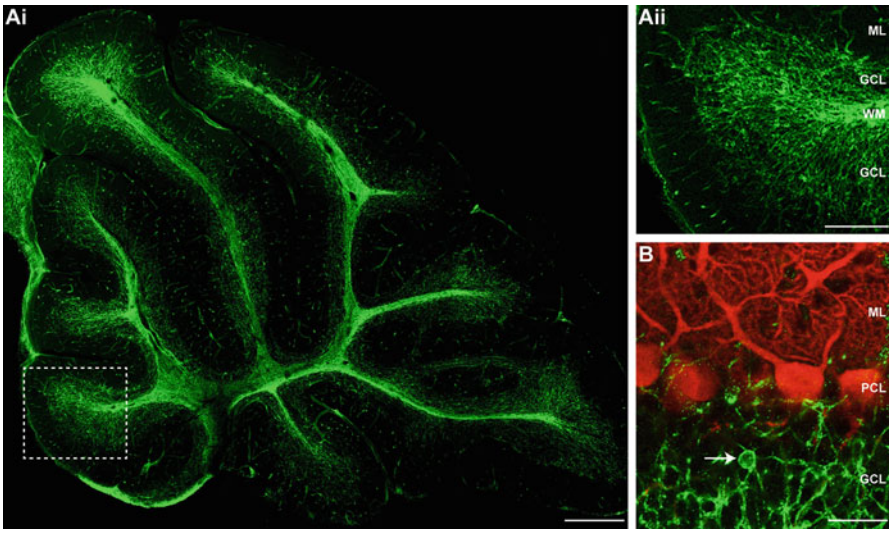

Fig. 2 *C. perfringens* epsilon toxin (ETX) binds to white matter and oligodendrocytes from (Wioland et al. 2015). (**a**) ETX immunostaining (*green*) in rat cerebellar slices (ETX 10^{-7}M for 5 min before fixation). This denotes binding of ETX on cerebellar white matter comprised of myelinated axon bundles. c. Ai. Whole rat cerebellum; scale bar = 500 μm. Aii. Magnification of region denoted by *dotted* frame in Ai, scale bar = 250 μm. (**b**) ETX immunostaining (*green*) in the granular cell layer. Immunostaining against the 28 kD protein (*red*) reveals the Purkinje cells, which are large inhibitory neurons. The *arrow* denotes an oligodendrocyte soma decorated by ETX, scale bar = 50 μm. *WM* white matter, *GCL* granule cell layer, *ML* molecular layer, *PCL* Purkinje cell layer

sheath. Indeed, white matter (Fig. 2) formed by bundles of myelinated axons is the prominent CNS component labeled by ETX (sheep, cattle, mouse, rat, and human) (Soler-Jover et al. 2007; Dorca-Arevalo et al. 2008; Lonchamp et al. 2010; Wioland et al. 2015). ETX binds to myelinated axons in peripheral nerves, too (Dorca-Arevalo et al. 2008). These nerves are myelinated by the Schwann cells, the functional analogues of oligodendrocytes in the peripheral nervous system. At low concentration ($<10^{-9}$M), ETX binds to oligodendrocytes in rodent cerebellar slices and primary cultures (Linden et al. 2015; Wioland et al. 2015). This concentration is much lower than that needed to induce cellular effects in renal cells (10^{-8} M) (Petit et al. 1997; Miyata et al. 2001, 2002; Chassin et al. 2007; Fernandez Miyakawa et al. 2011; Fennessey et al. 2012a) or neurons (10^{-7} M) (Lonchamp et al. 2010). This suggests that, similar as the renal cells (Dorca-Arevalo et al. 2012), oligodendrocytes express a high-affinity ETX receptor that may correspond to the single class of ETX binding sites with high affinity (Kd ~ 2.5–3.3 nM) detected in rodent brain (Nagahama and Sakurai 1992). MAL (Rumah et al. 2015) could be a specific ETX receptor on oligodendrocytes, since oligodendrocytes from MAL-deficient mice are resistant to ETX (Linden et al. 2015). Therefore, oligodendrocytes are likely preferentially attacked by the toxin when low amounts of ETX gain access to the brain parenchyma.

ETX Damages Neurons and Oligodendrocytes by Different Mechanisms

Pore formation in renal cell membranes causes marked decrease in the electrical membrane resistance, R_m (Chassin et al. 2007). Similarly, application of 10^{-7}M ETX to cerebellar granule cell neurons causes dramatic decrease in neuronal R_m likely due to formation of ETX pores, albeit of lower conductance (Petit et al. 2001 and Nestorovitch et al. 2010) than those reported in artificial membranes (Lonchamp et al. 2010). This causes membrane depolarization associated with an intracellular rise in Ca^{++} concentration along with bursts of action potentials propagating along axons with an ensuing increase of excitatory neurotransmitter release (Lonchamp et al. 2010). The ETX mechanism on oligodendrocytes differs strongly from that determined in renal cells and neurons, and electrophysiological (patch-clamp) experiments reveal that ETX does not form a pore in their plasma membrane (Wioland et al. 2015). Thus, binding of ETX to a specific receptor directly activates an intracellular pathway that mediates all the manifestations induced by the toxin (glutamate release, Ca^{++} waves, and demyelination) (Wioland et al. 2015). This pore-independent mechanism may explain why ETX can affect oligodendrocytes at much lower concentrations $<10^{-9}$M) than on other cell types.

ETX Induces Glutamate Release and Demyelination

In rodents, intra-hippocampal injection of ETX causes neuronal lesions (Miyamoto et al. 1998) and release of glutamate (Miyamoto et al. 1998, 2000), an amino acid contained in high quantities by all cell types and serving as an excitatory neurotransmitter in the CNS. Since ^{125}I-ETX binds to rat synaptosomes, which are comprised of isolated nerve terminals (Nagahama and Sakurai 1992, Miyata et al. 2001, 2002), it has been first proposed that ETX might trigger release of glutamate by acting on the nerve endings. However, it has been shown later that in the cerebellum, ETX does not bind to nerve terminals and the ETX-induced increase in glutamatergic transmission results from the firing of neurons (Lonchamp et al. 2010; Wioland et al. 2013). Moreover, ETX does not bind to brain synaptosomes (Dorca-Arevalo et al. 2008). The discrepancy noted between all of these reports might be due to the fact that synaptosomes are very often contaminated by myelin debris sticking to the isolated nerve endings. These debris can bind ETX (Dorca-Arevalo et al. 2008) and might act as a source of glutamate (see below).

ETX (10^{-9}M) binding at such a low concentration to oligodendrocytes induces a markedly rapid increase in extracellular glutamate, resulting from an alteration of the membrane glutamate transporter (Wioland et al. 2015). Thus, given the much higher affinity of ETX for oligodendrocytes, versus neurons, the ETX-induced glutamate release in the CNS may result primarily from ETX action upon oligodendrocytes rather than on neurons.

The secondary effects of elevated extracellular glutamate, which is an excitatory neurotransmitter, are multiple. Glutamate activates many types of neurons leading to

firing of the whole neuronal network and release of glutamate. An excess of glutamate induces excitotoxic damage/death in many cell types, provided they express a subtype of glutamate receptor (*N*-methyl-D-aspartate receptor) as do neurons and glial cells (reviewed by Slemmer et al. (2005) and Matute et al. (2006)). Moreover, ETX oligodendrocyte-mediated increase in extracellular glutamate can induce demyelination by autocrine activation of the type 1 metabotropic glutamate receptors present on oligodendrocytes and ensuing activation of intracellular [Ca^{2+}] signaling (Wioland et al. 2015). Such a mechanism is fully consistent with the notions that oligodendrocytes and myelin are highly vulnerable to glutamate-dependent insults (reviewed in Matute et al. (2006) and Matute (2011)). Note that demyelination has been proposed resulting from the oligodendrocyte death induced directly by ETX (Linden et al. 2015).

Does ETX Play a Role in Initial Triggering of Multiple Sclerosis?

Multiple sclerosis is a chronic inflammatory demyelinating disease of CNS white matter, with an etiology that is multifactorial and largely unknown. Environmental factors and other determinants (genetics other bacterial or viral underlying infections, etc.) are implicated. Oligodendrocyte apoptosis, permeabilization of BBB, and microglial activation are detected in the early stages of the disease (Trapp and Nave 2008). A remarkable correlation between the prevalence of multiple sclerosis and the geographical concentration of sheep farming has been pinpointed (Murrel et al. 1986). However, antibodies to ETX were not found in the patients at the time of this early study (Murrel et al. 1986). Recently, an ETX-secreting *C. perfringens* type B strain has been isolated from a patient suffering from multiple sclerosis (Rumah et al. 2013). Moreover, ETX immunoreactivity has been detected in the sera of approximately 10% of multiple sclerosis patients, as compared to only 1% of healthy controls (Rumah et al. 2013). The possibility that an initial exposure to ETX might be causative for nascent white matter lesions in multiple sclerosis is now open (Rumah et al. 2013).

Prospects for Developing Vaccines and Therapeutics

Vaccines against bacterial toxins are among the most efficient against bacterial diseases, resulting from toxin effects. Thereby, vaccines against enterotoxemia due to *C. perfringens* ETX are extensively used in veterinary medicine (Titball 2009). These vaccines are classically based on chemically detoxified toxins using formalin. Recombinant ETX inactivated by formaldehyde efficiently protects ruminants against enterotoxemia (Lobato et al. 2010). New approaches consist of genetically detoxified toxins such as toxin mutants or subunits, which are non-biologically active but retain the toxin's immunogenicity. These include recombinant ETX

vaccines under investigation (Oyston et al. 1998; Titball 2009; Mathur et al. 2010). Several recombinant-inactive ETX molecules have been proposed as vaccine candidates. For example, cysteine substitutions at Ile51-Ala114 and Val56-Phe118 yield non-cytotoxic ETX mutants by formation of disulfide bonds which prevent oligomerization (Pelish and McClain 2009). Mutation of surface-exposed tyrosines at positions 30 and 196 prevents ETX binding to cells. When combined with mutation of His149, involved in pore formation through the cell membrane, these changes result in inactive mutants (Bokori-Brown et al. 2013). The double mutant Tyr30Ala-Tyr196Ala has been proposed as a candidate vaccine (Bokori-Brown et al. 2014). Additional amino acids playing a role in ETX binding to receptor, like His106 and Phe199, have also been used to generate nontoxic mutants retaining ETX immunogenicity (Oyston et al. 1998; Li et al. 2013).

Polyclonal and monoclonal antibodies neutralizing ETX have been developed but are ineffective in treating animal diseases caused by ETX, due to the extreme rapidity of intoxication (Odendaal et al. 1989; McClain and Cover 2007; Garcia et al. 2014; Sully et al. 2014). Three small molecule inhibitors of ETX have been identified by high-throughput screening. These compounds neither prevent ETX binding to target cells nor halt toxin oligomerization, but seem to specifically block ion fluxes induced by ETX. They could be the basis for developing efficient therapeutic tools against ETX in the future (Lewis et al. 2010).

Conclusion

ETX belongs to the heptameric β-PFTs family that includes *Aeromonas* aerolysin and *C. septicum* alpha toxin, which are characterized by the formation of a pore consisting of a β-barrel resulting from the arrangement of 14 amphipathic β-strands (Knapp 2010b). Although these toxins share a similar mechanism of pore formation, ETX is much more potent than aerolysin and *C. septicum* alpha toxin. A main difference is that aerolysin and *C. septicum* alpha toxin recognize GPI-anchored proteins as receptors, whereas the ETX receptor, although localized in lipid rafts, is distinct from GPI-anchored proteins and is distributed in a limited number of cell types. The specific ETX receptor possibly accounts for the high potency of ETX, which also might depend upon a specific intracellular signaling induced by the toxin. Another particularity of ETX compared to the other β-PFTs is its ability to cross the blood-brain barrier, likely mediated by specific receptor interaction. ETX can be considered a neurotoxin, since it targets specific glutamatergic neurons. In contrast to the other bacterial neurotoxins which inhibit the release of neurotransmitter, ETX has an opposite effect by stimulating glutamate release and also acting upon other non-neuronal cells in the CNS. Recently, ETX has been found acting on oligodendrocytes (by a non-pore-forming mechanism) resulting in demyelination. This raises ETX as a possible cause of demyelinating diseases like multiple sclerosis.

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Clostridium perfringens Iota Toxin: A Successfully Shared Template for Common Enteric Pathogens

5

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Abstract

Clostridium perfringens produces a myriad of protein toxins with various modes of action. One of these toxins is iota and composed of two separate proteins (iota A or Ia and iota B or Ib), produced by type E strains, as well as historically associated with animal enteric disease. Other spore-forming bacilli use a similar binary mechanism for intoxicating the intestines of insects, animals, and humans that include: *Clostridium botulinum* (C2 toxin), *Clostridium difficile* (*C. difficile* toxin or CDT), *Clostridium perfringens* (iota toxin and binary enterotoxin), *Clostridium spiroforme* (*C. spiroforme* toxin or CST), as well as *Bacillus cereus* (vegetative insecticidal protein or VIP). These toxins form an AB complex on a cell's surface that consists of ADP-ribosyl transferase (A) and cell-binding (B) components, initially released as separate proteins from the bacterium. Following receptor-mediated endocytosis and endosomal trafficking, the A components mono-ADP-ribosylate globular actin that in turn destroys the cytoskeleton and causes death. The fact that different species, involving two genera, possess iota-like toxins suggests microbial sharing of a common virulence factor evidently important for these bacteria. This review presents the fundamental workings of iota, and other related, binary protein enterotoxins produced by some *Clostridium* and *Bacillus* species.

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Contents

Introduction

Clostridium and *Bacillus* are found throughout nature, particularly in soil, water, and gastrointestinal tracts of diverse mammals and insects (Table 1). Like many other organisms found in varied niches, these bacteria are remarkably adaptable and exploit different biological tools. Both genera grow in low-oxygen environments, but the clostridia are considered "strict" anaerobes with varying aerotolerance, depending upon the species. A distinct characteristic of these genera is spore formation when "times are tough" (i.e., insufficient food, atmosphere, water, etc.), thus enabling long-term survivability. Additionally, these bacteria can be rather prolific at producing different protein toxins with different modes of action.

Pathogenic *Clostridium* and *Bacillus* species cause various diseases that can be similar, yet possess uniqueness as per affected hosts (Table 1). A common pathogenic trait includes protein toxins and spores involved in gas gangrene (severe myonecrosis), food poisoning, antibiotic-associated diarrhea, pseudomembranous colitis, and enterotoxemia (Barth et al. 2004; Borriello and Carman 1983; Lindback and Granum 2015). One group of bacterial binary enterotoxins, related in multiple ways to *C. perfringens* iota toxin, is the primary focus of this review.

The protein components of *C. botulinum* C2 toxin (Ohishi et al. 1980a, b), *C. difficile* toxin (CDT) (Perelle et al. 1997a), *C. perfringens* iota toxin and binary enterotoxin (BEC) (Stiles and Wilkins 1986a, b; Yonogi et al. 2014), *C. spiroforme* toxin (CST) (Popoff et al. 1989), as well as *B. cereus* vegetative insecticidal protein

Table 1 Binary enterotoxin-producing *Clostridium* and *Bacillus*

Organism	Locations found	Disease(s)
C. perfringens	Soil, water, mammalian intestines	Gas gangrene, food poisoning, enterocolitis, antibiotic-associated diarrhea
C. difficile	Mammalian intestines, meat products, pets, water, health-care facilities	Enterocolitis, antibiotic-associated diarrhea
C. spiroforme	Mammalian intestines	Enterocolitis, antibiotic-associated diarrhea
C. botulinum	Soil, water, meat products, honey, mammalian intestines	Food poisoning, infant and adult botulism
B. thuringiensis / *cereus*	Soil, water, insect and mammalian intestines, meat and grain products	Food poisoning, insecticidal

(VIP) (Han et al. 1999) are produced as separate A and B molecules not associated in solution (Barth et al. 2004). This differs from other AB toxins (i.e., *Vibrio cholerae* cholera, *Escherichia coli* heat-labile enterotoxin, and *Shigella dysenteriae* shiga) released by bacteria as holotoxins (Heggelund et al. 2015; Sandvig et al. 2015). Upon binding of the *Clostridium* / *Bacillus* B components to cell-surface receptors, and forming an oligomer, the A component(s) then docks and is brought into the cell as an AB toxin via endocytosis. Once inside the cytosol, enzymatic modification of globular (G) actin by the A component destroys an intoxicated cell via disruption of the filamentous (F) actin-based cytoskeleton (Barth et al. 2015).

The gene locations for these toxins are plasmid (iota, BEC, C2) or chromosome (CST, CDT, VIP), and molecular weights plus modes of action for these toxin components are quite similar: A components (44–52 kDa) and B components (60–81 kDa) (Barth et al. 2004; Yonogi et al. 2014). As just one example, the iota toxin gene contains two open reading frames with 243 noncoding nucleotides separating the Ia and Ib genes. The A and B components, of *Clostridium* and *Bacillus* binary enterotoxins, are secretion products synthesized with a signal peptide of 29–49 and 39–47 residues, respectively (Popoff 2000). However, the C2 and BEC toxins are uniquely linked to sporulation, devoid of a signal peptide, and released into the environment following sporangium lysis (Nakamura et al. 1978; Yonogi et al. 2014). It remains a curious mystery as to why similar, intestinal-acting toxins like the bacterial binary enterotoxins portrayed in this review are produced under quite different conditions (sporulation versus vegetative growth), by the same genus. Further work detailing BEC and the intoxication process remains, as it was only recently described in the literature (Yonogi et al. 2014).

Comparisons of amino acid sequences among the *Clostridium* and *Bacillus* binary enterotoxins reveal structural commonality, as they share: (1) 80–85% identity within the iota toxin family (iota, CDT, CST); (2) 31–40% identity between C2 and iota-family toxins; and (3) 29–31% identity between VIP and equivalent clostridial toxin components, which might suggest common ancestral genes (Barth et al. 2004). In contrast, BEC appears unique and not simply a variant of these

other toxins (Yonogi et al. 2014). Sequence identities between the sporulation-linked C2 and BEC components are only 29% and 41% for their A and B components, respectively (Yonogi et al. 2014). Similar, relatively low sequence identities exist between BEC and the other binary enterotoxins. Overall, the prevalence of binary enterotoxins from two different bacterial genera and diverse species suggests a shared evolutionary success. Furthermore, toxin effects in the intestinal tracts of different mammals and insects are remarkable. The following section briefly describes each bacterium and their binary enterotoxins.

Sporeforming Bacteria and Their Binary Enterotoxins

Clostridium perfringens: Iota Toxin and Binary Enterotoxin of *C. perfringens* (BEC)

Having been described throughout the scientific literature as *Bacillus aerogenes capsulatus*, *Bacillus welchii*, and *Clostridium welchii*, the now known *C. perfringens* was first discovered in 1892 by George Nuttall and William Welch using techniques pioneered by Robert Koch's laboratory in Berlin. Classically, the five serotypes (A–E) of *C. perfringens* are based upon production of one or more lethal, dermonecrotic toxins (alpha, beta, epsilon, and iota) neutralized by type-specific antiserum in animal (mouse lethal, guinea pig dermonecrotic) assays (Barth et al. 2004; Bosworth 1943; McDonel 1980; Stiles and Wilkins 1986a, b). The sporulation-linked enterotoxin (*C. perfringens* enterotoxin or CPE) is a single-chain protein that is also lethal in mice, causes guinea pig erythema upon injection, but is not related to the clostridial binary enterotoxins in this review (Shrestha and McClane 2015). CPE is not part of the typing scheme for *C. perfringens*; however, recent work with BEC might suggest a reconsideration of the classic typing scheme (Yonogi et al. 2014). Genetic methods, instead of animal assays and toxin-neutralizing antisera, involving polymerase chain reaction (PCR) detection of toxin genes are now commonly used by diagnostic laboratories for typing of *C. perfringens* isolates.

The iota toxin was initially described in 1943 (Bosworth 1943), and its binary nature elucidated in the mid-1980s by exploiting cross-reaction (crossed-immuno-electrophoresis) and neutralization (mouse lethal and guinea pig dermonecrotic assays) with *C. spiroforme* antiserum (Stiles and Wilkins 1986a, b). Iota toxin consists of the electrophoretically slow iota a (Ia; 45 kDa) and electrophoretically faster iota b (Ib; 81 kDa) that when combined form a potent cytotoxin (Sakurai and Kobayashi 1995; Stiles and Wilkins 1986a, b). Ia is an ADP-ribosyltransferase using nicotinamide adenine dinucleotide (NAD) as substrate to transfer a mono-ADP-ribosyl group onto arginine, specifically R177, found in muscle and nonmuscle types of G-actin (Barth et al. 2015; Schering et al. 1988). Ultimately, this enzymatic activity disrupts the cytoskeleton and destroys normal cell functions. The many roles of Ib include: (1) binding to a cell-surface protein(s) on a targeted cell,

(2) forming an oligomeric platform, (3) docking with Ia, and (4) subsequently translocating the latter into the cytosol via acidified endosomes (Barth et al. 2004; Blöcker et al. 2001; Papatheodorou et al. 2011; Stiles et al. 2000; Wigelsworth et al. 2012). Once in the cytosol, containing NAD and G-actin, the A component is quite efficient and can cause in vitro cell rounding within hours.

Historically, the iota toxin is exclusively produced by type E strains implicated in some diarrheic outbreaks among calves and lambs (Bosworth 1943; McDonel 1980; Redondo et al. 2015). Besides releasing nutrients from iota-intoxicated cells lining the gastrointestinal tract, recent studies show that the iota toxin and/or metabolites (bacteriocin?) of *C. perfringens* type E promote its own colonization while preventing growth (in vitro)/adherence (in vitro and in vivo) of *C. perfringens* type A (Redondo et al. 2015). This mechanism, which is inactivated to varying degrees by proteases or heat, likely shares some attributes with that described below for *C. difficile* CDT (Gerding et al. 2014; Schwan et al. 2009, 2014).

Like the other binary enterotoxins described in this review, iota toxin requires proteolytic activation (Gibert et al. 2000). It was subsequently discovered, after cloning and sequencing of the iota toxin gene, that proteolytic activation of Ib protomer (Ibp) into Ib occurs at A211 (Perelle et al. 1993) which then facilitates Ia docking (Stiles et al. 2000), formation of temperature- and voltage-dependent, cation-selective channels (K^+ efflux, Na^+ influx) (Knapp et al. 2002), as well as SDS-stable heptamers on cell membranes (Stiles et al. 2002) and in solution (Blöcker et al. 2001). Furthermore, the Ia molecule is also proteolytically activated with a resultant loss of 9–13, N-terminal amino acids (Gibert et al. 2000). However, the purpose of proteolysis upon Ia is unknown but may include increased enzymatic activity and/or docking to Ib, more efficient passage into the cytosol from the endosome, etc. Proteolysis has not been described for A components from other *Clostridium* and *Bacillus* binary enterotoxins. It is known that secreted AB holotoxins not related to the *Clostridium* and *Bacillus* binary enterotoxins, such as *E. coli* heat labile, *S. dysenteriae* shiga, and *V. cholerae* cholera enterotoxins, also have enzymatic A components processed by serine-type proteases. Following proteolysis, these other A components form A_1 and A_2 subunits linked by a disulfide bond not characteristic of Ia or other *Clostridium* and *Bacillus* binary enterotoxins (Heggelund et al. 2015; Sandvig et al. 2015).

Interestingly, the binding of only Ib (100 ng/ml) to select cell types can cause rapid loss of ATP (80% loss within 60 min), swelling, and necrotic death (Nagahama et al. 2011). Why other cell types (i.e., Vero and Caco-2) susceptible to iota toxin (Ia + Ib) show no ill effects with Ib alone is not understood. Overall, this is a unique finding not fitting the classic synergy (A + B)-based paradigm for *Clostridium* and *Bacillus* binary enterotoxins. Furthermore, this discovery is not dependent upon lipid-raft based oligomerization, unlike that previously described for iota toxin or CDT by different groups (Hale et al. 2004; Nagahama et al. 2004; Papatheodorou et al. 2013). Yet unexplored (at least not reported to date), it is possible that other *Clostridium* / *Bacillus* binary enterotoxins might yield equivalent results and promote new avenues of research.

In addition to the iota toxin, Yonogi and colleagues describe a novel, binary enterotoxin designated as BEC that is produced by different type A isolates implicated in separate outbreaks of food-borne gastroenteritis in Japan (Yonogi et al. 2014). BEC contains two different proteins [BECa (47 kDa) and BECb (80 kDa)] lacking sequence homology with the single-chain CPE, so commonly associated with type A food-poisoning outbreaks; however, like CPE, BEC is produced during sporulation and devoid of a leader sequence. Cultural conditions for BEC production involve Duncan-Strong medium used specifically for sporulation of *C. perfringens*. Crude culture supernatants from BEC-producing isolates, which lack CPE, cause fluid accumulation in rabbit intestinal loops. Purified BECa and BECb when combined, or administration of BECb alone, causes fluid accumulation in suckling mice.

BEC is encoded on a large (54.5 kb) plasmid containing 55 open reading frames (ORFs), in which 39 ORFs encode for proteins of unknown function. The authors screened 36 other *C. perfringens* isolates (human intestinal origins) for BEC genes and found only one positive, suggesting to date minimal spread of BEC genes within this *C. perfringens* population. There is respectively 36–43% and 28–44% sequence identity of BECb and BECa with complimentary components of other *Clostridium* and *Bacillus* binary enterotoxins, suggesting BEC to be unique. BEC, like all other *Clostridium* and *Bacillus* binary enterotoxins presented in this review, modifies G-actin via ADP-ribosylation using NAD as substrate.

Clostridium spiroforme Toxin (CST)

During the late 1970s, *C. perfringens* was erroneously tagged as the lethal culprit for some rabbit diarrheal outbreaks because type E antiserum neutralized the cytotoxic effects of cecal contents (Borriello and Carman 1983; Carman and Evans 1984). However, *C. perfringens* type E was never isolated, and in 1983 a strong correlation was firmly established between disease and presence of *C. spiroforme* (Borriello and Carman 1983; Carman and Evans 1984).

The distinctly coiled *C. spiroforme* also causes diarrhea like *C. perfringens*, but in rabbits and not other animals or humans, which is ironic as *C. spiroforme* was first isolated from human feces (Borriello and Carman 1983; Kaneuchi et al. 1979). Rabbits are most susceptible to *C. spiroforme*-induced diarrhea while experiencing stress that includes lactation, old age, weaning, and changes in diet (Borriello and Carman 1983). The Sa (44 kDa) and Sb (76 kDa) components of CST are, respectively, analogous to Ia and Ib of the iota toxin, as first determined in the mid-1980s by crossed-immunoelectrophoresis and neutralization studies with *C. perfringens* type E antiserum (Borriello and Carman 1983; Popoff et al. 1989; Stiles and Wilkins 1986a, b). Furthermore, the components of CST and iota toxin are interchangeable and form biologically-active chimeras that suggest analogous structures (Perelle et al. 1997b).

Clostridium difficile Toxin (CDT)

C. difficile was first described in 1935 as *Bacillus difficilis*, because of difficulties in isolating the bacterium in the laboratory, and at the time deemed normal intestinal flora in healthy infants (Hall and O'Toole 1935). Thoughts of this newly described bacillus being a pathogen, especially for adults, were nonexistent at the time but would change with increasing reliance upon antibiotics to fight bacterial disease. It was not until the mid-1970s that the toxin-linked pathogenicity of *C. difficile* enterocolitis was associated with antibiotics (clindamycin in particular) and adults, costing the health-care industry and patients billions of dollars every year (Bartlett et al. 1978). Akin to the *C. spiroforme* story above, *Clostridium sordelli* was initially thought to be the pathogenic culprit as antiserum towards large-molecuar weight toxins (not related to binary enterotoxins reviewed here) of *C. sordelli* neutralized the cytotoxic effects of stool samples actually containing cross-reacting *C. difficile* toxins (Bartlett et al. 1978).

The rise of *C. difficile* as a nosocomial and community-acquired pathogen can be attributed to multiple factors that include increased awareness, dynamic bacterial genome, prevalence of spores in an environment, long-term care residency, and use of proton pump inhibitors as well as antibiotics (Loo et al. 2011). Hypervirulent strains of *C. difficile* have emerged over the past 15 years, causing increased morbidity and mortality particularly in health-care settings (Gerding et al. 2014). Traits of these hypervirulent strains can include those that: (1) possess CDT; (2) are more antibiotic resistant; (3) more readily sporulate; and (4) have increased production of large molecular weight toxins A and B, not related to CDT. A hallmark of severe disease caused by *C. difficile* consists of a pseudomembrane containing white blood cells, fibrin, mucin, and bacteria. Recurring bouts of *C. difficile* colitis are quite problematic, and life-threatening, for some unfortunate patients.

Besides humans, animals used for food production (cattle, chickens, pigs, rabbits, sheep), wildlife (elephants), and even pets (cats, dogs) can be colonized by *C. difficile* (Keessen et al. 2011). However, transmission of *C. difficile* via food or pets is still not confirmed to date. Treatment options (in practice and experimental) are few but include ironically antibiotics (i.e., vancomycin, metronidazole, or more recently fidaxomicin), fecal flora transplant, as well as intravenous immunoglobulins and vaccines that target *C. difficile* toxins (Seekatz et al. 2014; Shah et al. 2014).

Like iota toxin and CST, there is much structural homology [i.e. 80% and 82% sequence identity of CDTa (48 kDa) and CDTb (75 kDa) with *C. perfringens* Ia and Ib, respectively] (Perelle et al. 1993, 1997a). These three toxins (iota, CST, and CDT) represent the iota family that does not include *C. perfringens* BEC, *C. botulinum* C2 toxin, or *B. cereus* VIP (Barth et al. 2004). Additional structural commonalities of CDT with other iota-family toxins involve interchangeable protein components and biologically active chimeras (Gülke et al. 2001;

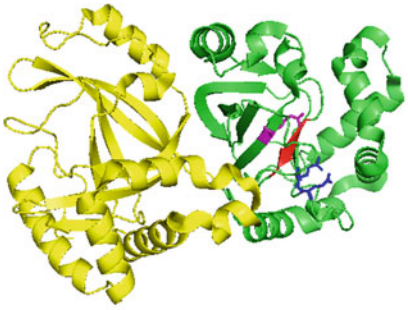
Perelle et al. 1997b; Popoff et al. 1989). Interestingly, *C. difficile*, *C. perfringens*, and *C. spiroforme* are all linked to toxin-induced, gastrointestinal diseases in humans and/or animals (Borriello and Carman 1983; Bosworth 1943; Gerding et al. 2014; McDonel 1980). Although not described in the literature, it is plausible that co-colonization by two species of binary enterotoxin-producing clostridia could result in chimeric-toxin damage to the gastric epithelium in vivo.

Clostridium botulinum C2 Toxin

C. botulinum (*Bacillus botulinus*) was first described in 1895 by van Ermengem following human consumption of contaminated sausage (Devriese 1999). Like *C. perfringens* toxins (alpha, beta, epsilon, and iota), the neurotoxin types (A–G) of *C. botulinum* are produced during vegetative growth and classically determined by mouse lethal assays with toxin-specific antisera (McDonel 1980; Poulain et al. 2015). The non-neurotoxic C2 toxin is produced during sporulation by types C and D (Nakamura et al. 1978), like CPE and BEC of *C. perfringens* (Shrestha and McClane 2015; Yonogi et al. 2014). Pioneering work on the cell-binding and translocation component (C2II; 60 kDa), as well as enzyme component (C2I; 49 kDa), of C2 toxin was initiated in the late 1970s and the first describing synergy of any *Clostridium* or *Bacillus* binary enterotoxin (Nakamura et al. 1978; Ohishi et al. 1980b). Another scientific leap first attributed to C2 toxin was made in 1986 when the intracellular target was identified as G-actin, via ADP-ribosylation of R177 (Aktories et al. 1986; Ohishi and Tsuyama 1986). These collective findings from different groups in Japan and Germany were ground breaking, further promoting a deeper understanding of how bacterial toxins can alter cell functions. With additional knowledge comes the opportunity to harness these proteins for medicinal purposes, largely unrealized to date and certainly a fertile field for the future (Aktories and Schmidt 2015).

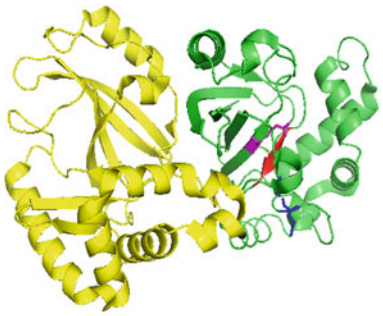
Bacillus cereus / *Bacillus thuringiensis* Vegetative Insecticidal Proteins (VIPs)

The *B. cereus* / *B. thuringiensis* VIPs kill corn pests (i.e., Northern and Western corn rootworms) but do not affect animals or humans (Bravo et al. 2015; Han et al. 1999; Lindback and Granum 2015). The *B. cereus* / *Bacillus thuringiensis* VIPs are composed of VIP1 (~86 kDa B component) and VIP2 (~54 kDa A component) produced during the growth, versus sporulation, phase (Bravo et al. 2015; Han et al. 1999; Yu et al. 2011). In addition to its insect killing properties, *B. cereus* can cause human food poisoning via other protein toxins not pertinent for this review (Lindback and Granum 2015). Relative to the clostridial binary enterotoxins, there is less published literature for the VIPs.



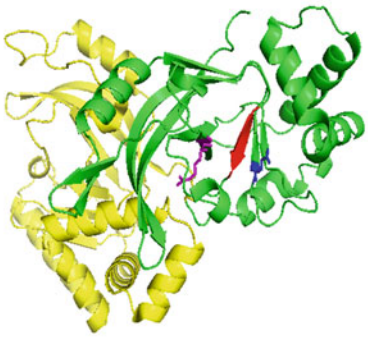
Iota A

N-terminal (yellow) and C-terminal (green) domains. S338-T-S340 motif (red), R295 (magenta), and E378-X-E380 motif (blue) involved in enzymatic activity.



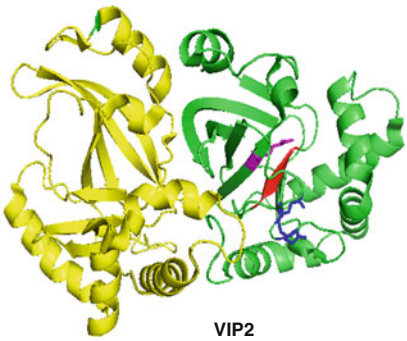
CDTa

N-terminal (yellow) and C-terminal (green) domains. S345-T-S347 motif (red), R302 (magenta), and E385-X-E387 motif (blue) involved in enzymatic activity.



C2-I

N-terminal (yellow) and C- terminal (green) domains. S348-T-S350 motif (red),R299 (magenta), and E387-X-E389 motif (blue) involved in enzymatic activity.



VIP2

N-terminal (yellow) and C-terminal (green) domains. S386-T-S388 motif (red), R349 (magenta), and E426-X-E428 motif (blue) involved in enzymatic activity.

Fig. 1 Crystal structures for iota a [Sakurai et al. 2009; 1.8 Å resolution, Protein Data Bank (PDB) ID = 1GIR]; CDTa (Sundriyal et al. 2009; 2.0 Å, PDB = 2WN6); C2-I (Schleberger et al. 2006; 2.3 Å, PDB = 2J3Z); and VIP2 (Han et al. 1999; 1.5 Å, PDB = 1QS1). The only B component available to date is C2II (not shown here) and of lower quality versus aforementioned A components (Schleberger et al. 2006; 3.1 Å, PDB = 2 J42). Molecular Modeling Database (MMDB) provided by the National Center for Biotechnology Information was used to generate these structures (Madej et al. 2014)

Basic Structure of *Clostridium* and *Bacillus* Binary Enterotoxins

Crystal structures are available for the A components of iota, CDT, C2, and VIP toxins but in contrast, there is data for only one B component: C2II (Han et al. 1999; Sakurai et al. 2009; Schleberger et al. 2006; Sundriyal et al. 2009) (Fig. 1). The structure and function of these toxins have been further explored by different groups using various techniques as briefly described below.

C. botulinum C2II and C2I

The C-terminus of C2II (and other *Clostridium* / *Bacillus* binary enterotoxins) facilitates receptor-mediated binding on the cell surface, as deletion of only seven C-terminal residues effectively prevents C2IIa interactions with cells (Blöcker et al. 2000). In this same study, antisera specific for the C-terminus (domain 4; residues 592–721), but not domains 1 (residues 1–264) or 3 (residues 490–592), block C2IIa binding to cells. Deletion studies focused upon the N-terminus of C2II reveal that residues 1–181, naturally cleaved from the C2II precursor to generate C2IIa, may assist in proper folding. Replacements within domain II (amino acids 303–331) can negate voltage-gating of C2II-induced pores in membranes, but do not affect translocation of C2I into the cytosol (Blöcker et al. 2003).

There are minimal conformational effects upon C2I when crystallized at either pH 3 (a value that mimics the acidic environment of an endosome) or 6.1 (Schleberger et al. 2006). The Stokes radius minimum of C2I is 40 Å, while the inner diameter of oligomeric C2II is maximally 32 Å, suggesting an unfolding (or threading) of C2I through the C2II-pore and into the cytosol with the help of host-cell provided chaperones (Ernst et al. 2015; Haug et al. 2003; Kaiser et al. 2011, 2012). Positioning of the N- and C-terminal domains of C2I are quite similar to that found on Ia and VIP2 (Han et al. 1999; Sakurai et al. 2009; Schleberger et al. 2006).

C. perfringens Ib and Ia

Like C2II, there are also four domains on Ib as determined by similar techniques involving deletion mutagenesis and antibodies (Marvaud et al. 2001; Stiles et al. 2002). Cleavage of the C-terminal ten amino acids (part of domain 4) or use of Ib peptides ($\geq$200 C-terminal residues) as competitive inhibitors prevent Ib binding to Vero cells (Marvaud et al. 2001). A commonality of the *Clostridium* and *Bacillus* binary enterotoxins is that the C-terminal region of B components interacts with cell-surface receptor, even if the latter is different (as per C2 and iota-family toxins). Furthermore, two monoclonal antibodies (Mabs) recognizing unique Ib epitopes within C-terminal residues 632–655 afford protection against iota cytotoxicity via different mechanisms. One Mab prevents Ib binding to cells while the other does not, yet the latter efficiently prevents Ib oligomerization on the cell surface (Marvaud et al. 2001; Stiles et al. 2002). On the other end, deletion of just 27 N-terminal residues (domain 1) from Ib prevents docking of Ia.

There is no crystal structure available for Ib, unlike Ia (Sakurai et al. 2009) (Fig. 1). Ia consists of two domains that share conformational, but little sequence, similarity. Other A components in this binary enterotoxin family also have this same shared structure, typical of ADP-ribosyl transferases targeting actin. Actin is highly conserved throughout the biosphere (Barth et al. 2015), and enzymes that ADP-ribosylate actin at a similar site (R177) likely have many common attributes. For example, the catalytic C-domain of Ia (residues 211–413) (Perelle et al. 1993) and VIP2 (266–461) (Han et al. 1999) contains 40% sequence identity and similar

surface charges. However, the bacteria (*Clostridium* versus *Bacillus*) that make these ADP-ribosyl transferases, and their residing niches (mammalian versus insect intestines), are quite different. One obvious difference between Ia and VIP2 is the spatial orientation of the first glutamic acid found within the conserved catalytic motif, EXE. An STS motif is also located near the active site of Ia and found in other ADP-ribosyltransferases (Sakurai et al. 2009). Amino acid changes within Ia focused upon the NAD binding cavity reveal that Y246 and N255 are important for ADP-ribosyltransferase, but not NAD-glycohydrolase, activity. All ADP-ribosyltransferases within the *Clostridium* and *Bacillus* binary enterotoxin family (BECa, C2I, CDTa, Ia, Sa, and VIP2) target G-actin, a conserved protein involved in multiple cellular functions critical for life (Aktories et al. 1986; Barth et al. 2015; Ohishi and Tsuyama 1986; Perelle et al. 1997a; Popoff and Boquet 1988; Sakurai et al. 2009; Schering et al. 1988; Yonogi et al. 2014).

C. difficile CDTb and CDTa

In contrast to the C2 and iota toxins, less biochemical work has been done with the CDT components which are more recently discovered. Structure-function studies show that the same amino acids in Ia are necessary for enzymatic activity of C2I and CDTa, which, respectively, share 40% and 84% overall sequence identity with Ia (Gülke et al. 2001; Perelle et al. 1993; Popoff 2000). The EXE motif of ADP-ribosyltransferases, part of the ADP-ribosyl turn-turn (ARTT) loop important for stabilizing substrate-enzyme complexes, differ regarding substrate contact made by Ia and CDTa (Sakurai et al. 2009; Sundriyal et al. 2009). The E385 and E387 (EXE motif) of CDTa do not make contact with NAD or NADPH, unlike the equivalent E378 and E380 of Ia. The STS motif found in CDTa is important in ligand binding and possibly catalysis.

Unlike the commonalities within the C-terminal, enzyme-critical regions of *Clostridium* and *Bacillus* binary enterotoxins, the N-terminal domains of these A components can be more variable in structure. The N-terminal region of an A component is generally used for docking to its B. For example, Ia (residues 1–210) and an equivalent region in VIP2 (residues 60–265) contain only 20% sequence identity, dissimilar surface charges, and different conformations (Han et al. 1999; Perelle et al. 1993; Sakurai et al. 2009; Yu et al. 2011). Relative to enzymatic components of other binary toxins, the Ib docking region on Ia is more centrally located (residues 129–257) (Marvaud et al. 2002; Sakurai et al. 2009), versus C2I (residues 1–87) for C2II (Barth et al. 2002; Schleberger et al. 2006) or CDTa (residues 1–240) for CDTb (Gülke et al. 2001).

Like Ib, CDTb depends upon similar C-terminal (domain 4) residues (757–868) for low-nanomolar affinity binding to cell-surface receptor (lipolysis-stimulated lipoprotein or LSR) via the latter's N-terminal, immunoglobulin-like region (Hemmasi et al. 2015; Marvaud et al. 2001). Unlike the Ib studies using His-tag constructs, the CDTb constructs were GST derivatives, and this might explain varying results with C-terminal truncates. For example, Ib binding is lost with

deletion of just ten C-terminal residues which in turn decreases, but does not abrogate, CDTb binding to receptor.

Basic Modes of Intoxication for *Clostridium* and *Bacillus* Binary Enterotoxins

Step One: Binding to Cells

To access the cytosol and G-actin, the *Clostridium* and *Bacillus* binary enterotoxin family must first bind to a targeted cell using B components and their receptor(s)-specific qualities, forming an oligomeric complex (Barth et al. 2004, 2015) (Fig. 2). The latter acts as a platform for docking of A that generates a biologically active holotoxin ready for cell entry. Surface plasmon resonance studies show nicely that C2I docks only with oligomeric, not monomeric, C2IIa (Nagahama et al. 2009). Important for B oligomerization are lipid rafts (cholesterol-rich microdomains) naturally found on the eukaryotic cell membrane (Hale et al. 2004; Nagahama et al. 2004; Papatheodorou et al. 2013). Results suggest that *C. perfringens* Ib, but not Ibp, localizes into these membrane microdomains on Vero cells that are

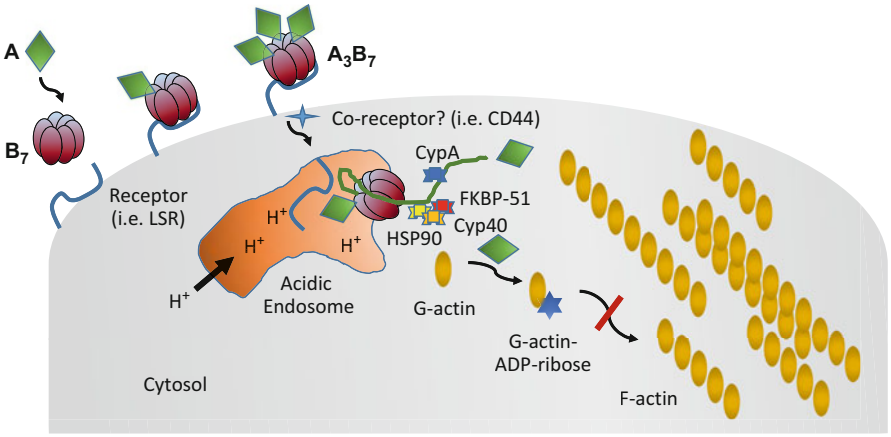


Fig. 2 Schematic showing cell-surface binding and internalization of *Clostridium* and *Bacillus* binary enterotoxins. The heptameric B components bind to their respective receptor (i.e., LSR) on the cell surface. A components (maximally 3 per heptamer) bind to B_7 and the toxin complexes are internalized via receptor-mediated endocytosis. CD44 might play a role as a coreceptor for the iota-like toxins. In acidic endosomes, the B_7 proteins insert as pores into endosomal membranes and the unfolded A components translocate into the cytosol. This step is facilitated by host-cell chaperones that include Hsp90, cyclophilins (Cyps) A and 40, as well as FK506 binding protein (FKBP)-51. In the cytosol, the enzymatic A components then ADP-ribosylate G-actin which results in depolymerization of F-actin, cell rounding, and finally cell death

susceptible to iota toxin (Hale et al. 2004; Nagahama et al. 2004). Similar studies with C2IIa yield results akin to Ib and include activation of the phosphatidylinositol 3-kinase pathway (Nagahama et al. 2009). This same pathway is also exploited for cell entry by various intracellular pathogens.

Of all the binary toxins portrayed in this review, the first definitive efforts for binding to cells were published with C2IIa which possesses unique hemagglutinating properties competitively inhibited by *N*-acetylgalactosamine, *N*-acetylglucosamine, L-fucose, galactose, or mannose (Sugii and Kozaki 1990). Pretreatment of human erythrocytes with proteases prevents C2IIa-induced hemagglutination and suggests a glycoprotein receptor. Chemical mutagenesis of CHO cells generates C2 (not iota) resistance because the cells subsequently lack *N*-acetylglucosaminyltransferase I, necessary for asparagine-linked carbohydrates (Eckhardt et al. 2000). Additionally, pretreatment of cells with various lectins or glycosidases does not affect Ib binding, suggesting that the receptor (or part of) for Ib is not a carbohydrate (Stiles et al. 2000).

In contrast to C2IIa, which binds and facilitates C2I-mediated cytotoxicity in all tested vertebrate cells (Barth et al. 2015; Eckhardt et al. 2000), the cell-surface receptor for Ib is not as ubiquitous (Stiles et al. 2000). Recent studies by Papatheodorou and colleagues elegantly reveal LSR as cell-surface receptor for the iota-family toxins (CDT, CST, and iota) (Papatheodorou et al. 2011, 2012, 2013). LSR is a transmembrane lipoprotein found in various tissues and naturally facilitates lipoprotein clearance and tight junction formation, as well as plays a critical role in cell development. It is to date unknown if LSR plays a further role during iota-family intoxications. For example, does LSR actively participate in pore formation with iota-family B components? It is known that CDTb and nonproteolytically activated CDTb promote clustering of LSR into lipid rafts on both HeLa and Caco-2 cells (Papatheodorou et al. 2013). Normally, LSR is evenly distributed throughout the cell membrane.

Additional studies provide evidence that CD44 [also known as homing cell adhesion molecule (HCAM)] promotes intoxication of iota-family toxins, although there is no physical link evident between CD44 and LSR (Wigelsworth et al. 2012). Like LSR, the CD44 glycoprotein is also a single-pass transmembrane protein with multiple functions that include: (1) receptor for multiple ligands; (2) signal transduction; and (3) being exploited by some bacteria and viruses for cell entry. There are ten known isoforms of human CD44, which vary within the exteracellular stem via gene splicing. Identification of LSR and CD44 involvement in iota-family toxin entry of cells was made using different methodologies (Blonder et al. 2005; Papatheodorou et al. 2011, 2012, 2013; Wigelsworth et al. 2012), and how these proteins might interact to affect intoxication remains a mystery. Like LSR and CD44, the two known receptors (tumor endothelial marker 8 and capillary morphogenesis gene 2) for *B. anthracis* protective antigen are also type I transmembrane proteins, which differ approximately three-log fold in binding affinity (Liu et al. 2015; Wigelsworth et al. 2012). To date, receptor-binding studies for *B. cereus* VIP1 have not been reported in the literature.

Step Two: AB Complex Formation and Entry

The N-terminal regions of A and B components for each *Clostridium* and *Bacillus* binary enterotoxin facilitate AB docking and complex formation on the cell surface. The mole ratio of A:B is unclear for these binary enterotoxins, but a 3(4):7(8) mole ratio of edema or lethal factors to protective antigen is respectively present for the *B. anthracis* edema and lethal toxins (Liu et al. 2015). After binding to a surface receptor, intracellularly-acting bacterial toxins have employed two major pathways for internalization. Retrograde routing through the Golgi apparatus and endoplasmic reticulum is used by other AB toxins like *S. dysenteriae* shiga (Sandvig et al. 2015) and *V. cholerae* cholera (Heggelund et al. 2015). Secondly, translocation from acidified early endosomes is employed by diphtheria toxin (Gillet and Barbier 2015), *B. anthracis* edema/lethal toxins (Liu et al. 2015), and the *Clostridium* / *Bacillus* binary enterotoxins (Fig. 2). Translocation of A components for C2, iota, or the edema/lethal toxins across the endosomal membrane is blocked by bafilomycin A, a small molecular weight compound which inhibits vacuolar-type ATPases that acidify the endosome (Barth et al. 2000, 2015; Blöcker et al. 2001; Liu et al. 2015). Decreased pH evidently induces conformational changes that promote membrane insertion of the heptameric B component, followed by translocation of the A component(s) through the B-induced pore across the endosomal membrane.

To enter the cytosol, A components of C2, CDT, and iota toxins use chaperones such as heat shock protein 90 (Hsp90, an essential ATPase that regulates cell signaling) and protein-folding enzymes that include the peptidyl-prolyl *cis/trans* isomerases like cyclophilin A (CypA) and cyclophilin 40 (Cyp40) (Barth et al. 2015; Ernst et al. 2015; Haug et al. 2003; Kaiser et al. 2011), as well as FK506 binding protein (FKBP) 51 (Kaiser et al. 2012) (Fig. 2). Cell entry by *Clostridium* and *Bacillus* binary enterotoxins is covered much more extensively in another chapter of this book.

Step Three: The Coup de Grace Applied Via ADP-Ribosylation and Destruction of the Actin Cytoskeleton

Once A components of any *Clostridium* or *Bacillus* binary enterotoxin enter the cytosol, G-actin becomes mono-ADP-ribosylated at R177 which in turn disrupts F-actin formation and the cytoskeleton (Barth et al. 2015). This mechanism for cell destruction is quite conserved by diverse bacteria, and in fact there can be different targets in a cell modified by ADP-ribosylation depending upon the toxin. All known ADP-ribosylating toxins use NAD as a source of ADP-ribose. Four bacterial groups of ADP-ribosylating toxins exist, and their eukaryotic targets include: (1) elongation factor 2; (2) heterotrimeric G-proteins; (3) Rho and Ras GTPases; and (4) G-actin (Barth et al. 2015; Heggelund et al. 2015; Sandvig et al. 2015). Actin (~42 kDa, G monomer) is found in all eukaryotic cells from diverse species (Barth et al. 2015) and critical for cytoskeletal formation (F-actin filaments) involved in maintenance of cell

structure, homeostasis, as well as the immune system. Bacteria also possess an actin-like molecule that forms intracellular filaments, although its role is less well defined versus actin's role in eukaryotes. Microbes exploit the actin cytoskeleton of eukaryotes in various ways that include cell entry, intra- and inter-cellular movement, and causing cell death that releases nutrients into the environment (Barth et al. 2015). Also, from a research perspective, actin-modifying toxins can be used as biochemical tools for studying the cytoskeleton and homeostasis of eukaryotic cells (Aktories and Schmidt 2015).

The actin-ADP-ribosylating enterotoxins of *Clostridium* and *Bacillus* form two groups. C2 toxin, the first bacterial toxin discovered to mono-ADP-ribosylate G-actin, exclusively modifies R177 on beta/gamma-nonmuscle, as well as gamma-smooth muscle, isoforms (Aktories et al. 1986, 2012; Barth et al. 2015; Ohishi and Tsuyama 1986; Schering et al. 1988; Vandekerckhove et al. 1987). The iota-family toxins are less discriminating and mono-ADP-ribosylate R177 found on all G-actin isoforms (Barth et al. 2015). Modification of actin by the recently discovered *C. perfringens* BEC likely occurs at R177, but remains unconfirmed to date (Yonogi et al. 2014). F-actin does not represent a substrate target for any of these bacterial binary toxins. Furthermore, the iota, CDT and C2 toxins induce microtubule-/calcium signaling-based protrusions from intestinal epithelial cells in just 90 min after toxin exposure (Schwan et al. 2009, 2014). Length of these cellular extensions is concentration dependent, as low amounts of toxin cause longer protrusions versus high toxin concentrations that elicit more rapid protrusions. Results with CDT reveal vesicles containing fibronectin traveling from the basolateral membrane to apical protrusions (namely base and tip), ultimately promoting bacterial adherence (presumably via a fibronectin-binding adhesin) that then delays bacterial expulsion from the host (Schwan et al. 2014). The intracellular fibronectin concentrations are also decreased in Caco-2 cells following CDT or C2 toxin exposure.

Conclusions and Future Directions

The *Clostridium* and *Bacillus* binary enterotoxins possess common characteristics that target the actin cytoskeleton of eukaryotic cells, primarily those found in the gastrointestinal tracts of mammals and insects. The discovery of *C. perfringens* iota toxin in 1943 was the first for any *Clostridium* or *Bacillus* binary enterotoxin (Bosworth 1943). The binary nature of *C. botulinum* C2 toxin, *C. perfringens* iota toxin, *C. spiroforme* CST, *C. difficile* CDT, *B. cereus* VIP, and *C. perfringens* BEC were respectively discovered in 1980 (Ohishi et al. 1980a), 1986 (Stiles and Wilkins 1986a, b), 1988 (Popoff and Boquet 1988), 1997 (Perelle et al. 1997a), 1999 (Han et al. 1999), and 2014 (Yonogi et al. 2014). As per the recent discovery in Japan of BEC from human food-poisoning strains of *C. perfringens* type A, there are likely similar toxins produced by other bacterial species not yet found. Further exploration by researchers employing gene probes and specific toxin antibodies will no doubt unveil new binary enterotoxins composed of separate AB components produced by

other bacteria, perhaps from different genera. Use of these binary enterotoxins as tools for further studying the biochemistry of cells, and as therapeutic vehicles, represent just two research areas ripe for future advancements (Aktories and Schmidt 2015).

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Role of *Clostridium perfringens* Alpha, Beta, Epsilon, and Iota Toxins in Enterotoxemia of Monogastrics and Ruminants

6

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Abstract

Clostridium perfringens produce several virulence factors to increase colonization and improve nutrient availability. Enterotoxins are among these virulence factors, and while some only have local effects, others can act at a distance from the bacterial colonization site. Enterotoxemias are defined as diseases caused by toxins generated in the intestine and absorbed into systemic circulation with systemic consequences. The term enterotoxemia is generally used indistinctly to name enteric and/or systemic diseases, but under the definition of enterotoxemia, several aspects of intestinal bacterial diseases include pathogenesis and toxin modes of action. The present aim is to describe some enterotoxemia-associated toxins, focusing on those which clearly produce systemic and enteric effects, as well as those commonly thought to produce enterotoxemia but remain questionable upon further consideration of the existing evidence.

Keywords

Clostridium perfringens • Enterotoxemia • Enteritis • Alpha toxin • Beta toxin • Epsilon toxin • Iota toxin

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Contents

Introduction

Toxin production by bacteria represents one of the most potent and widespread mechanisms of pathogenicity. Enteropathogenic bacteria, like *Clostridium perfringens*, produce several virulence factors that include adhesins and enterotoxins, which increase mucosal colonization and cause cell alterations. Enterotoxins are produced by bacteria in the lumen of the gastrointestinal tract, and while some of these toxins have only local digestive effects, others can have systemic effects and act at a distance from the bacterial colonization site. By definition, enterotoxemias are diseases produced by enterotoxins absorbed into systemic circulation, which acts on distant organs such as the brain.

Although different toxinogenic bacteria can produce enterotoxemias, the ordinary use of the term "enterotoxemia" is a synonym of clostridial intestinal disease. The bacterial genus *Clostridium* includes several enteric pathogens, and while it is true that many clostridial intestinal diseases are mediated by specific toxins with an intestinal origin, most of them are doubtfully or incorrectly called enterotoxemias. In particular, *C. perfringens* is a widely spread enteropathogen with an intimidating toxin arsenal; many of these toxins are produced in the intestinal lumen. While some of these toxins act only locally, others act both locally and systemically. Despite enterotoxemia being generally used indistinctly to name clostridial/*C. perfringens* enteric and/or systemic diseases, enterotoxemia includes several pathogenic aspects of intestinal bacterial diseases and toxin modes of action.

For example, epsilon toxin (ETX) is produced by intestinal *C. perfringens* type D and generates an enterotoxemia in sheep, since in this species ETX exerts damage on distant organs such as the brain and lungs, with no evident intestinal alterations. In goats the same ETX, also secreted by type D strains, causes enterocolitis without systemic effects. Therefore, precisely defined enterotoxemias, meaning diseases produced by systemically acting toxins absorbed from the intestines, should be limited to a particular group of diseases caused by certain bacterial toxins in determined animal species under particular conditions.

Under these concepts, a true enterotoxemia should be defined as a disease caused by an individual toxin with the ability to induce both intestinal and systemic effects and where the intestine alterations contribute to the onset of systemic disease. These systemic diseases of intestinal origin are mainly produced by an identified, and usually potent, protein toxin. From a strict point of view, an "enterotoxemic" toxin, when present in the intestinal lumen, should induce mild to severe modifications of the intestinal functions, with or without the requirement of a determined host or environment conditions. Intestinal changes should favor toxin translocation into the systemic bloodstream. Once in the circulatory system, the toxin should reach target organs, such as kidney or brain, causing damage.

Among enterotoxemic toxins, *C. perfringens* ETX and *E. coli* Shiga toxin (STX) are two of the most potent toxins known, responsible for important diseases with major economic and health impacts. The following pages aim to review the role of *C. perfringens* major toxins in associated diseases, questioning disease definition of those commonly thought to produce enterotoxemia.

Clostridium perfringens Major Toxins and Enterotoxemias

Clostridia produce the highest number of toxins of any bacterium known, and these toxins are involved in severe diseases of both humans and animals. *Clostridium perfringens* is considered the most widely occurring pathogenic bacterium and the most important cause of clostridial enteric disease in domestic animals (McClane et al. 2006; Songer 1996). Virulence of *C. perfringens* is mostly mediated by its intimidating toxin arsenal. Differences in toxin production between different strains allow classification of *C. perfringens* isolates into five toxinotypes (A, B, C, D, and E) (Table 1), based upon the production of four typing (major) toxins (Table 2): alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) (McClane et al. 2006; Uzal et al. 2014).

In the literature, all toxinotypes of *C. perfringens* are considered responsible for enterotoxemias in ruminants and monogastrics. *C. perfringens* type A are regularly recovered both from the intestinal tract of animals and environment, while others (types B, C, D, and E) are less common in the intestinal tract of animals and can occasionally be found in the environment in areas where diseases produced by these organisms are enzootic (McClane et al. 2006). Because it is a frequent postmortem

Table 1 Classification of *Clostridium perfringens* strains base on the production of major toxins

Toxin	Gene location	Toxinotype				
		A	B	C	D	E
Alpha (CPA)	Chromosome	+	+	+	+	+
Beta (CPB)	Plasmid	–	+	+	–	–
Epsilon (ETX)	Plasmid	–	+	–	+	–
Iota (ITX)	Plasmid	–	–	–	–	+

Table 2 Properties of major toxins produce by *Clostridium perfringens*

Toxin	Molecular mass (kDa)	Mouse LD_{50}	Route (ref.)	Biologic activity
Alpha (CPA)	43	1 μg	i.p. (Titball et al. 1991)	Phospholipase C and sphingomyelinase activity
Beta (CPB)	35	0.06 μg	i.v. (Sakurai and Fujii 1987)	Pore-forming toxin
Epsilon (ETX)	33	0.0015 μg	i.v. (Minami et al. 1997)	Pore-forming toxin
Iota (ITX)	Ia: 48 Ib: 72	Ia: 1.5 μg + Ib:3 μg	i.p. (Stiles and Wilkins 1986)	Actin-specific ADP-ribosyltransferase

i.p. intraperitoneally, *i.v.* intravenously

invader from the gut, isolation of *C. perfringens* from tissues of dead animals must be viewed with caution when making a diagnosis.

Intestinal diseases produced by *C. perfringens* in animals are indistinctly called "enteritis/enterotoxemias," suggesting that most toxins which affect the gastrointestinal tract can be absorbed into the general circulation with systemic effects. The scientific evidence and abundance of experimental and clinical evidence, together with the molecular Koch's postulate for enterotoxemia and ETX produced by *C. perfringens* type B and D, is strong (Garcia et al. 2013). However, the role of other *C. perfringens* major toxins in enteritis/enterotoxemia awaits full experimental confirmation.

Alpha Toxin (CPA)

Alpha toxin (CPA) is produced by all toxinotypes of *C. perfringens*. Although it has been generally assumed that type A strains produce higher amounts compared with the other toxinotypes, Fernandez-Miyakawa et al. (2005) described that other toxinotypes can produce also comparatively high levels of CPA in vitro. Type A is the most commonly found toxinotype in nature, and it is usually isolated from the intestine of apparently healthy humans and animals (McClane et al. 2006). Because of high prevalence in the intestines of healthy individuals, controversy exists about the real pathogenic role of this toxinotype. An exception to this uncertainty, however, is necrotic enteritis in poultry, where there is considerable evidence that *C. perfringens* type A is responsible for the disease (Prescott et al. 2016). As type A strains produce only CPA as a major toxin, a similar controversy exists about the role of this toxin in type A-associated diseases. While several studies with different approaches define a clear association between CPA and histotoxic diseases of humans and other animals (Flores-Díaz and Alape-Girón 2003), the role of CPA in enteric disease is not fully understood. Currently many nontyping toxins are described for pathogenic type A strains, like NetB (Keyburn et al. 2008).

Structure and Mode of Action

CPA has phospholipase C activity and a calcium-dependent phospholipid binding domain (Sakurai et al. 2004). This toxin is related to several *Clostridium sp*. phospholipases C (Flores-Díaz and Alape-Giron 2003) and other bacterial phospholipases C, including Zn^{2+}−metalloenzymes from *Bacillus cereus* and *Listeria monocytogenes*. CPA is the most toxic among bacterial phospholipases C and has platelet-aggregating, hemolytic, cytotoxic, and myotoxic activities (Titball et al. 1999).

CPA displays both lecithinase and sphingomyelinase activity and degrades the two major components of the outer leaflet of eukaryotic plasma membranes in the presence of calcium ions (Sakurai et al. 2004). Hydrolysis of cell membrane phospholipids by CPA results in damage on the cell membrane and activates several other membrane and internal cell mechanisms that contribute to cytotoxicity (Titball et al. 1991). For example, CPA activates the arachidonic cascade resulting in the formation of thromboxanes, leukotrienes, and prostaglandins, which activate the inflammation cascade and produce vasoconstriction (Flores-Díaz and Alape-Girón 2003).

Associated Diseases

A condition like enterotoxemia in lambs associated with *C. perfringens* type A, known as "yellow lamb disease," has been clinically characterized by depression, anemia, icterus, and hemoglobinuria, with animals dying after a clinical course of 6–12 h (Songer 1998). Necropsy findings include pale and friable liver and spleen, and the presence of red urine in the urinary bladder (Songer 1998). Histopathological changes include periacinar necrosis of the liver, splenic congestion, nephrosis with hemoglobin casts, pulmonary congestion, and edema (Songer 1998). A similar condition has been reported in goats and calves (Songer 1996). It is generally assumed that most clinical signs and lesions are due to the effects of CPA, and it is considered a virulence factor in cases of presumed enterotoxemia.

CPA and *C. perfringens* type A strains have been, and are still frequently, blamed for enteritis, abomasitis, and/or enterotoxemia in cattle (Lebrun et al. 2010), horses, goats, and pigs (Songer 1996). The most important gross changes observed in natural cases are characterized by hemorrhage and ulceration of the abomasal mucosa, and necrohemorrhagic enteritis of the jejunum, ileum, and sometimes colon (Songer 1996). In pigs, infection by type A strains is an important cause of enteritis in neonatal pigs. Affected animals develop watery diarrhea that continues in untreated pigs for about 5 days (Songer and Uzal 2005). At necropsy, the small intestine is flaccid, thin walled, and often gas filled with watery contents devoid of blood. Microscopic lesions can include mild necrotizing enterocolitis and equally mild villous atrophy (Songer and Uzal 2005), but microscopic examination often reveals no lesions. Unfortunately little information about pathogenesis of type A-associated diseases has come to light. Although CPA may be the determinant virulence factor as the experimental evidence suggest, most in vivo studies have

been based on the use of crude preparations of CPA which can contain other potentially toxic molecules.

Avian necrotic enteritis is an important veterinary disease because of the economic consequences for poultry producers (Van Immerseel et al. 2004). This disease was initially attributed to CPA produced by specific type A strains. However, a recent work has described that CPA null mutants retained full virulence in a chicken disease induction model. These data suggest that CPA is not responsible for disease, likely involving another toxin or virulence factor (Keyburn et al. 2008). The discovery of the pore-forming toxin NetB, and the growing evidence supporting an essential role in the ability of chicken *C. perfringens* isolates to cause necrotic enteritis, has been a paradigm shift in understanding the pathogenesis of necrotic enteritis (Keyburn et al. 2008).

Intestinal Effects

Although CPA's role in histotoxic diseases is clear (Flores-Díaz and Alape-Girón 2003), its role in enteric disease is not fully understood. Previous works described intestinal alterations after CPA treatment, suggesting an important role for this toxin in the pathogenesis of *C. perfringens* type A-induced enteritis or abomasitis. However, the information available is contradictory.

Different studies report that CPA induces morphologic alterations in the small and large bowels of ruminants. In lambs, a crude preparation of CPA inoculated into ligated small intestinal loops induced fluid accumulation. Further works report that CPA-inoculated colonic loops of lambs developed physiological and morphological changes (Fernandez-Miyakawa and Uzal 2005). Ovine ileal and colonic loops incubated with CPA retained more fluid than control loops, and there was a mild to moderate multifocal infiltration of neutrophils into the lamina propria and submucosa. In vitro measurements of water transport also revealed inhibition of net epithelial water absorption in ileum and colon incubated with CPA on the mucosal side (Fernandez-Miyakawa and Uzal 2005). Experiments from the same group in cattle also report fluid accumulation in small and large intestine loops (Morris et al. 2012). In CPA-treated ileal loops, morphological alterations include shortening and blunting of villi, together with epithelial cell detachment and hemorrhage of the lamina propria. Congestion and mild hemorrhage in the lamina propria were observed in the colon loops. Subsequent studies with null *cpa/pfo* mutants show that *cpa* complementation was necessary for *C. perfringens* type A strains to induce histological damage to bovine intestinal loops (Valgaeren et al. 2013). These results suggest that CPA could be responsible, or at least play an important role, in enteric diseases in ruminants.

Experiments in monogastric animal models also report CPA-induced histologic and physiologic alterations. Intragastric inoculation of CPA to neonatal piglets causes edema of villi and neutrophilic inflammation of the small intestine (Songer and Uzal 2005), although this toxin inoculated into ligated intestinal loops of adult pigs does not produce substantial lesions or fluid loss. Explants of rabbit small intestine incubated in vitro, with CPA, cause detachment of the epithelial cells at the

villi tips. Injection of CPA into small intestinal loops of rats causes neutrophilic enteritis. In birds, intraduodenal infusion or oral inoculation of CPA induces necrosis of the small intestinal epithelium. Examination of the CPA effect upon the electrophysiology of chicken jejunum reveals electrogenic secretion of anions, probably by stimulation of chloride secretion. CPA also diminishes electrogenic cotransport of Na+/glucose from the mucosa to the serosa (Rehman et al. 2006).

Results from other works, intended to explain pathogenesis of *C. perfringens* toxins different from CPA, involving isogenic mutants, suggest that this toxin is not important in the pathogenesis of intestinal diseases. For instance, neither enterotoxigenic type A *cpe* null mutants nor type C *cpb* null mutants that still produce CPA induce damage to the intestinal mucosa in rabbit ileal loops (Sayeed et al. 2008). However, it is important to consider that wild-type strains expressing additional virulence factors are probably poor CPA producers.

Systemic Effects

CPA can induce several in vivo systemic alterations which depend on its capacity to hydrolyze membrane phospholipids (Titball et al. 1999). CPA decreases cardiac contractility; increases capillary permeability; and induces platelet aggregation, hemolysis, myonecrosis, and lethality in different animal species (Titball et al. 1999). The CPA-mediated hydrolysis of phospholipids has different consequences, depending upon concentrations. At high concentrations, extended phospholipid degradation causes membrane disruption and cytolysis (Titball et al. 1999). With low concentrations, CPA causes a limited hydrolysis of phosphatidylcholine and sphingomyelin, generating second messengers like diacylglycerol and ceramide. This triggers various signal transduction pathways and leads to the uncontrolled production of several intercellular mediators (Titball et al. 1999).

Several works suggest that CPA is a major virulence factor in *C. perfringens*-mediated gas gangrene/malignant edema. Studies using purified CPA report that intramuscular injection in mice causes myonecrosis and reproduces many of the histopathological features of gas gangrene (Bunting et al. 1997). Further works show that immunization with the recombinant C-terminal domain of CPA protects mice from challenge with *C. perfringens* vegetative cells. Final evidence comes from works with a *C. perfringens* mutant strain, in which the *cpa* gene has been inactivated by homologous recombination. This CPA-deficient mutant was unable to produce gas gangrene (Awad et al. 1995). Furthermore, the CPA-deficient strain recovers virulence upon complementation with a plasmid carrying the wild-type *cpa* gene (Awad et al. 1995).

Yellow lamb disease has been considered a CPA-associated, enterotoxemia-like syndrome (Songer 1998; Uzal et al. 2014) because of the intravascular hemolysis and capillary damage, platelet aggregation, hepatic necrosis, and cardiac effects observed in lambs. Although these findings are consistent with the action of a circulating hemolytic toxin such as CPA, the disease is very rare and there are not many published reports of similar findings in other ruminant species. The absence of evidence about confirmatory clinical cases, the fact that disease was not reproduced,

and Koch's postulates have not been satisfied do not allow one to define a role for CPA in enterotoxemia. Further work is needed to demonstrate CPA translocation to general bloodstream and define the mechanisms involved in CPA intestinal alterations.

Beta Toxin (CPB)

Beta toxin (CPB) is produced by *C. perfringens* types B and C, and it is associated with diseases in several animal species. Type B isolates often cause fatal hemorrhagic dysentery in sheep, and possibly in other species, while type C isolates cause enteritis necroticans (also called Darmbrand or Pig-bel) in humans and necrotic enteritis and/or enterotoxemias in almost all livestock species. Both types B and C animal disease are often accompanied by sudden death or acute neurological signs (McClane et al. 2006; Songer 1996). The experimental evidence employing a rabbit intestinal loop model and oral or intestinal inoculation models of mice show that CPB is an important virulence factor of type B and C strains, as described by usage of purified CPB or isogenic null mutants of type C isolates (Fernandez-Miyakawa et al. 2007; Sayeed et al. 2008).

Structure and Mode of Action

CPB belongs to a β pore-forming toxin family, which includes *Staphylococcus aureus* alpha toxin, leukocidin, and gamma toxin. The *cpb* gene encodes a prototoxin of 336 amino acids that includes a 27 amino acids signal sequence removed during secretion, resulting in a mature toxin of ∼35 kDa (Sakurai and Duncan 1978). Purified CPB is thermolabile and highly sensitive to protease treatment in vitro and in vivo (Sayeed et al. 2008; Uzal et al. 2009).

CPB is known to shift to a multimeric complex in vitro; these CPB polymers form pores in the membrane of susceptible cell lines. After CPB binds to specific receptors, it forms an oligomeric pore of 228 kDa that is linked to its cytotoxic effect. These channels induce K^+ efflux and Ca^{2+}, Na^+, and Cl^- influxes, which then produce cell swelling and lysis (Nagahama et al. 2003a), and can be related to neuromuscular effects observed in type C-associated diseases (Shatursky et al. 2000). Receptor binding and pore formation is related to membrane cholesterol in lipid raft microdomains of HL-60 cells (Nagahama et al. 2003b). Site-directed mutagenesis emphasizes the importance of pore formation for CPB-associated cytotoxicity, since the mutation of arginine 212 to glutamine or glutamic acid reduces CPB lethality and channel formation (Steinthorsdottir et al. 1998). Incubation of porcine endothelial cells with CPB results in the typical biochemical and morphological behaviors of cells that had died due to necrotic cell death (Schumacher et al. 2013). Other effects of purified CPB include the release of TNF-alpha and IL-1 β, as well as activation of tachykinin NK1 receptors by a still unknown mechanism (Nagahama et al. 2008; Nagahama et al. 2003b).

Associated Diseases

CPB is elaborated by *C. perfringens* type B and C strains, and is the essential virulence factor involved in type C hemorrhagic enteritis (McClane et al. 2006; Uzal et al. 2009). Toxinotype C strains produce lethal infections ranging from necrohemorrhagic enterocolitis to enterotoxemia in pigs, horses, cattle, sheep, and goats. Even though mature animals can contract such illness, young animals are most vulnerable (Songer 1996). Piglets seem to be the most sensitive to type C infectious diseases (Songer and Uzal 2005), although similar infections occur in newborn calves, foals, and goats (Songer 1998). In unvaccinated herds, mortality can reach 100%, causing significant economic losses (Songer 1996).

During type C infection, vegetative cells proliferate in the small bowel and elaborate toxins (McClane et al. 2006), which causes extensive hemorrhagic intestinal lesions leading to the passage of CPB from the small bowel into systemic circulation. Neurological symptoms such as tetanic contraction and opisthotonos have been recognized in affected animals prior to death (Uzal et al. 2009), and those neurological symptoms are attributed to CPB elaborated in the gastrointestinal tract. In naturally occurring hemorrhagic enteritis in piglets, CPB binds to vessel endothelial cells in the enteric mucosa (Schumacher et al. 2013).

In humans, type C strains induce food-borne necrotizing enterocolitis (also named as Darmbrand or Pig-bel), which is an endemic disease in the highlands of Papua New Guinea (McClane et al. 2006; Uzal et al. 2009). Darmbrand, also known as "fire-belly," was a severe human illness that occurred in malnourished people of northern Germany in post-World War II (McClane et al. 2006). The disease was often fatal because of necrotic inflammation of the small intestine, especially in the jejunum. Pig-bel is historically the type C infection most strongly related to human disease. In the highlands of Papua New Guinea, the disease occurs in individuals after the ingestion of insufficiently cooked pork during certain ritualistic ceremonies (McClane et al. 2006; Uzal et al. 2009). Affected individuals present with serious bloody diarrhea, abdominal pain, distension, and emesis. As Pig-bel results from an increased consumption of pork, it is proposed that the illness is related to the intake of a high-protein food, together with decreased trypsin activity either due to chronic protein nutritional deficiency (Darmbrand or Pig-bel) or ingestion of food with trypsin inhibitors like sweet potatoes (Pig-bel). Each of these risk factors plays a role in the persistence of CPB in the alimentary tract during infection of *C. perfringens* type C. At the present, cases of necrotizing enterocolitis associated with type C isolates has been described in several countries (Matsuda et al. 2007). In those cases, risk factors include delayed gastric emptying and reduced intestinal mobility, diabetes, and other pancreatic diseases. Immunohistochemical studies of tissue samples from a diabetic patient who died of necrotizing enterocolitis showed endothelial binding of CPB to enteric lesion sites (Matsuda et al. 2007).

At necropsy, the predominant lesions are most frequently observed in small intestine of most species, but the cecum and colon can sometimes be involved. Gross and histological lesions are similar in all segments of the intestine. Gross

lesions in peracute cases consist of diffuse or segmental and extensive necrotizing and hemorrhagic enteritis, with emphysema and bloody gut contents (Songer 1996; Uzal et al. 2014). In acute and subacute cases, fibrinonecrotic enteritis with the presence of pseudomembranes is usually observed.

Histologically, the hallmark of *C. perfringens* type C infection is hemorrhagic necrosis of the intestinal wall, which starts in the mucosa but usually progresses into all layers of the intestine. Fibrin thrombi occluding superficial arteries and veins of the lamina propria and submucosa are characteristic of this condition (Songer and Uzal 2005; Uzal et al. 2009).

Intestinal Effects

Evidence from natural type C enteritis cases, together with experimental works, has demonstrated that CPB is the main virulence factor of *C. perfringens* type C strains. In piglets, naturally occurring *C. perfringens* type C enteritis consists of CPB binding to vascular endothelium in lesion sites, suggesting that CPB could cause vascular necrosis, hemorrhage, and subsequent hypoxic necrosis (Popescu et al. 2011). In a piglet jejunal loop model, CPB was recognized in microvascular endothelial cells in intestinal villus in sites with necrohemorraghic lesion development. A direct binding of CPB to endothelial cells might induce damage to endothelial cells and vascular necrosis, contributing to the pathogenesis of type C necrotizing enterocolitis (Popescu et al. 2011).

Studies from the McClane group evaluated the role of CPB in the pathogenesis of type C disease, using isogenic CPB null mutants of a type C strain (CN3685) (Sayeed et al. 2008; Uzal et al. 2009; Vidal et al. 2008). As type C isolates typically produce CPA, CPB, and perfringolysin O, single and double mutants of these toxins were also used for this study. In rabbit intestinal loops, inoculation of wild-type CN3685 induced necrosis of villous tips, which showed that there was early intestinal epithelial injury. On the other hand, the *cpb* null mutant induced neither intestinal necrosis nor accumulation of bloody fluid in rabbit intestinal loops (Vidal et al. 2008). Additionally, complementing the *cpb* null mutant to recover CPB production notably elevated intestinal pathogenesis. Mutants of CN3685 that did not produce CPA or perfringolysin O retained sufficient virulence, as probed in rabbit ileal loops. Furthermore, purified CPB in the presence of trypsin inhibitor reproduced the intestinal injury of wild-type CN3685, and this damage was blocked by anti-CPB antibody (Sayeed et al. 2008; Uzal et al. 2009). Taking these findings altogether, the above studies suggest that CPB plays a crucial role in the pathogenesis of type C isolates.

Systemic Effects

The lethality of type C infection has long been attributed to toxin(s) absorption from the intestine to the circulation. Several studies have proven that purified CPB is highly lethal for mice (Fisher et al. 2006; Sakurai and Duncan 1978; Shatursky et al. 2000; Steinthorsdottir et al. 1998), with a calculated LD_{50} of 3.2 μg/kg of body

weight when administered intravenously (Sakurai and Fujii 1987). However, type C isolates typically produce at least three, and sometimes up to five, different lethal toxins (Fisher et al. 2006). The role of CPB in type C lethality was clarified by means of single and double isogenic mutants of a type C strain (CN3685) in a mouse intravenous injection lethality model (Sayeed et al. 2008). In this work, a wild-type C strain was 100% lethal, yet an isogenic *cpb* null mutant showed largely decreased lethality (25%), and the isogenic CN3685 double mutant, unable to produce CPA or perfringolysin O, exhibited only a modest reduction in lethality (76%). Also, in a previous work, lethality induced by type C culture supernatants or purified CPB could be completely blocked by preincubation with a monoclonal anti-CPB antibody, but not one against CPA (Fisher et al. 2006).

In natural type C diseases, CPB originates in the intestine but often systemic alterations are observed, probably due to absorption of toxins into the general circulation (enterotoxemia). Type C cultures or purified CPB reproduced neurological signs and lethality in mice after intragastric and intraduodenal challenge (Uzal et al. 2009). It is evident that CPB produced in the intestines of infected animals can reach the general bloodstream, causing systemic alterations. Further works are needed to clarify CPB translocation mechanisms, but is possible that extensive damage to the intestinal epithelium might allow CPB and other toxins to translocate into the circulation to induce systemic/lethal effects.

The molecular basis of CPB-induced neurological signs during type C disease is under investigation. CPB attacks control of the autonomic nervous system and then causes arterial contraction by catecholamine liberation, thus elevating blood pressure. Other works describe CPB causing substance P liberation, an agonist of tachykinin NK1 receptor, involved in subsequent neurogenic plasma extravasation (Nagahama, et al. 2003b). Furthermore, substance P liberated by CPB from sensory neurons causes the liberation of TNF-α, and these agents are responsible for plasma extravasation (Nagahama et al. 2008). These results indicate that CPB has a direct, or indirect, effect upon the central and peripheral nerves.

Epsilon Toxin (ETX)

Epsilon toxin (ETX) is synthesized by *Clostridium perfringens* types B and D. Type B is rare and is the causative agent of lamb dysentery which is endemic in some countries such as the UK (Songer 1996). Sheep and goats affected by type D enterotoxaemia could have a disease ranging from a peracute form with neurological signs and sudden death up to a chronic disease including hemorrhagic diarrhea and colitis (Songer 1998). The cause for this variation in clinical signs remains unknown. ETX contributes with CPB to the pathogenesis of toxinotype B (Fernandez-Miyakawa et al. 2007), and it is considered the main virulence factor of toxinotype D (Garcia et al. 2013).

Structure and Mode of Action

ETX is expressed as a prototoxin from *C. perfringens* vegetative cells. To exert significant cytotoxic activity, the secreted prototoxin must be proteolytically processed, which increases its activity nearly 1,000-fold (Freedman et al. 2014). Purified trypsin or chymotrypsin can activate ETX prototoxin in vitro (Freedman et al. 2014), as well as other proteases present in the intestines of mammals (elastase, enteropeptidase, and carboxypeptidases) (Freedman et al. 2014).

Specific activity of ETX is also observed in cultured cells. Only very few cell lines, including renal cell lines from various species such as MDCK (dog), mpkCCDcl4 (mouse), and to a lesser extent the human leiomyoblastoma (G-402) cells, are sensitive to ETX (Popoff 2011). ETX binding to the surface of sensitive cells is mediated by a specific membrane receptor. ETX cytotoxicity can be prevented by the prior administration of the prototoxin or formalin-inactivated prototoxin (Manni et al. 2015), probably by competitive binding, supporting the existence of specific receptor sites.

Binding of ETX to its receptor leads to the formation of large membrane complexes. These complexes correspond to the heptamerization of toxin molecules within the membrane and pore formation (Popoff 2011). The structure of an ETX pore has been defined as a cone shape (Popoff 2011), and thus its insertion in lipid bilayers might be favored by a specific lipid membrane organization. Therefore, although ETX does not directly bind to a lipid receptor, the lipid composition and physical properties of membrane influence ETX pore formation and insertion into the membrane. ETX cytotoxicity is associated with a rapid loss of intracellular K^+, and an increase in Cl^- and Na^+, whereas the increase in Ca^{2+} occurs later. In addition, the loss of viability also correlates with the entry of propidium iodide, indicating that ETX forms large pores in the cell membrane. Although ETX causes rapid cell death by necrosis, cell signaling leading to necrosis is not yet fully understood.

Associated Diseases

ETX produced by types B and D strains is responsible for a highly fatal enterotoxemia in livestock (Uzal et al. 2014). Type D strains and ETX produce acute, subacute, or chronic diseases in sheep, characterized by sudden death or neurologic and respiratory signs, including blindness, opisthotonos, convulsions, bleating, frothing from the mouth, and recumbency with paddling immediately before death. Digestive clinical manifestations like diarrhea are occasionally observed, although this is not a common clinical presentation in sheep (Uzal et al. 2004). In goats, type D produces acute, subacute, or chronic disease as well. The acute form occurs more frequently in young, unvaccinated animals and is clinically similar to the acute disease in sheep (Songer 1998). The subacute form is more frequently seen in adult goats, (Songer 1998; Uzal et al. 2004) vaccinated or not, and is characterized by hemorrhagic diarrhea, abdominal discomfort, severe shock,

opisthotonos, and convulsions. The disease may result in death 2–4 days after onset, but some animals recover (Garcia et al. 2013; Songer 1998).

Most cases of type D enterotoxemia in ruminants are related to sudden changes in diet, usually those rich in highly fermentable carbohydrates (Popoff 2011). Such alimentary conditions induce a perturbation in the microbial balance in the gut and massive passage into the small intestine of undigested fermentable carbohydrates, like starch, which are normally metabolized in the rumen and represent an excellent substrate for bacterial growth together with ETX production. Other predisposing factors which cause intestinal stasis will contribute to the accumulation of *C. perfringens* vegetative cells and ETX in the intestinal loops.

Gross lesions may be absent in some cases of acute or subacute type D ovine enterotoxemia, and lack of such lesions should not, therefore, be considered sufficient to rule out this condition. Intestinal changes, although rarely present, consist of hyperemic small intestine mucosa with slight to marked red fluid contents. Colitis may occur, but it is not a consistent finding in sheep enterotoxemia. Pathognomonic gross changes in sheep are rarely observed in brain and consist of herniation of the cerebellar vermis (cerebellar coning) in acute or subacute cases, and focal symmetrical encephalomalacia in chronic cases. The focal symmetrical encephalomalacia is characterized by dark hemorrhagic foci in corpus striatum, thalamus, midbrain, and cerebellar peduncles, as well as white matter (Garcia et al. 2013; McClane et al. 2006; Songer 1998). Kidney lesions, from which one of the common names of the disease is derived (pulpy kidney disease), is likely a postmortem change (Popoff 2011). It is widely believed that autolysis occurs faster in animals dying of type D/ETX enterotoxemia versus other causes, but no data support or refute this contention. This change in kidney is highly subjective, and relying upon the presence of lesions can lead to diagnostic errors. Gross changes in acute caprine type D enterotoxemia are similar to those in the ovine disease. In the chronic form of the disease in goats, fibrino-hemorrhagic colitis with occasional involvement of the distal portion of the small intestine seems to be the most consistent lesion described (Uzal et al. 2004). A combination of necropsy findings typical of acute and chronic forms of disease is frequently seen in subacute forms. Neither the so-called pulpy kidney has been reported in caprine enterotoxemia nor has cerebellar herniation or focal symmetrical encephalomalacia.

Microscopic changes in the brain of sheep with type D infection are unique and pathognomonic, although they are not present in all cases (Buxton and Morgan 1976; Uzal et al. 2004). The most consistent change, observed in approximately 90% of cases, is perivascular proteinaceous edema (microangiopathy) in the brain (Buxton and Morgan 1976), which presents as acidophilic accumulations of protein surrounding small- and medium-sized arteries and veins (Uzal et al. 2004). These lesions are first evident a few hours after onset of clinical signs. Apparently, no other condition in sheep produces this highly proteinaceous perivascular edema in brain, and this change is therefore diagnostic for type D enterotoxemia. In chronic disease, necrosis of white matter, grossly known as focal symmetrical encephalomalacia, can be observed (Uzal et al. 2004). This lesion is usually multifocal and characterized by degeneration of white matter, hemorrhage, as well as astrocyte and axonal swelling.

Perivascular edema, with necrosis of brain parenchyma, are always bilateral and symmetrical, and they have been described most frequently in corpus striatum, thalamus, midbrain, cerebellar peduncles, and cerebellar white matter (Uzal et al. 2004). These areas are not exclusively affected, and lesions can sometimes be seen in other parts of the brain, such as cortex and hippocampus (Uzal et al. 2004). Most descriptions of microscopic lesions in the brain of sheep with enterotoxemia are based on experimental inoculations (Uzal et al. 2004), and there is little information about distribution of lesions in natural cases. These changes are a valuable criterion for diagnosing enterotoxemia, particularly when intestinal content is not available for examination of ETX. However, histologic changes are useful indicators of enterotoxemia, but the absence of these lesions does not preclude a diagnosis in sheep. In goats, there are few descriptions of histologic changes in type D enterotoxemia, and changes in brain are not considered a consistent feature of caprine enterotoxemia.

Intestinal microscopic alterations in subacute and chronic type D enterotoxemias in goats are characterized by fibrinonecrotic (pseudomembranous) colitis, with large numbers of intralesional Gram-positive bacilli (Songer 1998; Uzal 2004). However, this lesion is suggestive but not specific, and it cannot be used to establish a definitive diagnosis of enterotoxemia. No significant histologic changes are usually found in the intestines of sheep dying from enterotoxemia (Songer 1998). Histologic changes were not observed in kidneys of experimentally inoculated lambs necropsied immediately after death (Popoff 2011), supporting suggestions that these lesions are due to postmortem changes. Thus, microscopic lesions in kidney should not be considered a diagnostic indicator of ovine or caprine enterotoxemia.

Intestinal Effects

The sequential events leading to clinical enterotoxemia start with the elaboration of prototoxic ETX in the gut, which is activated by intestinal trypsin or a metalloproteinase produced by *C. perfringens* (Minami et al. 1997). ETX accumulates in the intestinal lumen. In sheep, accumulation of ETX up to 10^2–10^3 mouse lethal dose/mL remarkably do not induce any associated clinical signs of enterotoxemia. In experimental mice and rat intestinal loops, ETX at a concentration of 10^3 mouse lethal dose/ml and higher are necessary to produce accumulation of fluid in the intestinal lumen, decrease in transepithelial electrical resistance, and an increase in macromolecule passage across the intestinal barrier (Goldstein et al. 2009). The absence of histological and ultrastructural changes in the small intestinal epithelium, and the indirect detection of macromolecules in the gap junction between enterocytes of this intestinal section, suggest increased passage of relative high molecular-weight molecules through the paracellular pathway independent of morphological damage (Goldstein et al. 2009). The only lesions observed in the small intestine are perivascular edema and apoptotic cells in the lamina propia (Fernandez-Miyakawa and Uzal 2003). In the colon, obvious damage to the epithelial mucosa has been observed in mice, rats, sheep, goats, cows, etc. (Fernandez-

Miyakawa and Uzal 2003; Garcia et al. 2013). Although binding of ETX has been observed on enterocytes at the top of small intestinal villi and surface colonocytes of large intestine (Goldstein et al. 2009), the precise mechanism of ETX-dependent permeability of the small intestinal barrier and epithelial damage of the colon is unknown. It has been postulated that massive ETX absorption from the intestine occurs paracellularly after changes in the organization of the tight junction, produced by ETX (Goldstein et al. 2009). Subsequently, ETX absorption into the general circulation can occur from the small and large intestines (Losada-Eaton et al. 2008). However, clinical and experimental evidence from mice, sheep, and goats (Garcia et al. 2013) suggest that during lethal and acute disease, most of the ETX absorption occurs in the small intestine. It seems that the toxin also modifies gastrointestinal motility (Losada-Eaton and Fernandez-Miyakawa 2010), apparently by an indirect action via the nervous system. Inhibition of gastrointestinal motility could be a key event for the progression of enterotoxemia, potentiating bacterial overgrowth and toxin accumulation in the intestinal lumen to lethal levels which finally boosts toxin absorption.

Systemic Effects

Endothelium

Once ETX gains access to the systemic circulation, several organs are at risk of damage, including brain, lungs, and kidneys (Popoff 2011; Uzal et al. 2004). The accumulated evidence suggests that ETX targets endothelial cells and alters the integrity of the vascular barrier. It has been observed that ETX binds to the luminal surface of the endothelium of most blood vessels after intravenous injection of mice (Soler-Jover 2004, 2007). Edema and petechia have been observed in several tissues from naturally or experimentally intoxicated animals (Garcia et al. 2013; Uzal et al. 2004). It has been evidenced that a direct interaction of ETX with endothelial cells increases the vascular permeability of rat mesentery microvessels (Adamson et al. 2005). Also, intradermal ETX injection produces an increased permeability of skin vessels. The observation of necrotic cells and gaps in endothelium a few minutes after toxin exposure shows that ETX modifies directly the integrity of the endothelial barrier by destroying cells rather than causing disassembly of the intercellular junctions (Adamson et al. 2005). However, primary cultures of endothelial cells obtained from various animal species are not sensitive to ETX (Uzal et al. 1999). Although it is possible that these primary culture cells obtained from a major vessel could lose the specific ETX receptor (Uzal et al. 1999), significant morphological and functional differences exist between cells obtained from different vascular endothelium (Popoff 2011). On the other hand, culturing endothelial cells in vitro deprive them of their microenvironment which could be important for ETX effects (Goldstein et al. 2009). Therefore, the action of ETX on endothelial cells and the posterior increase of endothelial barrier permeability appear to be one of the crucial steps of intoxication in exposed animals.

Kidney

Clostridium perfringens type D/ETX enterotoxemia in lambs has been called traditionally "pulpy kidney disease" (McClane et al. 2006). Rapid postmortem autolysis of kidneys is characteristic of lamb type D enterotoxemia and is less evident in sheep and other animal species. Immediately after death, only a variable degree of congestion in the kidneys is observable in lambs intoxicated with ETX, which is more pronounced at 2 h, and after 4 h post mortem, kidneys show interstitial hemorrhage between tubules and degeneration of the proximal tubule epithelium. Similar findings were described in mice injected with ETX-GFP (fluorescent-labeled ETX), which showed severe kidney alterations. The latter include hemorrhagic medullae and selective degeneration of distal tubules congestion and hemorrhage in the medulla, as well as severe degeneration of the distal tubule epithelium (Soler-Jover 2004). It has been shown that ETX binds specifically to the basolateral surface of distal tubule epithelial cells of many species (Soler-Jover 2004), which is in agreement with the degenerative changes observed in this epithelium. These findings suggest that an ETX receptor is expressed on renal distal tubules of mammalian species, including human. ETX also binds to the luminal surface of proximal tubules, although in a nonspecific manner, probably as a result of toxin filtration by the glomerules (Soler-Jover 2004; Tamai et al. 2003). It is also supported by experiments of nephrectomy in mice that shortens the time to death of animals injected with ETX, suggesting that the kidneys play a protective role by trapping the toxin from the circulation and eliminating it from the organism (Tamai et al. 2003). Also, the few cultured cell lines that are sensitive to ETX are kidney-derived cells like MDCK. Taken together, these results strongly suggest that the kidney is one of the main target organs for ETX. However, Uzal et al. (2004) stated that histology of the kidney should not be a diagnostic indicator in sheep enterotoxemia, following the hypothesis that the so-called pulpy kidney lesion is a postmortem phenomenon.

Brain

Following systemic dissemination, ETX also reaches the central nervous system. The brain is the second organ, after the kidneys, where ETX accumulates massively (Popoff 2011). It has been described that ETX-GFP can be detected on the luminal surface of the vascular endothelium, after the intravenous injection of mice (Soler-Jover et al. 2007). Once ETX reaches brain blood vessels, ETX induces alterations to endothelial cells, altering the integrity of the blood–brain barrier, permitting not only its own passage but also that of other macromolecules like serum albumin. ETX-induced alterations in the blood–brain barrier, and the concomitant extravasation of macromolecules, are extremely fast when lethal ETX concentrations are injected in mice (<1–2 min), and it is reduced (about 20 min) when less than one lethal dose is injected. Diffusion of ETX into the brain parenchyma is greater than that of albumin, which remains confined around the damaged vessels (Soler-Jover et al. 2007).

ETX then causes cerebral microvascular endothelial damage by severe, diffuse vasogenic edema and a marked increase in intracranial pressure (Finnie 2003).

Cerebral lesions of acute and subacute *C. perfringens* type D/ETX enterotoxemia have been produced experimentally in sheep (Buxton and Morgan 1976), mice (Fernandez-Miyakawa et al. 2007; Finnie 2003), and rats (Finnie 2003). It seems that formation and the spread of edema depends on both ETX concentration and the time between ETX application and observation (Buxton and Morgan 1976; Finnie 2003; Uzal et al. 1997). When high doses of ETX cross the blood–brain barrier, the disease is very severe and leads to quick death. By contrast, when low doses of ETX are applied, the lethal outcome is delayed and numerous brain lesions develop (Fernandez-Miyakawa et al. 2007; Finnie 2003). Widening of the perivascular space is the most evident early change, observed as soon as 1 h after intraperitoneal injection of a sublethal dose in mice, progressing to stenosis of the capillary lumen (Finnie 2003). Perivascular edema is mainly distributed in white matter and accompanied by swelling of the perivascular astrocytic cells, predominantly in the cerebellum, a sensitive site for the induction of early central nervous system damage (Finnie 2003). Swelling is also observed in axon terminals and dendrites, with the myelin sheath being distended by edema (Morgan et al. 1975). In animals exposed to comparatively low doses of toxin, focal, bilaterally symmetrical encephalomalacia may also develop after a more delayed clinical progress. The localization and severity of cell damage seems dependent upon ETX doses, the time between ETX injection and animal euthanasia (Finnie 2003; Miyamoto et al. 2000), as well as on the repetition of ETX injection (Finnie 2003; Uzal et al. 2004). Altered neurons are scattered among apparently normal nerve cells in the cerebral cortex, hippocampus, thalamus, basal ganglia, and cerebellum. In rats, injection of ETX at a sublethal dose seems to cause neuronal damage predominantly in the hippocampus (Miyamoto et al. 2000), a brain region that plays important roles in the consolidation of information from short- to long-term memory and spatial navigation.

The damage induced by ETX against neural cells could be produced by direct and indirect cellular actions of ETX. Indirectly, as stated above, alterations of cells in brain tissue may be a consequence of impaired blood–brain vessels that leads to vasogenic edema and reduced perfusion of the tissues and, therefore, to tissue hypoxia and cell necrosis. Directly after ETX spreads into the brain parenchyma, there are effects upon neurons and other cell types. It is highly probable that a combination of both processes might be involved.

Direct action of ETX on neuronal tissue is suggested by the observation of bilateral symmetry of the damaged brains. It has been shown that ETX accumulates in brain areas that have been described as the main sites of histological changes in animals suffering the action of ETX (Soler-Jover et al. 2007).

Examination of the cellular localization of ETX in mouse cerebellum has revealed toxin binding to the cell body of cerebellar granule cells, which are glutamatergic neurons. This identification is confirmed by the observation that ETX colocalizes with specific granule cell markers, such as the alpha-6-GABAA receptor subunit or potassium channel subunit Kv3.1b. In the granule cell layer of the cerebellar cortex, ETX colocalizes with MAP-2 (microtubules-associated protein-2) denoting that ETX concentrates not only in the somata but also the dendritic trees of granule

cells. It is significant that ETX binds to the cell body of granule cells or other target cells, but not to axons or nerve terminals. This finding suggests that a specific ETX interaction with a cell body membrane receptor must occur. It has been found that ETX-GFP binds to only a subset of astrocytes and microglia cells and is cytotoxic (Soler-Jover et al. 2007). A more detailed analysis of ETX binding to mouse cerebellum has identified granule cells and oligodendrocytes, but not Purkinje cells and astrocytes. ETX binding to myelin probably accounts for ETX staining of oligodendrocytes, which are involved in myelin synthesis in contrast to astrocytes, which participate in blood–brain barrier function, regulation of local pH and electrolytes, and probably recapture of neurotransmitter.

It has been shown that ETX specifically targets the myelin-forming cells of the central nervous system and oligodendrocytes, leading to cell death. ETX also binds to the myelin structure of the peripheral nervous systems of mammals (Dorca-Arévalo et al. 2008). However, myelin does not seem to be the primary target of ETX since intravenously injected toxin in mice does not show a correlation between the ETX staining pattern and myelin-containing structures. The selectivity of ETX for oligodendrocytes is remarkable, as other cells of the CNS are unaffected. The possibility that ETX can act on oligodendrocytes, thereby causing demyelination, agrees with the concept of a direct action of ETX (Wioland et al. 2015). Moreover, it suggests that for certain cell types such as oligodendrocytes, ETX can act without forming pores, namely through the activation of an undefined receptor-mediated pathway (Wioland et al. 2015). Albeit, no direct interaction between ETX and MAL has been evidenced, recent studies with knock-out mice suggest that ETX-induced oligodendrocyte death is dependent on expression of MAL (Linden et al. 2015).

Both in vitro and in vivo experimental approaches describe different and probably complementary mechanisms. However, to understand the pathogenesis of ETX-induced neural damage, it is important to consider that cellular manifestations (binding, cell damage or death) caused by ETX and cell types affected by this toxin ultimately depend on the actual concentration of ETX in neural tissue. The actual concentration of ETX in neural tissue still remains unknown and may be nonhomogenous. Future works should be directed to describe the limiting steps of ETX passage through the different epithelial barriers.

Iota Toxin (ITX)

Iota toxin (ITX) is a clostridial binary toxin produced by *C. perfringens* type E strains. These toxins have a common structure consisting of two independent protein components that are not covalently linked, one being the binding component (Ib,100 kDa) and the other an enzymatic component (Ia, 45 kDa) (Stiles and Wilkins 1986). Both components are required for biological activity. Although ITX is only produced by *C. perfringens* type E strains, structurally related binary toxins are widely spread among other species of enterotoxic clostridia and *Bacillus* species (Stiles et al. 2014). Clostridial binary toxins include CDT from *C. difficile* (Geric et al. 2006), CST from *C. spiroforme* (Stiles and Wilkins 1986), and C2 from

C. *botulinum* (Ohishi et al. 1980). It has been generally assumed, but not directly proven, that the pathogenesis of type E enteritis/enterotoxemia largely involves ITX.

Structure and Mode of Action

The binding component of ITX (Ib) recognizes a cell-surface receptor and mediates internalization of the enzymatic component (Ia) into the cytosol. Alone, each component is nontoxic, but together, Ia and Ib are cytotoxic to various cultured cells, lethal to mice, and dermonecrotic in guinea pigs (Sakurai and Kobayashi 1995; Stiles and Wilkins 1986).

Cell intoxication starts with the binding of Ib component to cell-surface receptor, the lipolysis-stimulated lipoprotein receptor (LSR) (Papatheodorou et al. 2011) which forms clusters in lipid rafts following binding of Ib. It appears that lipid rafts are necessary for heptamer formation, although monomeric Ib components can evidently bind to receptors located outside of these microdomains. Ib heptamers trigger the internalization of Ia into the cell by receptor-mediated endocytosis. After internalization of the receptor-Ib-Ia complex, an acidic pulse in endocytic vesicles induces Ib heptamers to form ion permeable channels, allowing the enzymatic component to escape into the cytosol (Nagahama and Yamaguchi 2004; Sakurai et al. 2009). Once in the cytosol, Ia component mediates ADP-ribosylation of G-actin at Arg-177 (Stiles et al. 2014) which causes accumulation of G-actin monomers leading to depolymerization of actin filaments. Cytoskeleton disorganization includes alterations of the intermediate filaments, and whereas the microtubules are not directly affected by Ia, changes in actin filaments have indirect consequences upon microtubule regulation (Schwan et al. 2009).

The final result of actin depolymerization includes changes in morphology (rounding); inhibition of migration and activation of leucocytes (Stiles et al. 2014); inhibition of smooth muscle contraction; and impairment of endocytosis, exocytosis, and cytokinesis. Deregulation of the cytoskeleton by ITX also induces disorganization of tight and basolateral intercellular junctions, with a subsequent increase in permeability of cultured intestinal cell monolayers (Richard et al. 2002). After inducing nonreversible cytopathic effects on eukaryotic cells, ITX triggers delayed, caspase-dependent death of epithelial cells (Hilger et al. 2009).

Associated Diseases

ITX is solely produced by *C. perfringens* type E strains which, since its first description more than 50 years ago, has been considered an uncommon cause of enteritis. However, recent reports suggest that this toxinotype could be more common than previously considered (Songer and Miskimmins 2004). *C. perfringens* type E strains are associated with enteritis in cattle (Redondo et al. 2013; Songer and Miskimmins 2004), rabbits (Baskerville et al. 1980), lambs (Songer 1996), and dogs (McClane et al. 2006). Disease in ruminants is characterized by hemorrhagic enteritis that is often fatal, affecting calves and lambs. Lesions observed at necropsy reveal hyperemia and edema in the intestinal mucosa and abomasum, with foci of hemorrhage, acute inflammation, as well as submucosal edema (Redondo et al. 2013; Songer and Miskimmins 2004). In rabbits, clinical signs include diarrhea

and weight loss (Baskerville et al. 1980). At the moment, only one report from Japan describes the occurrence of type E-associated foodborne disease in humans. In this work the authors describe the isolation of a new kind of type E strain which can produce both ITX and enterotoxin (Miyamoto et al. 2011). Subsequent works from a different group, also from Japan, describes foodborne outbreaks caused by *C. perfringens* strains; however, these strains produce novel binary enterotoxins different from ITX, BEC, and CPILE (Yonogi et al. 2014). Further work is needed to determine if these strains should be classified as novel type E strains.

Available information about *C. perfringens* type E pathogenesis is scarce, and the mechanisms involved in the onset of this disease are poorly understood. Despite the lack of information about ITX, the actual information of the intestinal effects of other clostridial binary toxins may suggest an important role for ITX in type E-associated digestive diseases.

Intestinal Effects

ITX and *C. perfringens* type E strains seem to be associated solely with often fatal hemorrhagic enteritis in different animal species (Uzal et al. 2010). Actual information regarding the systemic or intestinal effects of ITX on mammals is scanty, based upon descriptions of clinical cases (Baskerville et al. 1980; Redondo et al. 2013; Songer and Miskimmins 2004). In ruminants, type E disease is clinically characterized by severe diarrhea and sudden death. Necropsy findings include abomasal and small intestinal hyperemia and edema, with multifocal mucosal hemorrhage, acute inflammation, and edema of the submucosa (Redondo et al. 2013; Songer and Miskimmins 2004).

Evidence of the importance of ITX as a virulence factor in *C. perfringens* type E enteritis was first provided when a relatively crude toxoid of *C. spiroforme* was shown to protect rabbits against type E hemorraghic enteritis (Songer 1996). Recent work with different binary toxins (CDT from *C. difficile* and BEC from *C. perfringens*) shows that this group of toxins can induce fluid accumulation in rabbit intestinal loops (Geric et al. 2006; Yonogi et al. 2014), suggesting an enterotoxic effect. More recent work shows that the intraintestinal inoculation of purified ITX into ligated loops of mice reproduces the microscopic alterations found in natural cases of type E hemorraghic enteritis in cattle (Redondo et al. 2015). Treatment of ileal loops with low concentrations of toxin causes necrosis in the enterocytes of the villus tips and degenerative changes among the enterocytes of the middle region of the villi. Ileal loops treated with higher concentrations of toxin reveal sloughing of the epithelium.

Several works describe changes in intestinal fluid and ion permeability induced by ITX and other binary toxins (Redondo et al. 2015; Geric et al. 2006); however, no existing works describe changes in macromolecular permeability. Actin depolimeryzation induced by ITX causes disruption of the actin cytoskeleton and intercellular junctions (Richard et al. 2002). The morphological changes and posterior detachment of enterocytes could cause increased permeability observed in advanced stages of type E enteritis. This damage to the intestinal epithelium might allow ITX and other toxins to be translocated into the circulation to induce systemic effects.

Systemic Effects

Information about changes produced by systemically circulating ITX in mammals is sparse. To differentiate between enterotoxemia and enteritis is essential for clearly defining systemic effects produced by a suspected enterotoxin such as ITX. According to previous studies, ITX is lethal when administered intravenously or intraperitoneally to mice (Sakurai and Kobayashi 1995). The reported LD_{50} for ITX (above 1 μg/mouse) is relatively high compared to other toxins that produce "true" enterotoxemia (i.e., ETX 0.0015 μg/mouse) (Minami et al. 1997), raising questions about the role of ITX in systemic clinical signs and death described in natural cases of type E disease.

Sakurai and Kobayashi (1995) propose that injection of separate components of ITX (Ia and Ib over time) will reproduce lethal or dermonecrotic properties of ITX. Such data suggest that individual components can be distributed throughout the bloodstream until they bind to their specific cell receptor, in the case of Ib, or a receptor/Ib complex for Ia. In vitro experiments show that ITX binds to different cell lines (Stiles et al. 2000), including kidney derived (Vero and MDCK) or lung derived (MRC-5). However, further work is needed to define the association between the in vitro results and the origin of the macroscopic lesions observed in natural cases of ITX-associated disease.

Further Directions

Clostridium perfringens is an important etiological agent of a wide range of diseases in humans and animals. The ability of these bacteria to cause such a diverse range of diseases is closely related to differential production of toxins/virulence factors. Among the diverse virulence factors, toxins probably represent the most powerful and fast-acting way to affect target cells. Bacterial toxins with effects upon the gastrointestinal tract have a broad variety of structures (single-chain, oligomeric or binary toxins) and modes of action, including signal transduction through the membrane, pore formation, alteration of a particular mechanism of eukaryotic cells, that then leads to cell death. The mechanism of cell intoxication produced by the toxins reviewed in the present work is well documented and, in some cases, fully understood. However, relations between these cell changes and clinical disease is not as well documented, and further works would be necessary to define the validation of those mechanisms in host species.

In *C. perfringens*, it is clear that there are many different toxins capable of producing enteritis and/or enterotoxemia. Although each toxin has its own particularities with different surface receptors, trafficking routes, and translocation compartments, most of these toxins share a common mechanism of action. With the exception of CPA, which is also an enzyme, the most important *C. perfringens* toxins are pore-forming toxins. A brief analysis highlights an apparent redundancy in the function of these toxins and promptly becomes interesting to determine the origin of these convergent evolutionary processes. The study of these toxins from an

evolutionary perspective is a valuable tool since it is possible that functional and structural data from one pore-forming toxin may be often applicable to other members of the group. Consequently, the study of a relatively minor toxin may have an impact on economically or socially important diseases produced by other toxins. Such comparative studies have the potential to offer new insights upon the modes of action of these toxin groups.

On the other hand, it is interesting that toxins like ETX share several common functional features with toxins from other bacterial genera, as Shiga toxins of *Escherichia coli*. These toxins produce analogous damage in the same target organs but have different structures and cellular intoxication mechanisms. The comparative study of both toxins from an evolutionary point of view can provide innovative concepts about the host-pathogen interactions. For example, during their evolution, enteropathogenic bacteria have developed protein toxins that affect the digestive systems of several vertebrates, obtaining benefits inherent to enterotoxicity, as more efficient gut colonization and outgrowing gut microbiota. In contrast, it is not very obvious why toxins produced by enteric bacteria can act on distant organs such as the brain, with such a specific mode of action. Together with the lack of an obvious advantage for the bacteria, these toxins are codified in mobile genetic elements which represent a high metabolic cost for host bacteria. For these reasons, it is evident that production of enterotoxemic toxins is favored by natural selection. But, what is the underlying selective pressure for such evolution? A possible answer is that common features between ETX and Shiga toxin result as alternative responses from two different bacteria, as *C. perfringens* and *E. coli*, in a similar ecological niche defined by the same challenges.

A possible interpretation for the maintenance of highly expensive, metabolic virulence factors in pathogenic bacteria is the possibility of increased transmission rate between infected and susceptible hosts. Some of the physical or behavioral manifestations of disease, for example diarrhea, are probably consequences of pathogen survival strategies. Eventually such pathogens could acquire a combination, or just one particular, characteristic with lethal consequences for the host. Although pathogen transmission could partially explain the production of ETX by *C. perfringens* or STX by *E. coli*, this is an extremely simplified point of view and overlooks the complexity of any host-pathogen relationship.

Furthermore, it is not evident how systemic alterations described for ETX, and probably for the other reviewed toxins, can be related to an increase in bacterial transmission. Also, it is difficult to define the relations between highly potent toxins and biological fitness of microorganisms. Considering that most of the toxins described in this chapter are present in mobile genetic elements, it would be possible that natural selection discerns more positively a potent specific virulence factor than the whole bacterium, which is only a temporary reservoir. These mobile elements, with an inherent high cost for bacterial cells, would produce an important benefit only under certain conditions. This new perspective can probably help improve actual strategies for control and prevention of enterotoxemic diseases.

Cross-References

- ► *Clostridium perfringens* Epsilon Toxin: Structural and Mechanistic Insights
- ► *Clostridium perfringens* Iota Toxin: A Successfully Shared Template for Common Enteric Pathogens
- ► Role of *Clostridium perfringens* Toxins in Necrotic Enteritis in Poultry

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Role of *Clostridium perfringens* Toxins in Necrotic Enteritis in Poultry

7

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Abstract

Poultry products represent over 30% of animal protein consumption worldwide, and its demand is growing considerably. The current global annual production of poultry meat and eggs is more than 115 million tons and 70 million tons, respectively. Necrotic enteritis (NE) in poultry is a reemerging infectious disease caused by certain strains of *Clostridium perfringens*. This bacterium is found in limited quantities as a normal inhabitant of the birds' gut; however, under certain circumstances, a pathogenic strain proliferates and secretes a variety of bacteriocins, mucins, and adhesins that favors bacteria colonization and establishment. Once bacterial population reaches a certain density, toxin production is triggered

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which induces mucosal damage. NE generates a dramatic reduction of production levels and a significant increase in mortality in flocks of broilers and laying hens, leading to annual economic losses for the poultry industry estimated to be over $6 billion. NE may manifest as an acute or chronic enterotoxemia. Acute infection is associated with higher rates of mortality and chronic infection, with loss of weight and productivity in sick animals. There is evidence that immunization with formalin-inactivated crude supernatants, native or modified toxins, or other proteins induces partial protection against NE. This review summarizes the findings concerning virulence factors associated with NE pathogenesis and the efforts oriented to develop rational strategies to prevent and control this disease.

Keywords

Bacterial toxins • Bacterial phospholipase • Pore-forming toxin • Necrotic enteritis • *C. perfringens* • Enterotoxemia

Contents

Introduction

During the last two decades, the global consumption of poultry meat and eggs has increased considerably, which reflects the dynamics of the poultry industry (FAO 2015, 2016). Poultry meat and eggs are protein-rich products available at relatively low price and therefore are considered basic food in many societies (FAO 2015, 2016). The increasing demands of poultry meat have forced the global poultry industry to improve genetic selection for faster growth, enhance feed conversion, promote higher meat yields, and lower mortality rates (Cooper et al. 2013).

One of the major concerns in poultry meat production is that the birds have healthy intestinal tracts to grow to proper weights. Therefore, the indiscriminate use of antibiotics as growth promoters favoring weight gain of broilers, and helping to control bacterial infections, was allowed for many years (Van Immerseel et al. 2016). However, the risk of antibiotic resistance among human pathogens is favoring the ban of their use as growth promotors around the world, leading to NE reemergence

in poultry (Van Immerseel et al. 2016). Currently NE is causing annual economic losses for the poultry industry estimated to be over $6 billion, on a global scale, due to an impaired growth performance and veterinary treatments (Wade and Keyburn 2015).

NE in poultry is caused by the overgrowth of pathogenic *C. perfringens* which induces intestinal mucosal necrosis (Prescott et al. 2016a). However, the presence of a pathogenic *C. perfringens* strain is not sufficient to precipitate this disease, as predisposing factors are necessary to generate the optimal conditions that favor its proliferation (Moore et al. 2016).

C. perfringens is a Gram-positive, anaerobic, spore-forming, mesophilic bacterium widespread in nature and commonly found in the intestines of humans and animals (Uzal et al. 2014). This bacterium produces more than 15 toxins and it is responsible for a variety of diseases in humans, domestic animals, and livestock, even though its ability to cause diseases is strain dependent (Uzal et al. 2014). On poultry farms, *C. perfringens* can be isolated from soil, bird feces, litter material, live insects, poultry food, water pipes, nipple-drinker drip-cups, floors, wall, fans, and from the clothes and shoes of farm staff (Lee et al. 2011a). *C. perfringens* spores are very resistant to desiccation, chemicals, and temperature, which causes great risk of infection and reinfection in NE (Uzal et al. 2014). Thus, pathogenic *C. perfringens* strains could be transmitted by the fecal-oral route as well as through contaminated food, water, housing structures, and insects, as well as by vertical transmission from hens to newly hatched chicks (Lee et al. 2011a).

Necrotic Enteritis: Symptoms, Pathological Findings, and Diagnosis

NE has been identified in broiler chickens, laying hens, turkeys, ducks, quails, waterfowl, and ostriches in association with certain predisposing factors (Moore et al. 2016). Outbreaks of NE represent the endpoint of a complex series of events, and it occurs either as an acute clinical or as a mild subclinical enterotoxemia (Prescott et al. 2016a).

Birds suffering from clinical NE are depressed, inappetent, huddling, somnolent, diarrheic, dehydrated, and have ruffled feathers (Cooper et al. 2013). The onset of disease is sudden, with birds dying within 1–2 h after the first appearance of clinical signs, and even without premonitory clinical signs (Cooper et al. 2013). Mortality can reach up to 50%, and productivity losses of up to 1% per day can last several days (Cooper et al. 2013).

Postmortem findings in clinical cases of NE include the small intestine (usually middle to distal) very relaxed and fragile (Cooper et al. 2013). The duodenum, jejunum, ileum, and cecum are frequently thickened, friable, distended, and filled with gas (Cooper et al. 2013). As the disease progresses, there is a large accumulation of necrotic debris that constitute a dark brown diphtheritic membrane which adheres tightly to the jejunum mucosa and sometimes to the duodenum, ileum, and ceca (Cooper et al. 2013).

Although clinical outbreaks of NE may cause high levels of mortality, the subclinical form of the disease is more relevant. Sick birds remain untreated because the disease is undetected, generating great economic losses for the poultry industry (Wade and Keyburn 2015).

Subclinical NE occurs without a substantial increase in mortality, but with clear signs of intestinal disorders (Cooper et al. 2013). There is damage of the intestinal mucosa, problems with digestion and absorption, lower performance resulting in weight loss, increased feed conversion rate, and decrease in production (Cooper et al. 2013). Gram-positive rods are found in the lesions and can be isolated by differential anaerobic cultures (Cooper et al. 2013). Necropsy findings of subclinical cases include ulcers of focal necrosis in the mucosal surface, with a watery intestinal content that has a greenish brown color (Cooper et al. 2013). Histologically, there are necrotic foci in the villi of the duodenum and ileum mucosa, accompanied by congestion of blood vessels in the lamina propria and submucosa (Cooper et al. 2013). Often the liver is enlarged and there is cholangiohepatitis or hepatitis. It is proposed that the large number of *C. perfringens* residing in the intestine allows it to get into the bile ducts (Cooper et al. 2013). Livers are distended, have a pale appearance with extensive red or white foci of coagulative necrosis, and contain clusters of Gram-negative bacilli in necrotic areas (Cooper et al. 2013).

Microscopic examination of early stages of NE shows that the lamina propria is hyperaemic and infiltrated with numerous inflammatory cells, mainly granulocytes (Olkowski et al. 2008). There is extensive edema and substantial disturbance of the structural integrity between the lamina propria and the enterocytes (Olkowski et al. 2008). Severe coagulative necrosis of the luminal part of the mucosa is observed in later stages, with the massive presence of Gram-positive bacilli which do not invade the epithelium, nor are they attached to the viable mucosal epithelial cells (Cooper et al. 2013). The presence of a pseudomembrane consisting of numerous Gram-positive bacilli suspended in mucus, necrotic epithelial cells, erythrocytes, granulocytes, mononuclear cells, and fibrin occurs in severe cases (Cooper et al. 2013). If the birds survive, the regenerated mucosa epithelium is characterized by a decrease in goblet and columnar epithelial cells and an increase in cuboidal cells, which results in a short flat villus with a reduced absorptive surface (Cooper et al. 2013). In cases of cholangiohepatitis, histopathological lesions include extensive multifocal coagulative necrosis in the liver, bile duct hyperplasia, fibrinoid necrosis, cholangitis, and occasionally focal granulomatous inflammation (Cooper et al. 2013).

Since *C. perfringens* is part of the intestine microbiota of birds, their presence is not indicative of disease. However, an increase up to 10^9 *C. perfringens*/g intestinal content may occur in sick animals, whereas in healthy birds, it increases to not more than 10^5 *C. perfringens*/g (Cooper et al. 2013). The diagnosis of NE in chickens has been done historically via clinical, macroscopic, and microscopic findings characteristic of the infection (Cooper et al. 2013). A standardized scoring system based on macroscopic lesions is common (Cooper et al. 2013). Currently, new molecular techniques offer the possibility of detecting potentially pathogenic strains in an

accurate way, for example, identifying genes encoding for toxins relevant for NE pathogenesis in isolates from clinical samples (Bailey et al. 2013).

Clostridium perfringens Toxins Involved in Necrotic Enteritis

Although *C. perfringens* as a species produces more than 15 different toxins, individual strains produce only some of them. Based on four of the toxins produced by *C. perfringens* (α, β, ε, and ι), strains are classified into five different toxinotypes, from A to E (Uzal et al. 2014). Other toxins, such as perfringolysin O (PFO or θ-toxin), collagenase (κ-toxin), β2 toxin, CPE, and NetB, are not considered for toxinotyping (Uzal et al. 2014). NE in poultry is caused mainly by *C. perfringens* toxinotype A strains, which among other toxins produce phospholipase C (CpPLC or α toxin) and the pore-forming necrotic enteritis B-like toxin (NetB) (Uzal et al. 2014).

In *C. perfringens*, the VirR/VirS two-component regulatory system and the regulatory RNA (VR-RNA) cascade control expression of a regulon composed of 150 genes, including those for some toxins (such as *cpa* that codifies for CpPLC, *pfoA*, and *netb*), transporters, and energy metabolism (Ohtani and Shimizu 2015). The accessory growth regulator (Agr)-like quorum-sensing system monitors bacterial environments, regulates, and switches on the VirR/VirS system, and thus toxin production during NE is greatly influenced by different factors affecting bacterial density (Ohtani and Shimizu 2015).

C. perfringens Phospholipase C (Cp-PLC)

The *cpa* gene encoding CpPLC is present on the chromosome of all *C. perfringens* strains and shows only minor variations of sequence among strains (Uzal et al. 2014). However, the amount of CpPLC produced by different strains varies considerably, reflecting differences in gene expression (Uzal et al. 2014). Toxinotype A strains, which are associated with gas gangrene in humans and with NE in birds, produce the highest amount of CpPLC (Uzal et al. 2014).

The CpPLC is a zinc-dependent metalloenzyme with phosphohydrolase activity and a preference for phosphatidylcholine and sphingomyelin, two main components of the outer layer of the eukaryotic cell membranes (Flores-Díaz et al. 2016). This 43 kDa toxin is composed of an N- and a C-terminal domain. The catalytically active site is located in the N-terminal domain, while the C-terminal domain is important for anchoring and interaction with biological membranes (Flores-Díaz et al. 2016). CpPLC possibly anchors to membranes via Ca^{2+} ion binding sites in the C-terminal domain, and thus the active site is oriented to interact directly with membrane phospholipids (Flores-Díaz et al. 2016).

It has been shown that CpPLC affects the jejunal mucosa of laying hens, which suggests that it contributes to NE pathogenesis (Rehman et al. 2006). However,

CpPLC is not the main virulence factor in this disease, as it has been shown that a pathogenic *C. perfringens* strain with inactivated *cpa* gene still induces NE lesions comparable to those found in birds challenged with the wild-type strain (Rood et al. 2016).

C. perfringens NetB

Virulent *C. perfringens* strains associated with NE secrete into culture supernatant a pore-forming toxin, referred to as NetB, which has 30% sequence similarity with *S. aureus* alpha toxin (Rood et al. 2016). Treatment of chicken cells with NetB causes rapid cell blebbing and swelling, leading to cell lysis. To determine the role of NetB in NE, a mutant strain isolated from sick chickens lacking the *netb* gene was generated by homologous recombination (Rood et al. 2016). That mutant strain does not induce NE, while the wild-type strain and the mutant strain complemented with the wild type *netb* gene did, demonstrating that NetB is essential for causing experimental NE in chickens (Rood et al. 2016). More recent work has shown that the coding sequence of the *netb* gene is highly conserved and that strains producing the most severe injuries are those that produce greater amounts of NetB (Rood et al. 2016).

The crystal structure of monomeric NetB shows an overall β-rich fold similar to those of the alpha-hemolysin family, such as the delta toxin from *C. perfringens*, *S. aureus* Panton-Valentine leukocidin S, *S. aureus* leukotoxin LukE, and *S. aureus* alpha-hemolysin (Yan et al. 2013). NetB consists of 16 β-strands and an α-helix, arranged into a β-sandwich, latch, rim, and prestem domains (Yan et al. 2013). The β-sandwich domain is comprised of a five-stranded and six-stranded antiparallel β-sheets (Yan et al. 2013). The prestem region (residues 140–186) forms a three-stranded antiparallel β-sheet, which packs against the five-stranded antiparallel β-sheet of the β-sandwich (Yan et al. 2013). The rim region, which is involved in membrane recognition and binding, is comprised of a four-stranded antiparallel β-sheet and a well-ordered loop formed by residues 205 to 242 (Yan et al. 2013). The base of the rim domain contains a number of solvent-exposed aromatic groups predicted to form direct contacts with the lipid membrane. Structural comparisons with other members of the alpha-hemolysin family reveal significant differences in the conformation of the membrane binding domain, suggesting that NetB may recognize different membrane receptors or use a different mechanism for membrane-protein interactions (Yan et al. 2013). Entrance from either side of the NetB pore is lined with acidic residues (Asp-116, Glu-153, and Glu-132) forming two distinct negatively charged belts, which likely explains why the NetB pores differ in their ion selectivity, preferring cations over anions (Yan et al. 2013).

The crystal structure of the heptameric complex of NetB in detergent, which likely represents the membrane-inserted pore form, is known (Savva et al. 2013). A NetB oligomer is composed of seven monomers arranged in a ring, in which each monomer forms an extensive hydrogen bond network and salt bridges on both interfaces (Savva et al. 2013). The seven β-sandwich domains form the cap of the

mushroom-shaped heptamer, whereas the stem domains form the 14-strand β-barrel pore (Savva et al. 2013). This heptameric assembly shares structural similarity to that of the staphylococcal α-hemolysin, although important differences can be identified in the membrane binding and pore lumen regions (Savva et al. 2013).

Site-directed mutagenesis studies helped identify several residues critical for NetB-induced cell lysis, which are clustered in regions predicted to be required for oligomerization or membrane binding (Yan et al. 2013). Comparative analysis of the NetB structure indicates that the S254 residue is involved in oligomerization, as a NetB S254 L variant does not oligomerize in lipid bilayers and chicken erythrocytes (Yan et al. 2013). In contrast, the NetB R230Q and W287R variants form oligomers in a solution and also form single channels in artificial phospholipid bilayers; however, these protein variants were inactive against chicken erythrocytes (Yan et al. 2013). Since the R230 and W287 residues are located in the putative rim domain of NetB, it was postulated that these residues are required for binding to a cell-surface receptor (Yan et al. 2013).

C. perfringens TpeL

A large clostridial glucosylating cytotoxin referred to as TpeL, and homologous to TcdA and TcdB from *C. difficile*, also appears to have a role in the pathogenesis of NE (Chen and McClane 2015). TpeL is encoded on a conjugative plasmid (Chen and McClane 2015) and binds to LDL receptor-related protein 1 in the target cells (Schorch et al. 2014). Experimental infections with *C. perfringens* TpeL-positive strains in broilers induce more severe intestinal lesions and cause NE, with a more rapid course and higher fatality rate than those strains lacking this cytotoxin (Coursodon et al. 2012). Thus, TpeL likely potentiates the effect of other *C. perfringens* virulence factors associated with NE.

Other Toxins

A chromosomally encoded bacteriocin referred to as perfrin, present in several NetB positive strains of broiler origin, likely contributes to the virulence of NE associated *C. perfringens* strains (Timbermont et al. 2014). Perfrin has bactericidal activity over a wide pH-range and has no sequence homology to any currently known bacteriocin, suggesting that this is the paradigm for a new bacteriocin class (Timbermont et al. 2014). Perfrin inhibits the growth of commensal *C. perfringens* strains and seemingly confers an advantage to pathogenic strains during competition for nutrients (Timbermont et al. 2009).

Comprehensive studies of *C. perfringens* genomes from several NE-inducing strains have revealed up to four different, large conjugative plasmids encoding several known and putative virulence factors clustered in discrete pathogenicity loci (Lepp et al. 2010; Parreira et al. 2012; Lepp et al. 2013). The best characterized of them, referred to as NELoc-1, encodes the *netb* gene and 36 additional genes

including two encoding glycohydrolases, one metalloprotease, two leukocidins, one protein similar to internalin, and several putative adhesins (Lepp et al. 2010). Thus, besides the well-recognized toxins, NE pathogenesis likely involves several additional enzymes and adhesins encoded in those pathogenicity loci, and hence, variation in plasmid contents probably contributes greatly to interstrain pathogenicity variation (Lacey et al. 2016).

Predisposing Factors

Besides the presence of a *C. perfringens* virulent strain in the intestine, certain predisposing factors that affect gut microbiota or the immune state of the birds are required for the onset of NE (Moore 2016).

One of the most important predisposing factors to NE is the intestinal mucosa damage caused by the intracellular phase of coccidial lifecycle (Wu et al. 2014; Moore 2016; Tsiouris 2016). Coccidiosis induces extravasation of plasma proteins into the intestinal lumen which favors the overgrowth of pathogenic *C. perfringens* (Moore 2016). Furthermore, coccidial infections enhance intestinal mucus production which also creates a favorable environment for *C. perfringens* proliferation and toxin production (Moore 2016).

Feed composition is another important factor that could favor the onset of NE (Moore 2016; Tsiouris 2016). High content of proteins from animal sources is a dietary factor that predisposes broilers to NE (Wu et al. 2014). Diets with high viscosity, due to a high content of indigestible materials soluble in water, predispose the bird to intestinal stasis which enables proliferation of *C. perfringens* (Moran 2014). The incidence of NE in animals fed with diets rich in rye, wheat, and barley is 6 to 10 times greater than in those fed with diets based on maize (Moore 2016).

Stress in broiler chickens, such as that induced by high stocking density, predisposes the birds to NE in a subclinical experimental model (Tsiouris et al. 2015). Immunosuppressive agents such as the viruses that cause chick anemia, Gumboro disease or Marek's disease, or exposure to mycotoxins, reduce resistance to gut infections and may increase the severity of NE (Antonissen et al. 2014, 2015; Moore 2016).

Experimental Models of the Disease

NE produced by *C. perfringens* is a complex and multifactorial disease difficult to reproduce experimentally (Shojadoost et al. 2012; Prescott et al. 2016b; Van Waeyenberghe et al. 2016). Several factors should be considered for a successful reproduction of experimental NE, including induction of intestinal damage with a coccidial infection, genetic background of the animals, size of feed particles, animal protein content and non-starch polysaccharides in the birds'diet, and *C. perfringens* strains used in the challenge, as well as the route, dose, and frequency of the

infection (Stanley et al. 2014; Rodgers et al. 2015; Prescott et al. 2016b; Van Waeyenberghe et al. 2016; Oh and Lillehoj 2016).

To predispose for experimental NE, coccidial vaccines are often administered at 10 times the recommended vaccination dose, 4–5 days before the *C. perfringens* challenge, so that the coccidian-induced intestinal damage coincides with bacterial challenge (Stanley et al. 2014; Rodgers et al. 2015; Prescott et al. 2016b; Van Waeyenberghe et al. 2016).

There are different susceptibilities of Cobb, Ross, and Hubbard broilers toward developing experimental NE. When chickens are orally infected with *E. maxima* and viable *C. perfringens* and fed a high-protein diet to promote the development of experimental NE; Cobb chickens are more susceptible to NE than the Ross and Hubbard lines (Jang et al. 2013).

Diets that increase the digesta viscosity or enhance mucus production lead to prolonged transit times in the intestine, favoring *C. perfringens* growth (Prescott et al. 2016b). High ground feed; cereals that contain high percentages of water-soluble, indigestible non-starch polysaccharide; and diets with large amounts of animal protein, such as fish meal, allow bacteria to proliferate faster and predispose birds to experimental NE (Stanley et al. 2014; Rodgers et al. 2015; Prescott et al. 2016b).

The use of *netb*-positive *C. perfringens* strains with other virulence determinants, such as the toxin tpeL, is critical for reproducing NE experimentally. The plasmids associated to virulence factors can be lost on subcultures of the bacterium; therefore, maintaining a frozen master stock of the virulent strains, and confirming by PCR that the strain used for challenge has not lost *netb* carrying plasmid, is mandatory (Prescott et al. 2016b).

Fluid thioglycolate medium (with dextrose) is the most common culture medium used as the challenge inoculum. To induce NE experimentally, birds are usually starved overnight and fed with infected food, inoculated at a ratio of 1.25–1.5 fluid thioglycolate medium/feed (v/w), twice daily (Prescott et al. 2016b). Cultures of 15 h increase lesion scores, probably due to greater toxin production (Prescott et al. 2016b).

Pathogenesis

NE pathogenesis depends on the concerted expression of a variety of extracellular enzymes and toxins by *C. perfringens* which contributes to the colonization of the intestinal mucosa, acquisition of nutrients, evasion of host defenses, and the damage to host tissues (Parreira et al. 2016; Prescott et al. 2016a).

A higher number of pathogenic *C. perfringens* strains produce bacteriocins compared with commensal *C. perfringens* strains (Timbermont et al. 2014). By inhibiting other *C. perfringens* strains, the virulent strains obtain the maximum benefit of the increased nutrient availability due to the initial damage of the intestinal mucosa (Timbermont et al. 2014). Mucus secreted by intestinal epithelial cells is rich in glycosylated mucin glycoproteins and contains a variety of antimicrobial

molecules designed to add a barrier to bacterial colonization of the intestinal epithelial cells (Prescott et al. 2016a). Strains of *C. perfringens* associated to NE possess within NELoc-1 two genes encoding "chitinases" capable of degrading mucins (Prescott et al. 2016a). These enzymes probably hydrolyze the chitobiose core of glycoproteins present in the intestinal mucus and therefore participate in *C. perfringens* colonization (Prescott et al. 2016a). The sugars of glycoproteins and glycolipids represent potential binding sites for microbial adhesins, as well as an energy source (Prescott et al. 2016a). *C. perfringens* sialidases, which are regulated by the VirR/VirS system, could be involved in promoting bacterial adhesion through modification of epithelial surfaces which expose receptors on enterocyte cell membranes (Li and McClane 2014). The adhesion of *C. perfringens* to extracellular matrix-based collagen type III and types IV and V, fibrinogen, and vitronectin depends on a putative fimbrial adhesins VR-10B operon, always present in *netB*-positive isolates (Lepp et al. 2013). This operon is important for colonization because its inactivation abolishes collagen adhesion, as well as virulence (Wade et al. 2015).

Virulent *C. perfringens* strains either secrete collagenases at higher levels than commensal strains or induce the expression of host collagenolytic enzymes because higher collagenase activity is found in the intestines of broilers with NE than in controls (Olkowski et al. 2008). Ultrastructural analysis of NE found that primary changes occur at the level of basal and lateral domains of the enterocytes, whereas their apical domain remains intact even in the face of advanced necrotic changes (Olkowski et al. 2008). Thus, mucosal necrosis seems to result from the destruction of lamina propria, the extracellular matrix, and intercellular junctions (Olkowski et al. 2008). NetB may induce necrotic lesions by forming pores in enterocytes leading to cell death (Rood et al. 2016). Whether this is involved in the early, or later, stages of disease progression is not clear (Prescott et al. 2016a).

Prevention and Control

The strategy most commonly used in the control of NE in poultry includes administration of antibiotics (Caly et al. 2015). However, the European ban on antibiotics (feed additives regulation 1831/2003/EC), which took effect in January 2006, made necessary other alternatives to prevent NE occurrence (Caly et al. 2015). NE prophylactic treatments include improving the hygiene in poultry farms, coccidiosis prevention, diet modifications to include prebiotics and probiotics, or targeting directly *C. perfringens* virulent strains with bacteriophages (Caly et al. 2015).

Prebiotics are mostly indigestible oligosaccharides that will stimulate the commensal microbiota and enhance the beneficial effects of probiotics within the host, dependent upon dose, length of treatment, or even diet (Caly et al. 2015). Mannanoligosaccharides and Saf-Mannan are components within the yeast cell wall often used as feed additives not only to improve intestinal health and immune response, but also inhibit pathogen colonization by reducing adhesion (Thanissery et al. 2010; Caly et al. 2015).

Probiotics are a live microbial food supplement that improve immunity and increase broiler chicken performance (health, weight gain, feed conversion), thus reducing the risk of infection by opportunistic pathogens in the host (Caly et al. 2015). It has been shown that enterococci, lactic acid bacteria, and several *Bacillus* species (licheniformis, pumilus, subtilis, cereus) have antagonistic activity against *C. perfringens*, associated with the production of bacteriocins and antimicrobial peptides (Knap et al. 2010; Cao et al. 2012; Jayaraman et al. 2013; Caly et al. 2015).

Bacteriophage therapy was widely used to treat bacterial infections up until the 40s and has had a recent upsurge of interest with the growing need for alternatives to antibiotic treatments (Caly et al. 2015). Bacteriophages produce endolysins, which target peptidoglycans and lyse the bacterial cell wall, freeing the phages and allowing them to spread to other cells (Caly et al. 2015). Many bacteriophages isolated from *C. perfringens* strains of poultry origin, with specific anti-*Clostridium* activity, have been described and used to limit *C. perfringens* infection in field trials (Miller et al. 2010). A mixture of six bacteriophages reduces mortality in a NE challenge by over 90%, and overall performance was enhanced as assessed by weight gain and feed conversion (Miller et al. 2010).

Immunity and Prospects for Vaccination

The immune response to *C. perfringens* infection in poultry is not completely understood (Mot et al. 2014). Several *C. perfringens* antigens are recognized by antibodies present in sera from chickens after a NE infection, including pyruvate: ferredoxin oxidoreductase (PFOR), elongation factor G, perfringolysin O, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), fructose-1,6-biphosphate aldolase (FBA), endo-beta-N-acetylglucosaminidase (Naglu) and phosphoglyceromutase (Pgm), the elongation factor Tu, and a hypothetical unidentified protein (HP) (Kulkarni et al. 2006; Lee et al. 2011a, b).

Many trials with different approaches have been carried out to develop a vaccine able to induce a protective immunity against NE (Mot et al. 2014). The antigens have been administered as non-inactivated supernatants, formalin-inactivated crude toxoids, immunogenic proteins, and genetically modified toxins, using different routes of administration (intramuscularly, subcutaneously, orally), varying dosages, several boosters, and adjuvants. Furthermore, selected *C. perfringens* antigens have also been delivered by live-attenuated vaccine carrier strains. Multiple vaccination dosages are necessary for a good immune response, and one parenteral single vaccination, at the day of hatching, offers no protection (Jiang et al. 2009; Crouch et al. 2010; Lanckriet et al. 2010; Kulkarni et al. 2010; Saleh et al. 2011; Mot et al. 2013; Keyburn et al. 2013a, b; Jiang et al. 2015).

Chickens immunized intramuscularly two (or three) times with recombinant CpPLC/CpPLC toxoid, GAPDH, HP, FBA, or PFOR, were able to decrease the mean intestinal lesion score (Kulkarni et al. 2007). GAPDH and FBA protected only against mild challenge, whereas CpPLC, HP, and PFOR protected significantly against a heavy challenge (Kulkarni et al. 2007). In another study, double

subcutaneous vaccination regimens using CpPLC, NetB toxin, PFOR, or elongation factor Tu gave similar protection levels after experimental infection (Jang et al. 2012). Immunization with Naglu and Pgm also confers partial protection after challenge with two different strains (Jiang et al. 2009).

CpPLC has been considered a potential antigen for a vaccine to protect from NE in poultry (Kulkarni et al. 2006). Even though CpPLC has been shown not to be crucial for the pathogenesis of NE, immunization with this toxin confers at least partial protection against NE (Kulkarni et al. 2006; Zekarias et al. 2008). It has been postulated that secreted proteins of Gram-positive bacteria accumulate within the cytoplasm and can be recognized by antibodies that might block protein transport channels, thereby inhibiting bacterial proliferation (Zekarias et al. 2008). Different trials have shown that immunization of birds with CpPLC, or its carboxy terminal domain only, either intramuscularly or with *Salmonella* strains expressing those antigens, are partially protected against the mucosal damage induced experimentally by virulent *C. perfringens* strains (Kulkarni et al. 2006; Zekarias et al. 2008; Hoang et al. 2008; Cooper et al. 2009; Kulkarni et al. 2010; Jang et al. 2012, 2015; Fernandes da Costa et al. 2016).

Comparison of different vaccination schedules, different challenge models, and different scoring procedures to induce protection against NE has revealed that vaccination with NetB (using recombinant NetB, native NetB, or toxoids and bacterin vaccines containing NetB) induces an immune response that partially protects against NE. These results occur in directly vaccinated birds or in chicks derived from vaccinated hens (Jang et al. 2012; Keyburn et al. 2013a, b; Fernandes da Costa et al. 2013; Jiang et al. 2015; Fernandes da Costa et al. 2016). Recently, a novel attenuated *Salmonella* vaccine strain composed of the carboxyl-terminal fragment of CpPLC and a GST-netB fusion protein, designed to replicate 6–10 times before lysis in broilers, was able to generate a strong production of intestinal IgA, IgY, and IgM antibodies specific against those toxins that protect against mild and severe challenges of pathogenic *C. perfringens* (Jiang et al. 2015). All of the above-described data thus shows that the development of a practical and fully protective vaccine against NE is a difficult challenge and requires the combination of at least two different bacterial proteins.

Conclusion and Future Directions

The annual consumption of poultry eggs and meat for the global human population is respectively more than 70 million and 115 million tons, and the demand is growing. Therefore, an efficient and safe production of healthy poultry is essential for the future. The indiscriminate use of antibiotics as growth promoters favoring weight gain of poultry and helping to control bacterial infections has been banned in several countries, leading to NE reemergence and entailing enormous economic losses worldwide. Because there is a rising concern that the antibiotic-resistant pathogens could be transmitted to humans, it is expected that the use of antimicrobials in animal production will decrease during the coming years.

To ensure continuity in the supply of poultry-based food products, effective control measures against NE in the framework of the current situation are required. Under optimal conditions, pathogenic *C. perfringens* strains proliferate in birds' gut causing intestinal mucosal necrosis, diarrhea, malabsorption, dehydration, and, in severe cases, death. Several virulence factors, such as chitinases, bacteriocins, and toxins, likely play a role in colonization and intestinal mucosal damage induced by the bacteria. The new possibilities provided by genomic and proteomic techniques offer alternatives to achieve a comprehensive knowledge of the pathogenesis of NE. A better understanding of the complex interaction between pathogenic *C. perfringens* and nonpathogenic commensal bacteria in the birds' gut is also required to implement rational strategies to control NE. Further studies on the immunobiology of host-pathogen interaction will contribute toward identifying factors that determine the occurrence of NE outbreaks, allowing the development of integrated and effective strategies to affordably prevent and treat this disease.

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Structure Function Studies of Large Clostridial Cytotoxins

8

Joseph W. Alvin and D. Borden Lacy

Abstract

The large clostridial toxins (LCTs) are a group of homologous, high molecular weight proteins that include toxin A and toxin B from *Clostridium difficile* (TcdA and TcdB), the lethal and hemorrhagic toxins from *C. sordellii* (TcsL and TcsH), α-toxin from *C. novyi* (Tcnα), and a large cytotoxin from *C. perfringens* (TpeL). The LCTs share a glycosyltransferase enzymatic activity that results in the inactivation of specific Rho and Ras GTPases, essential signaling proteins and regulators within eukaryotic cells. The importance of these toxins in the context of disease has led many to apply structural and functional approaches to the understanding of LCT mechanism.

Keywords

Glycosyltransferase • Autoprocessing • Pore formation • Endocytosis • Receptor

Contents

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Introduction

The Clostridium genus is comprised of a highly diverse set of anaerobic, gram-positive, spore-forming organisms. Nevertheless, some share the capacity to produce a homologous family of proteins called the large clostridial toxins (LCTs) (Table 1). In each instance, the production of toxins is thought to contribute to the link each organism has with disease.

Clostridium difficile is a bacterial pathogen that causes nearly half a million infections in the United States each year (Smits et al. 2016). It infects the human colon and can cause diarrhea, pseudomembranous colitis, and, in some cases, death. As the major virulence factors in *C. difficile* infection (CDI), TcdA and TcdB have become the prototypical members of the LCT family. The toxins are responsible for the diarrhea, inflammation, and colonic tissue damage that occur with CDI. While strains containing only TcdB have been associated with severe disease in both human and experimental animal infections, most clinical isolates express both toxins. The respective role that each toxin plays in the context of infection is an active area of inquiry (see also ► Chap. 9, "Role of *Clostridium difficile* Toxins in Antibiotic-Associated Diarrhea and Pseudomembranous Colitis").

C. sordellii infections are most often associated with gynecological events, intravenous drug use, or musculoskeletal allografts (Aronoff 2013). While rare, these infections can cause a profound toxic shock syndrome characterized by treatment-refractory hypotension, extreme leukocytosis, and visceral effusions. Infections are associated with a high mortality rate. The major toxins for this organism are two members of the LCT family, TcsH and TcsL. Notably, TcsH shares greater sequence identity with TcdA of *C. difficile* than with TcsL, and a similar relationship exists between TcsL and TcdB (Table 1). The toxins have been shown to be important in animal models and in the evaluation of human infections, although there are a number of clinical isolates that only express TcsL.

Table 1 Comparison of amino acid sequence identity between LCTs

	TcdA	TcdB	TcsH	TcsL	Tcnα	TpeL
TcdA						
TcdB	48%					
TcsH	78%	48%				
TcsL	48%	76%	49%			
Tcnα	31%	30%	31%	30%		
TpeL	41%	41%	42%	42%	33%	

Infections by *C. novyi* are typically the result of soil contact with open wounds or injection drug use with soil contamination (Aronoff 2013). Symptoms of disease can include wound-associated gas gangrene or a clostridial toxic shock syndrome that is similar to what is observed in *C. sordellii* infections. Vaccination with Tcnα toxoid in guinea pigs significantly improved survival after challenge with *C. novyi* spores (Amimoto et al. 1998), and strains cured of the Tcnα-encoding bacteriophage show reduced virulence in animal models (Aronoff 2013). These observations underscore the importance of Tcnα in disease pathogenesis.

Although *C. perfringens* is an etiological agent in gas gangrene and foodborne illnesses, the role (if any) for TpeL in human disease is unclear. (For a broader view of *C. perfringens* pathogenesis please see ▶ Chaps. 7, "Role of *Clostridium perfringens* Toxins in Necrotic Enteritis in Poultry," and ▶ 6, "Role of *Clostridium perfringens* Alpha, Beta, Epsilon, and Iota Toxins in Enterotoxemia of Monogastrics and Ruminants.")

The presence of TpeL has been linked to an increase in the frequency and size of enteric necrotic lesions in broiler chicks, however (Coursodon et al. 2012). This suggests that TpeL may play an important role in necrotic enteritis of chickens and other agriculturally significant birds. Still, much of the current understanding of TpeL structure and function has come from a comparative analysis with other members of the LCT family.

LCT Structure

The LCTs are large single-chain proteins ranging from 191 to 308 kDa in molecular weight (Pruitt and Lacy 2012). They are considered AB-toxins in that a "B domain" is responsible for the binding and delivery of an enzymatic "A domain" into the cytosol of the host cell. In each LCT, the "A domain" is a glycosyltransferase that targets members of the Rho and Ras GTPase families. The "B domain" can be divided into three regions: the autoprocessing, delivery, and combined repetitive oligopeptide repeats (CROPS) domains (Fig. 1a). These discrete structural domains contribute to a multistep mechanism of intoxication that includes (1) receptor binding and endocytosis, (2) pore formation and delivery of the "A domain" across the endosomal membrane, (3) autoprocessing, and (4) glycosyltransfer (Fig. 1b).

Receptor Binding and the CROPS

With the exception of TpeL, the carboxy-terminal region of the LCTs encodes a series of repetitive sequences that together form the CROPS domain (Pruitt and Lacy 2012). The repeats are composed of multiple 19–24 amino acid short repeats interspersed with 31 amino acid long repeats (Fig. 2a). In electron microscopy (EM) images of the TcdA holotoxin, the CROPS is evident as an extended structure (Fig. 2b). Crystal structures of fragments from the CROPS domains of TcdA suggest an extended β-solenoid structure where the short and long repeats come together to

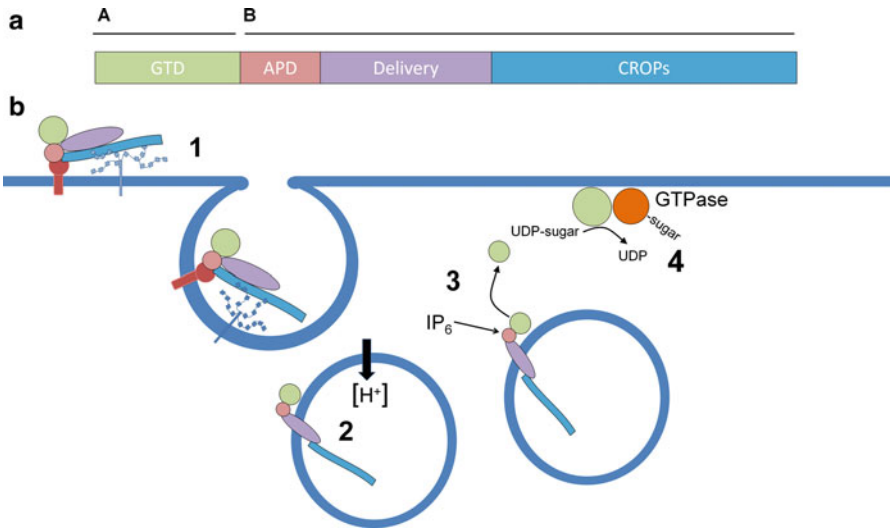

Fig. 1 Overview of domain organization and model of LCT intoxication. LCTs are comprised of (N- to C-terminus) a glycosyltransferase domain (GTD), autoprocessing domain (APD), delivery or pore-forming domain (PFD), and combined repetitive oligopeptides (CROPS). Step 1: the CROPS is thought to help mediate LCT cell binding through interactions with cell surface glycoproteins and glycolipids*. The LCT can also interact with a host receptor that contributes to specificity and endocytosis. Step 2: as the endosome acidifies, the delivery domain inserts into the lipid bilayer, forming a pore. The GTD (and likely the APD) passes through this pore into the host cytosol. Step 3: InsP6 binds to the APD, initiating autoproteolysis and the release of the GTD from the endosome. Step 4: the GTD localizes at membranes and glycosylates specific subsets of host GTPases. This glycosylation disrupts downstream signaling of the GTPase pathways and results in both cytopathic and apoptotic effects. (*)TpeL lacks the CROPS domain but can still bind and enter cells

form vertices (Fig. 2c). In TcdA, this vertex has been shown to bind an α-Gal-(1,3)-β-Gal-(1,4)-β-GlcNAc trisaccharide (Fig. 2d).

Historically, the CROPS has been thought to represent the receptor-binding domain of the LCTs (Pruitt and Lacy 2012). Much of the evidence in support of this view has come from the study of TcdA and the capacity of the TcdA CROPS to bind carbohydrates. The sugar binding properties of the TcdB CROPS appear to be distinct and have not been investigated in detail for the other LCTs. TcdA can bind to the human I, X, and Y blood antigens as well as a human glycosphingolipid, and all have a core β-Gal-(1,4)-β-GlcNAc structure. These interactions suggest a mechanism where TcdA forms multivalent interactions with intestinal cell surface carbohydrates. Other proposed TcdA receptors include rabbit sucrose isomaltase and human gp96, but the mechanisms of how these molecules interact with TcdA have not been reported. In addition to the capacity to bind carbohydrates, excess TcdA CROPS domain can compete with TcdA holotoxin for cell binding. Lastly, the CROPS domains are highly antigenic, and antibodies against the TcdA and TcdB CROPS can neutralize the effects of their respective toxins *in vitro.* In one example,

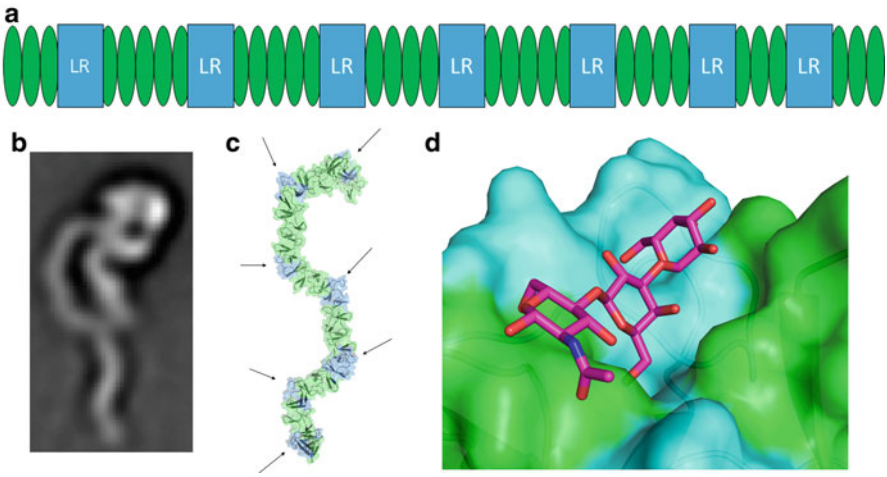

Fig. 2 Structural organization of the CROPS domain. (**a**) TcdA-CROPS consists of seven long repeat (LR) sections, separated by varying numbers of short repeats (SR). (**b**) Electron microscopy shows TcdA-CROPS adopts a sinusoidal structure, similar to what was observed in (**c**) a model derived from the crystal structure of a TcdA CROPS fragment. In TcdA, each of the LR vertices can bind carbohydrates (indicated by *arrows*). (**d**) A crystal structure of a CROPS SR-LR-SR fragment bound to a α-Gal-(1,3)-β-Gal-(1,4)- β-GlcNAc trisaccharide

such antibodies have shown efficacy in protecting people against recurrent CDI (Lowy et al. 2010). The mechanism of this protective effect is not currently known but could result from a block in receptor binding.

More recent studies have indicated that LCT receptor-binding function is not limited to the CROPS domain. Truncations of TcdA and TcdB that lack the CROPS domains are still capable of intoxicating cells, and TpeL lacks a CROPS domain entirely (Pruitt and Lacy 2012). LDL receptor-related protein 1 (LRP1) was shown to act as a TpeL receptor (Schorch et al. 2014), confirming the idea that specific binding and entry of the toxin does not rely on the presence of a CROPS domain. Similarly, chondroitin sulfate proteoglycan 4 (CSPG4) and poliovirus receptor-like protein 3 (PVRL3) were recently reported as TcdB receptors, and both bind in regions outside of the CROPS (Yuan et al. 2015; LaFrance et al. 2015).

In light of these observations, the role of the CROPS domain in LCT function is not entirely clear. It could be that it contributes to but is not essential for binding, consistent with a proposed two-receptor binding model (Schorch et al. 2014). It is also possible that the importance of the CROPS to receptor binding varies among the LCTs. For example, in contrast to what was observed with TcdA, the application of recombinant TcdB CROPS does not compete for TcdB binding. Finally, it could be that the CROPS is involved in processes outside of the host entry mechanism, for example, interaction with the producing bacterium or other components of the infected host. These possibilities merit investigation in the context of the lifestyle and pathogenesis associated with the different LCT-producing bacteria.

Delivery into the Cytosol

LCT receptor binding is followed by receptor-mediated endocytosis (Fig. 1b). As the endosome matures, the pH becomes more acidic. The low pH triggers a structural change in the LCT allowing it to insert into the endosomal membrane and form a pore. The pore can then serve as a conduit for the delivery of the glycosyltransferase domain (GTD) (and perhaps also the autoprocessing domain (APD)) into the cytosol. The requirement for an acidic pH is supported by the observations that bafilomycin, an inhibitor of the vacuolar H^+-ATPase, protects against the cytopathic and/or cytotoxic effects of TcdA, TcdB, and TcsL. Pore formation can also be triggered at the cell surface by acidification of the extracellular medium and quantified by a measure of cellular Rb^+ release. Other indications of pore formation and pH-dependent conformational changes have come from EM, fluorescence, planar lipid bilayer, and liposome fluorescence release assays (Pruitt and Lacy 2012).

The LCT central region that separates the APD from the CROPs has been termed the pore-forming or delivery domain based on the presence of conserved hydrophobic sequences that are thought to insert into the membrane with low pH. The recent crystal structure of a TcdA construct containing the first 1832 amino acids of TcdA ($TcdA_{1832}$) provides the first atomic model of the delivery domain in its soluble form (Fig. 3a) (Chumbler et al. 2016). The domain begins as a three-helix bundle at the GTD-APD interface (residues 767–841, Fig. 3a, blue) that leads into a small globular sub-domain (residues 850–1025, Fig. 3a, red). This sub-domain is then followed by an extended "hydrophobic helical stretch" containing four α-helices (1026–1135, Fig. 3a, yellow) that wrap around a series of β-sheet structures (1136–1802, Fig. 3a, purple). The β-sheet structures end at the base of the APD and are thus positioned to transition into the junction with the CROPs domain. An EM analysis of TcdA holotoxin structure at neutral and low pH indicates that the junction between the delivery domain and the CROPs is a point of flexibility (Fig. 3b) (Pruitt and Lacy 2012; Pruitt et al. 2010).

While the molecular details of how pore formation and translocation occur are currently unclear, mutational studies suggest that residues within the globular sub-domain and the hydrophobic helical stretch are important for pore formation (Zhang et al. 2014). Many of the residues within this hydrophobic stretch are conserved across the LCT family and have been demonstrated to be important for the formation of membrane pores in the context of TcdA and TcdB. The presence of a surface turn at the base of the helical stretch which is important for both pore formation and cytotoxicity, and which is conserved across the entire LCT family (Fig. 3a), suggests an attractive target for broad spectrum neutralization by antibodies or other therapeutic molecules (Chumbler et al. 2016).

Autoprocessing

Following the delivery of the GTD across the endosomal membrane, the APD mediates cleavage at a conserved leucine residue to release the GTD into the cytosol (Pruitt and Lacy 2012). In vitro, this cleavage is initiated by the binding of inositol

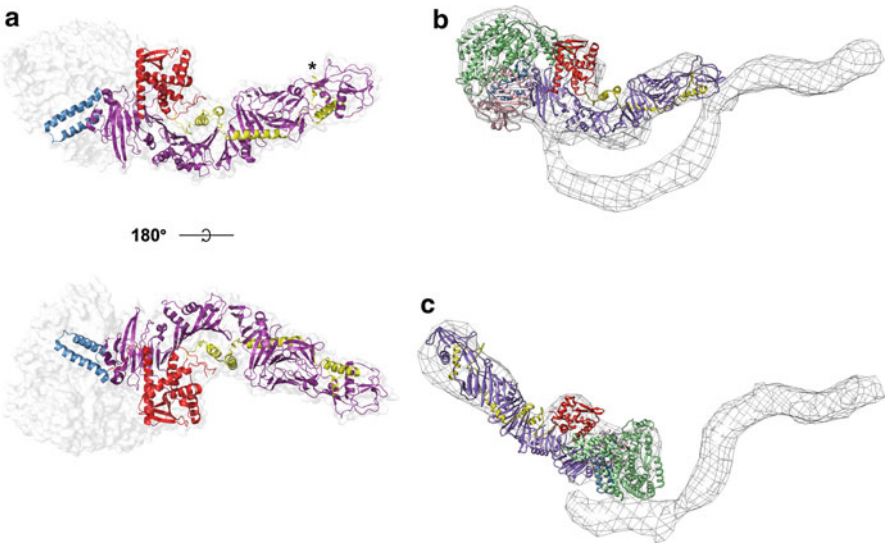

Fig. 3 (**a**) The crystal structure of TcdA 1–1832 is shown as a transparent surface model with the delivery domain highlighted in color. Key features of the delivery domain include a three-helix bundle (*blue*), a globular sub-domain (*red*), and the α-helical hydrophobic stretch (*yellow*). The conserved surface loop is indicated with an *asterisk*. The rest of the domain is colored in *purple* and is primarily composed of β-sheet structures. (**b**, **c**) The TcdA 1–1832 structure with the GTD in *green*, the APD in *pink*, and the delivery domain colored as in (**a**) was docked within TcdA EM structures obtained at (**b**) neutral (**c**) acidic conditions

hexakisphosphate (InsP6), a small molecule found at high concentrations within the eukaryotic cell cytosol. Intracellular concentrations of InsP6 in mammalian cells have been reported in ranges of 10 μM to 1 mM. Isothermal titration calorimetry experiments have been used to calculate the affinity of InsP6 for multiple LCT APDs in the form of an equilibrium dissociation constant (K_D): 4.4–5.1 μM for TcdB, 2.1–2.8 μM for TcsL, and 6.7–8.9 μM for Tcnα (Guttenberg et al. 2011). These data, along with *in vitro* experiments demonstrating holotoxin autoprocessing at InsP6 concentrations as low as 0.1 μM, support the idea that InsP6 acts as the small molecule activator in physiological contexts.

InsP6 binds the APD and causes an allosteric change that is propagated through a central β-flap region which separates the electropositive InsP6 binding site from the active site of the APD (Fig. 4) (Pruitt and Lacy 2012). Structures of the TcdA and TcdB APDs bound to InsP6 have revealed highly similar structures where the InsP6 is coordinated by several positively charged residues. While efforts to crystallize the isolated APD of either toxin in the absence of InsP6 have been unsuccessful, a comparison of the apo-APD structure (crystallized in the context of $TcdA_{1832}$) with that of InsP6-bound APD reveals the significant rearrangements that occur with InsP6 binding (Chumbler et al. 2016). Most notably, the domain's C-terminal helix (TcdA 766–778) uncoils at either end, and the central β-flap (TcdA 746–765) rotates toward the InsP6 binding site and away from the enzyme active site by ~90°

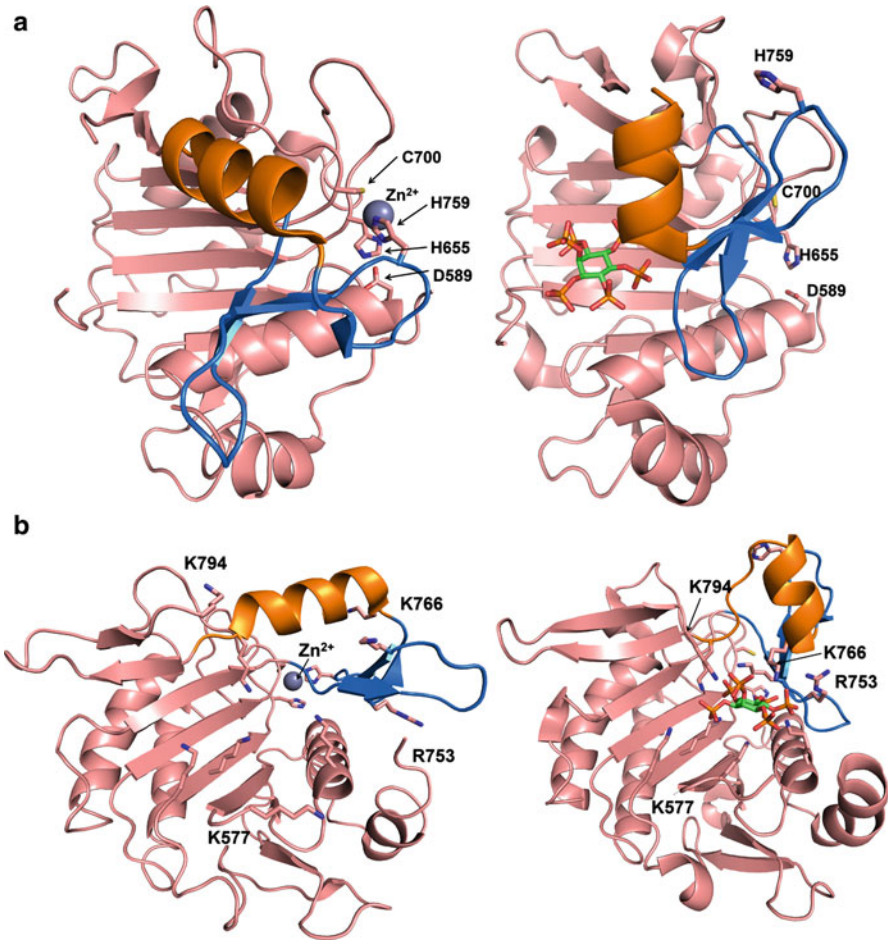

Fig. 4 TcdA-APD conformational changes induced by InsP6 binding (**a**) comparison of the TcdA APD in the absence of InsP6 (*left*, the *apo* state) versus the InsP6-bound structure (*right*) reveals conformational changes in the β-flap (*blue*) and a C-terminal α-helix (orange). H759 coordinates a Zn^{2+} ion in the active site of the apo-APD but moves out of the active site upon InsP6 binding. Residues C700, H655, and D589 are located within the active site in both structures and have been shown to have a role in autoprocessing activity. (**b**) An alternate view of the apo (*left*) and InsP6-bound structures (*right*) highlights the positively charged residues (K577, K766, R753, and K794) that undergo conformational changes in order to bind InsP6

(Fig. 4a). One effect of this rearrangement is that the side chains of four positively charged residues move into the InsP6 binding site to bind the InsP6 phosphate groups (Fig. 4b). In addition, the H759 residue, located at the tip of the β-flap, moves from being a part of the enzyme active site to a location 19 Å away (Fig. 4a). Mutation of H759 in TcdA to alanine (or H757 in TcdB) results in a protein whose efficiency of autoprocessing no longer depends on InsP6 concentration (Chumbler

et al. 2016). These data are consistent with a model where the structural changes imposed by InsP6 binding trigger allosteric changes in the enzyme active site that permit autoprocessing.

The active site has been described as a catalytic triad in that it has a conserved trio of cysteine, histidine, and aspartic acid residues (Pruitt and Lacy 2012). Mutation of any one of these residues (D589, H655, or C700 in TcdA) results in a loss or significant defect in autoprocessing and has led the field to describe the APD as a cysteine protease. While crystal structures of the TcdA and TcdB APDs in the presence of InsP6 did not indicate the expected arrangement of these three residues (Fig. 4a, right), the absence of substrate made it hard to evaluate the proposed mechanism of activity.

The $TcdA_{1832}$ crystal structure led to the unexpected discovery that both TcdA and TcdB have a zinc bound to the cysteine of their APD active sites (Fig. 4a, b, left), and this zinc is required for autoprocessing activity (Chumbler et al. 2016). In light of these observations, it is unclear whether autoprocessing results from an activated cysteine, a zinc-activated water, or some other nucleophile. Structures in the presence of substrate could help resolve this question. In the meantime, the more general reference to an autoprocessing domain (rather than a cysteine protease domain) seems warranted.

The presumed function of the APD is to release the GTD from the endosomal surface, thereby allowing it to migrate to GTPase substrates located at the plasma membrane (Fig. 1b). Studies in TcdA and TcdB have indicated that while autoprocessing is important for the kinetics of cytopathic responses, it is not essential (Kreimeyer et al. 2011; Chumbler et al. 2012; Li et al. 2013). TcdA and TcdB toxin mutants that are deficient in autoprocessing will still cause phenotypic changes suggesting that some GTPase substrates are associated with the endosomal membrane and/or that toxin-containing endosomes are recycled to the cell surface. It is interesting to note that when a TcsL-autoprocessing mutant is applied to cells, the rates of Rac1 and Ras modification differ (Craven and Lacy 2015). This is consistent with known differences in Rac and Ras localization and suggests that autoprocessing may be more important in LCTs that target Ras GTPase proteins exclusively localized at the plasma membrane.

Glycosyltransfer

The LCTs encode an N-terminal 63 kDa GTD that can transfer a sugar onto small Rho and Ras family GTPases (Pruitt and Lacy 2012). The specificity for different GTPase family members can differ among the LCTs (Genth et al. 2014) (Table 2). In general, the glycosyltransferase mechanism involves the nucleophilic attack of an activated nucleotide sugar that results in the transfer of the sugar on to the starting nucleophile. In the LCTs, the nucleophile is the conserved threonine in the Switch I region of the GTPase. Modification of the threonine prevents exchange of GDP for GTP and the capacity of the GTPase to bind regulatory molecules and downstream effectors, and therefore disrupts multiple downstream signaling pathways.

Table 2 Selectivity of LCT GTDs for disparate GTPases based on in vitro studies. This table represents the ability (denoted by "+") of LCT GTDs to glycosylate various GTPases. Asterisks represent cases where LCTs are capable of modifying a different set of substrates or where the GTPase is not a preferred substrate. This table was reproduced from data presented in Genth et al. (2014)

	Rho(ABC)	RhoG	Rac1	Cdc42	(HKN) Ras	R-Ras	Ral(ABC)	Rap1	Rap2
TcdA	+	+	+	+	*		*		*
TcdB	+	+	+	+			*	*	*
TcsH	+	+	+	+	*	*	*		*
TcsL	*	*	+	*	+	+	+	+	+
TcnA	+	*	+	+					
TpeL			+		+	*	*	+	

The modification of the Rac1 GTPase has been linked to changes in the actin cytoskeletal structure and a cytopathic rounding effect. The capacity of TcsL to inactivate Ras has also been linked to TcsL-induced cell death. Other phenotypic effects of toxin-induced GTPase inactivation include the disruption of tight junctions and the production of cytokines.

The LCTs use either uridine diphosphate (UDP)-glucose or UDP-N-acetylglucosamine (GlcNac) GlcNac as the glycosyl donor. UDP-glucose serves as the source of sugar for TcdA, TcdB, TcsH, and TcsL; Tcnα uses UDP-GlcNAc; and TpeL can use either UDP-glucose or UDP-GlcNAc as the co-substrate (Nagahama et al. 2011). The TpeL capacity to bind either UDP-glucose or UDP-GlcNAc is based on the primary sequence at amino acid positions 383–385. Where TcdA, TcdB, and TcsL have an isoleucine-asparagine-glutamine (INQ) sequence, TpeL and Tcnα have alanine-asparagine-glutamine (ANQ) or serine-asparagine-alanine (SNA), respectively. Substrate discrimination depends on steric clashes between the sugar C2 moieties and side chains of this trio. Mutations in Tcnα from 385-SNA to 385-INQ change the donor substrate from UDP-GlcNAc to UDP-glucose. Similarly, an A383I mutation in TpeL results in a preference for UDP-glucose (Ziegler et al. 2008). Measurements of intracellular UDP-glucose demonstrate concentrations approaching 100 μM (Laughlin et al. 1988). This exceeds the K_m for TcdB-GTD by >10-fold and implies the rate-limiting factor for GTD substrate engagement is the local concentration of the target GTPases (Jank and Aktories 2008).

Crystal structures are available for the TcdA, TcdB, TcsL, and Tcnα GTDs (Pruitt and Lacy 2012; Ziegler et al. 2008). All are similar in that they share a Rossman fold at their core and four helical sub-domains that are thought to contribute to target specificity (Fig. 5a). The core is similar to what has been observed for other members of the glycosyltransferase type A family of enzymes. A conserved DXD within this core (Asp285 and Asp287 in TcdA) is important for the coordination of Mn^{2+}, and there are two conserved tryptophans (Trp101 and Trp 519 in TcdA) that bind UDP and the glycosidic oxygen, respectively (Fig. 5b). Mutation of these conserved residues can lead to toxins that are highly attenuated in their glycosyltransferase activities.

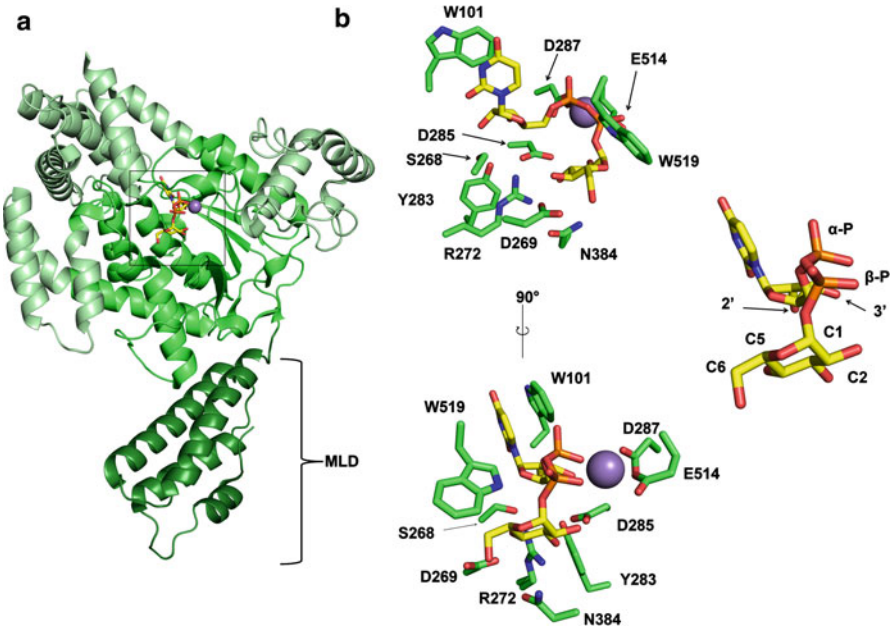

Fig. 5 The TcdA glucosyltransferase domain (GTD) bound to UDP-glucose and Mn^{2+}. (**a**) The LCT GTD has a Rossman fold at its core (*bright green*) that is involved in the coordination of UDP-glucose and catalysis. Four α-helical sub-domains emerge from this core: three in *light green* that are thought to contribute to GTPase binding and the N-terminal membrane localization domain (MLD) (*dark green*) that allows the GTD to bind the eukaryotic cell membrane. (**b**) UDP-glucose structure (*right*) and functionally important residues within the GTD active site (*left*). W102 coordinates the uridine base of UDP-glucose through pi-pi stacking. Y283 and D285 form hydrogen bonds with the 2′ and 3′ hydroxyl groups on the ribose. R272 and D285 help bind glucose by interacting with the C3 hydroxyl group. The acidic residues D287 and E514 chelate the manganese cofactor along with the diphosphate backbone and two water molecules (not pictured). Both W519 and N384 play important roles in catalyzing the proposed S_Ni rearrangement. N384 is thought to stabilize the transition state where partial bonds exist between the glucose C1 hydroxyl, β-phosphate, and GTPase threonine Cβ hydroxyl groups. The indole nitrogen of W519 also plays a role in catalysis through interactions with the oxygen linking the β-phosphate and glucose C1

The mechanism of how the sugar is transferred is not fully understood. While some glycosyltransferases use an inverting mechanism, one where the nucleophilic attack by the accepting atom results in a change of stereochemistry at the sugar's anomeric bond, the LCTs do not appear to function in this way. Work from Aktories and colleagues has shown that the stereochemistry of the anomeric bond is retained in the course of the reaction (Jank and Aktories 2008). Multiple mechanisms that could explain the retention in stereochemistry have been put forward, but none have been fully accepted. For example, a double displacement reaction could explain the retention in stereochemistry but implies the existence of a covalent sugar-GTD intermediate, for which there is no evidence. Another retaining mechanism could involve S_Ni (nucleophilic substitution with internal return). An S_Ni mechanism

permits simultaneous bond lysis and formation through a short-lived oxocarbenium transition state rather than a covalent intermediate. TcdB has been proposed to undergo an S_Ni mechanism where a conserved asparagine (N384) stabilizes a ringlike transition state and bonds are simultaneously broken and formed between UDP-glucose and a sterically constrained threonine. This hypothesis is supported by the observation that TcdB N384A has a 1200-fold reduction in glycosyltransferase activity (Jank et al. 2007). A crystal structure of an LCT GTD in complex with GTPase would be useful in resolving these mechanistic questions.

The four additional sub-domains that emerge from the Rossman fold core are unique to the LCTs (Fig. 5a). While it is assumed that one or more of these sub-domains contribute to the LCT specificity for Rho and Ras family GTPases, the molecular basis of this specificity and how GTPase specificity differs among LCT family members is not currently understood. Studies have demonstrated the effects of several mutations in TcdB-GTD that diminish its capacity to modify some GTPases (Jank and Aktories 2008). A series of charged residues are located between helix 16 and helix 17, which comprise residues ~440–480. Exchanging the sequence of helix 17 from TcsL into TcdB significantly impaired glucosylation of Rho, Rac, and Cdc42 by TcdB-GTD. However, this exchange was not sufficient to enable TcdB-GTD to glucosylate the TcsL substrates Ras or RalA. When TcsL was modified to include the entire helix 17 from TcdB, modification of Ras and Ral was not observed. However, a small amount of glucosylated RhoA was found, suggesting that some elements of TcdB-GTD helix 17 contribute to the capacity to recognize RhoA (Jank et al. 2007).

Most GTPases are modified at a C-terminal cysteine with an extended hydrophobic moiety such as myristoyl, palmitoyl, or prenyl groups. In the absence of regulatory proteins, these nonpolar groups localize GTPases to lipid membranes, where some function as regulators of the cytoskeleton and some function in receptor signaling. One of the four sub-domain structures in the GTD is a four-helix bundle that is formed by the first ~90 residues of each LCT (Pruitt and Lacy 2012). This sub-domain acts as a membrane localization domain (MLD) with specificity for lipids found on the inner leaflet of the plasma membrane domain. The MLD is thought to orient the GTD in a position to modify the membrane-associated GTPases. Mutation of conserved residues extending from the tip of the MLD four-helix bundle have been shown to impair TcsL glycosylation of substrates (Craven and Lacy 2015; Varela Chavez et al. 2016). While the TcsL GTD preferentially binds membranes enriched in phosphatidylserine, the TcdB-GTD has equivalent rates of glucosylation in liposomes containing phosphatidylserine or phosphatidylglycerol. Thus, the MLDs may also play a role in determining each LCT's specificity for GTPase substrates.

LCT Function

The functional outcomes of LCT intoxication can vary depending on what cell type and tissue is exposed to toxin. As an enteric pathogen, the LCTs from *C. difficile* (TcdA and TcdB) are thought to first encounter the epithelial cells of the colonic

epithelium. The same may be true for TpeL in the context of necrotic enteritis. In contrast, the *C. sordellii* and *C. novyi* toxins are thought to encounter endothelial cells and cause a vascular leak that contributes to edema, hypotension, and hemoconcentration (Coursodon et al. 2012). Endothelial and epithelial cells function as the basis for fluid, gas, nutrient, and waste exchange in nearly every tissue and are thus essential in maintaining homeostasis.

When LCTs intoxicate endothelial or epithelial cells, they cause changes in cytoskeletal structure that results in a cytopathic effect (CPE). The concomitant disruption of tight junctions permits fluid secretion and edema, as well as the release of immunomodulatory cytokines and chemokines that call innate immune cells to the site of infection. The toxins can also promote cell death by either apoptotic or necrotic mechanisms depending on the toxin and the toxin concentration.

Glycosylation-Dependent Cytopathic and Cytotoxic Effects

The cytopathic effect of cell rounding is a hallmark of LCT intoxication and is the result of GTPase glycosylation. The modification of the Rac1 GTPase has been linked to changes in the actin cytoskeletal structure and results in an arborized D-type CPE observed in response to TcdA and many forms of TcdB (Halabi-Cabezon et al. 2008). TcsL can modify Ras family GTPases to cause a spindle-like (S-type) cytopathic effect. These cells are characterized by intact focal adhesions despite a loss of central actin organization. Interestingly some $TcdA^{-}/B^{+}$ strains of *C. difficile* cause S-type CPEs, similar to the effects of TcsL (*C. difficile* strains 8864, J9965, WA151, 1470, and ES130) (Rupnik 2016). The GTDs of these TcdB-producing strains are capable of modifying broader arrays of GTPases, including those of the Ras family. It is currently unclear whether these discrete cytopathies play distinct roles in the context of infection. (More discussion of this topic can be found in ▶ Chap. 9, "Role of *Clostridium difficile* Toxins in Antibiotic-Associated Diarrhea and Pseudomembranous Colitis.")

A key consequence of the cytopathic effect is a cellular arrest at the G_2/M checkpoint. Intoxication by TcdA and TcsH leads to a rapid increase in RhoB expression in multiple cell types (Jank and Aktories 2008). RhoB is a proapoptotic transcript variant that functions through interactions with protein kinase B (Akt) and the p38 MAPK pathways. TcsL and TpeL are thought to cause apoptosis through inhibition of GTPase-mediated (e.g., Ras) regulation of the Akt/PI3K survival pathway (Schulz et al. 2009; Guttenberg et al. 2012). In sum, all LCTs have the capacity to stimulate apoptotic pathways of cell death as a result of their glycosyltransferase activities.

Glycosylation-Independent Necrosis

In addition to the apoptotic events that can be observed after 24–72 h of intoxication, TcdB can stimulate a rapid necrotic form of cell death when applied to cells or colonic tissue explants at concentrations of 100 pM and higher (Chumbler et al.

2016; Farrow et al. 2013). Notably, the necrotic mechanism does not depend on either the autoprocessing or glucosyltransferase activities of the toxin. Instead, TcdB can stimulate the assembly of the NADPH oxidase (NOX) complex on the surface of epithelial cell endosomes which leads to the production of reactive oxygen species (ROS) that go on to kill the cell (Frädrich et al. 2016). This mechanism of necrotic cell death may explain the presence of necrotic lesions in the colons of patients with severe forms of CDI and the link between TcdB and disease in animal models of infection (Carter et al. 2015). While it is known that TcdA does not stimulate the NOX response, there are not yet reports of whether this effect has been observed in response to other LCT family members.

LCT Mechanism as a Tool for Novel Therapeutic Discovery

While antibiotics are a first step in almost all strategies to treat LCT-associated bacterial infections, the LCTs themselves are attractive targets for the development of novel therapeutic and prevention strategies. Many studies have shown that toxoid vaccines of *C. difficile* or *C. sordellii* LCTs, or infusion with purified antibodies provide significant protection in animal infections and some human studies (Amimoto et al. 2007; Sougioultzis et al. 2005). In keeping with the theme of this review, the discussion below is focused on therapeutic concepts that are being driven by the understanding of LCT toxin structure and mechanism (Beilhartz et al. 2015).

The mechanism of action depicted in Fig. 1 suggests several mechanistic steps that could be targeted to block intoxication: receptor binding and entry, acidification of the endosome, pore formation, autoprocessing, and glycosylation of GTPases. In concept, one could block the initial interaction of the LCT with its cell surface receptor. This is the presumed mechanism of humanized monoclonal antibodies that have been shown to reduce the recurrence of CDI in clinical trials (Lowy et al. 2010). It is interesting to note, however, that while the antibodies from this study are known to bind the TcdA and TcdB CROPS, the newly discovered CSPG4 and PVRL3 receptors do not bind the TcdB CROPS. Further efforts to understand the mechanism of action for neutralizing antibodies and to specifically target LCT-receptor interactions are ongoing.

Several efforts at chemical screening have revealed compounds that link to known mechanistic steps of intoxication. For example, a cell-based phenotypic screen for small molecules that block TcdB toxicity identified inhibitors of endosome maturation including concanamycin A (an endosome acidification inhibitor) (Slater et al. 2013). The bile salts methyl cholate and taurocholic acid also protect against TcdB-mediated toxicity (Tam et al. 2015; Darkoh et al. 2013). While one could speculate that this results from an impact on membrane structure that affects receptor binding and/or internalization, there is also evidence that methyl cholate binds directly to TcdB to cause a conformational change (Tam et al. 2015; Darkoh et al. 2013).

Efforts to limit the autoprocessing activities of the toxins have centered around the conserved cysteine that serves either as nucleophile or in the coordination of

zinc. An electrophilic peptide mimetic was able to bind the cysteine and limit the cytopathic effects of the toxin (Shen et al. 2011). Similarly, the small molecule ebselen was found to be a potent inhibitor of TcdB autoprocessing through modification of the APD active site cysteine (Bender et al. 2015). Importantly, ebselen also showed efficacy in protecting against CDI disease pathology in vivo. While ebselen is known to have many activities in cellular function (including a disruption of NOX complex assembly) (Smith et al. 2012), the possibility that ebselen could be acting though inhibition of the autoprocessing activity is intriguing. In vitro studies indicate that the autoprocessing activity is not strictly required for TcdA and TcdB function (Kreimeyer et al. 2011; Chumbler et al. 2012; Li et al. 2013), but the role of autoprocessing in an in vivo context has not been evaluated.

Another target for inhibition is the glycosyltransferase activity of the LCT GTDs. Natural products such as castanospermine, swainsoline, and 1-deoxynojirimycin are broad inhibitors of glycosidases and glycosyltransferases (Jank et al. 2015). Castanospermine was used to obtain a crystal structure of TcsL and appeared to occupy the equivalent position of glucose or GlcNAc from similar LCT-GTD structures. Injection of castanospermine prior to LCT application prevented cytopathic and cytotoxic effects of TcsL and TcdB. Castanospermine was found to have K_i values of ~100 μM for Rac1 glucosylation and UDP-glucose hydrolysis by TcsL-GTD, but was less effective at inhibiting TcdB-GTD (K_i ~400 μM; 120 μM, respectively) (Jank and Aktories 2008). Interestingly, the K_i against these GTDs was significantly improved when UDP was included, which suggests occupation of both UDP and hexose sites enhances complex stability. Recently, a common plant flavonoid – phloretin – was shown to protect cells against TcdA- and TcdB-induced cell death (Tam et al. 2015). Subsequent in vitro studies found that phloretin inhibits the glucosyltransferase activity of the TcdA/B-GTDs with an IC_{50} ~2 μM (Tam et al. 2015). This effect was shown to be noncompetitive with UDP-glucose and had a much smaller effect on UDP-glucose hydrolysis ($IC_{50} > 200$ μM). These data suggest the flavonoid acts allosterically on the GTD or GTD-GTPase complex, significantly reducing the rate of glucosylation without affecting UDP-glucose binding.

Finally, the discovery that TcdB can trigger the aberrant production of ROS through the assembly of the NOX complex opens up opportunities for blocking TcdB-induced necrosis (Farrow et al. 2013). Toward this goal, one proof of concept experiment was to treat colonic explants with N-acetylcysteine (NAC), an FDA-approved antioxidant, and then challenge with high concentrations of TcdB. The observation that NAC prevented TcdB-induced tissue damage provides an encouraging sign that such strategies might be effective in animal models of infection and human patients. Efforts to identify inhibitors of mechanistic steps upstream of ROS formation are ongoing. While the affinity and specificity for these compounds would need to be significantly improved for them to be considered viable drug candidates, these data provide a proof of concept that mechanism-based inhibitors could be used to reduce or prevent LCT-mediated toxicity.

Conclusions and Future Directions

The last few years have seen significant advances in the understanding of LCT structure and mechanism. Toxins have long been appreciated for their capacity to reveal fundamental aspects of normal cellular function. The specificity of the LCTs for unique receptors and substrates provides an opportunity to dissect the processes of endocytosis and GTPase function. Mechanistic knowledge has also contributed to proof of concept that these toxins are *bonafide* targets in the development of novel therapeutic and preventative medicines. The future is likely to bring advances in this sounds odd to me proof of concept to clinical reality. This could be in the form of human and veterinary vaccines, biologic or small molecule therapeutics, and improved disease diagnostics.

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Role of *Clostridium difficile* Toxins in Antibiotic-Associated Diarrhea and Pseudomembranous Colitis

9

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Abstract

Clostridium difficile is a nosocomial agent affecting immunocompromised populations under antibiotic treatment. The clinical manifestations induced by this bacterium range from mild antibiotic-associated diarrhea to potentially fatal pseudomembranous colitis. Traditionally, all the signs and symptoms produced by *C. difficile* have been associated to the production of two toxins, toxin A and toxin B. Both toxins belong to the family of large clostridial cytotoxins (LCTs), and their mechanism of action relies on a series of complex steps. First, these toxins recognize cell-surface located receptors allowing the entrance in membrane-surrounded compartments. The toxins are then translocated through acid sensing-dependent conformational changes and the enzymatically active domain is released into the cytosol through an autoprocessing activity. This enzymatic domain modifies through glucosylation of small guanosine triphosphatase (GTPases) from the Rho and Ras families. Consequently, the signal transduction pathways mediated by these proteins are interrupted leading to the corresponding cytoskeletal alterations and different effects which might finally result in different types of cell death. In vivo, these toxins induce toxicity on epithelial cells lining the intestinal mucosa and induce a severe inflammatory reaction characterized by the recruitment of neutrophils and secretion of several cytokines. It is precisely a combination of dead intestinal epithelial cells combined with polymorphonuclear immune cells that constitutes the characteristic pseudomembrane observed in diarrheic depositions by patients suffering complications of this infection. In this chapter, the clinical manifestations induced by *C. difficile* toxins, the cellular consequences of their mechanism of action and the

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evolution of the pathogenicity locus where they are encoded are discussed in detail.

Keywords
Clostridium difficile infections • Glucosyltransferase toxins • Small GTPases • *C. difficile* pathogenicity locus

Contents

Introduction

Clostridium difficile Infections Are Toxigenic Diseases

Clostridium difficile is the infectious agent most frequently isolated in cases of antibiotic-associated diarrhea (AAD) and pseudomembranous colitis (CPM). *C. difficile* infections (CDI) or *C. difficile*-associated disease (CDAD) may occur sporadically or as healthcare-associated outbreaks. Symptoms associated with this infection usually begin after the patient has received antibiotic therapy. The incubation period of *C. difficile* is variable since symptoms may appear immediately after starting antibiotic therapy or several weeks after the treatment is completed.

Historically, the antibiotics commonly associated with AAD were clindamycin, cephalosporins, and broad-spectrum penicillins. However, in recent years, the use of fluoroquinolones has been strongly related with AAD, and the emergence of *C. difficile* epidemic strains has been associated to resistance to these antibiotics (Cohen et al. 2010).

The major virulence factors of *C. difficile* are two protein toxins, which are ultimately responsible for producing diarrhea and CPM. Toxin A (TcdA), also known as the enterotoxin, is a protein of 308 kDa and Toxin B (TcdB), also known as the cytotoxin, is a protein of 270 kDa. These two toxins have 63% identity at the amino acid level and belong to a family known as large clostridial cytotoxins (LCTs) that includes toxins of *Clostridium sordellii* and *Clostridium novyi* (Rupnik

2007). In addition, 5%–6% of *C. difficile* clinical isolates produce a binary toxin (CDT); however, the role of this toxin in the development of CDI has not been completely clarified.

Cellular Effects of *C. difficile* Glucosylating Toxins

The main effect of TcdA and TcdB on the host cells is the modification of small guanosine triphosphatase (GTPases). These large clostridial toxins target Rho and Ras GTPases, and modify them by mono-O-glucosylation. This mechanism of action is shared with toxins from *C. sordellii* and *C. novyi*. Through this mechanism of action, *C. difficile* toxins inhibit the signaling and regulatory functions mediated by small GTPases, resulting in cell morphological changes, alterations in secretion, apoptosis, and other actin cytoskeleton processes.

The loss of structural integrity of the colonocytes as a consequence of the actin depolymerization is one of the most striking and early effects of intoxication (Voth and Ballard 2005). This toxin-mediated disruption of the actin cytoskeleton and the subsequent death of colonocytes lead to the loss of the intestinal epithelial barrier function and the impairment of cellular tight junctions producing diarrhea in the host. In addition, intestinal epithelial cells also release a number of cytokines in response to toxin exposure leading to the activation of both the inflammatory response and neurons within the intestinal tissue.

Various small GTPases are involved in the regulation of numerous cellular events such as motility, organization of the actin cytoskeleton, apoptosis, cell cycle progression, and membrane trafficking (Jank and Aktories 2008). Predictably, modification of these proteins by TcdA and TcdB results in (i) the loss of the cytoskeleton structure which induces cell rounding and (ii) cell death. Cell rounding referred as the cytopathic effect (CPE) and cell death, known as the cytotoxic effect, are temporally distinct outcomes. While cell rounding is seen at earlier times of intoxication, cell death has been reported after 24 h post intoxication (Voth and Ballard 2005). In addition, TcdA and TcdB induce cell cycle arrest and cytokine secretion, which are also important characteristic of CDI.

Cytopathic Effect

Glucosylation events mediated by *C. difficile* toxins are indispensable for cytopathic-induced effects. In cultured cells, glucosylation of Rho-GTPases induces actin reorganization due to the decline of F-actin. The disruption leads to morphological changes which result in cell rounding and formation of neurite-like retraction fibers, even at very low toxin concentrations. This canonical arborizing effect, known as the classic CPE, includes the loss of actin-based structures such as stress fibers and focal complexes. RhoA inactivation results in disaggregation of adaptor proteins at focal adhesions like vinculin and in turn, the stress fibers disassemble and are released

from the complexes. Eventually, the neurite-like retraction fibers disappear and cells detach from the cellular matrix.

Differences in substrate specificity have been related to variations of the CPE induced. Indeed, RhoA glucosylation by TcdA and TcdB seems to be essential for the classical CPE as (i) transient overexpression of RhoA prevents the effect of toxins (Quesada-Gómez et al. 2016), (ii) microinjection of glucosylated RhoA induces the canonical effect, and (iii) *C. botulinum* C3 exoenzyme, which modifies RhoA by adenosine diphosphate (ADP)-ribosylation, also induces this type of CPE (Aktories 1997). On the other hand, TcdB from *C. difficile* strains 1470 and 8864 and a growing group of clinical isolates including strains that lack TcdA, strains that harbor TcdB, TcdA, and CDT or only TcdB and CDT, induce a variant CPE. Consequently, these proteins have been identified as variant TcdBs. Cells treated with variant TcdBs present rounding of the cell body and a rapid detachment from the substrate; this CPE is however not accompanied by protrusion formation and resembles the effect of *C. sordelli* lethal toxin TcsL. Initially, the variant CPE was attributed to the glucosylation of R-Ras, since this GTPase is not modified by toxins inducing classical CPE. R-Ras glucosylation leads to integrin inactivation, and focal adhesions disassemble causing detachment of the rounded cells (Chaves-Olarte et al. 2003). The variant CPE has also been related to transient RhoA (Chaves-Olarte et al. 2003) activation and none of the variant toxins, including TcsL, modify this GTPase. In this context, it seems that RhoA-modifying toxins induce an arborizing CPE and non-RhoA-modifying toxins induce a variant CPE (Quesada-Gómez et al. 2016). Other studies suggest that Rac1, rather than RhoA, is crucial for the classic CPE induced by TcdA and TcdB as all LCTs modify this protein. Glucosylation of Rac results in dephosphorylation of paxillin which regulates focal adhesions. Conversely, this might not be an effect of Rac1 modification since direct interaction of TcdA with colonocytes cause dephosphorylation of focal adhesion kinase and paxillin (Kim et al. 2009).

Both TcdA and TcdB can induce similar morphological changes in multiple cell lines, yet the sensitivities to intoxication vary probably as a result of receptor density. In line with this, TcdB has been reported to have 100–1000 times more cytotoxic potency in several cells lines; an exception for this is colonic cell lines which are 10 times more sensitive to TcdA. Furthermore, TcdA and TcdB seem to compete for cell receptors as recombinant TcdB that lacks glucosyltransferase activity delays the CPE induced by TcdA, while TcdA intoxication of colonic cells is thought to prevent TcdB from acting on these same cells. The in vitro autoprocessing of TcdA is less efficient than that of TcdB, which could also contribute to the differences in their potencies. Variations in cytopathic potencies have also been described for TcdB purified from epidemic NAP1/027 strains. TcdB from these strains possesses a higher cytotoxic potency over numerous cell types as a result of a more efficient intracellular autoprocessing (Lanis et al. 2012). Overall, these observations have contributed to the debate on the exact roles of TcdA and TcdB in CDI, as shall be discussed later on in this chapter.

Given that the assembly of tight junctions in epithelial cells is regulated by Rho and Ras GTPases, it is only consequential that intoxication events leading to cell

rounding increase tight junction permeability in polarized cell cultures. Modifications of apical and basal F-actin by TcdB have been associated to loss of occludin and ZO-1 in the tight junctions of polarized cell lines. Nonetheless, mechanisms independent of the glucosyltransferase activity could contribute to these cellular effects as well. TcdA has been reported to reduce epithelial resistance before cell rounding through pathways regulated by protein kinase C (Chen et al. 2002). Likewise, TcdB activates phospholipase A2 resulting in membrane alterations which are independent of cytoskeleton modifications. Regardless if this event is dependent on morphological changes or glucosylation of small GTPases, alterations of tight junctions by cytotoxins play a pivotal part in bacterial pathogenesis such as *C. difficile* associated diarrhea.

Cytotoxic Effects

The cytotoxic effects of TcdA and TcdB have been reported in distinct cell lines such as epithelial cells, endothelial cells, monocytes, and neurons. Since Rho GTPases have an important role in apoptosis associated with morphological changes, the appearance of the CPE is thought to precede apoptotic cell death. In addition, the temporal differences between cell rounding and cell death suggest that various mechanisms could contribute to the cytotoxicity triggered by the toxins (Voth and Ballard 2005). Recently, other studies have speculated on the relationship of GTPase glucosylation and cell death, as (i) nonsynchronized cultured cell lines treated with low toxin concentrations are highly sensitive to the cytopathic effect, but less sensitive to the cytotoxic effect, and (ii) TcdA and TcdB induce mechanisms of cell death different from apoptotic events.

TcdA and TcdB induce chromatin condensation and nucleus fragmentation in intoxicated cells by caspase dependent and independent mechanisms. Glucosylation of Rho induces apoptosis in intestinal epithelial cells by activation of caspase 3, 8, and 9 and triggers the release of Bid and cytochrome *c* (Nottrott et al. 2007), in an event that is independent of actin cytoskeleton modifications (Gerhard et al. 2008). In agreement with this, intoxicated cells respond to RhoA glucosylation by activation of RhoB, resulting in pro-apoptotic activities; in contrast, variant TcdBs, that do not glucosylate RhoA, do not induce apoptosis (Huelsenbeck et al. 2007). Furthermore, p53-dependent and p53-independent mechanisms of cell death in response to intoxication have been reported (Nottrott et al. 2007). In neuron cell cultures, Rac1 inhibition by TcdB stimulates cytochrome *c* release, Bax translocation to mitochondria, and c-Jun activation, resulting in apoptotic cell death.

On the other hand, TcdB-treated HeLa cells undergo caspase-3-dependent apoptosis as a consequence of vimentin loss and thus as a consequence of morphological changes, while caspase independent apoptosis is dependent on substrate modification. The role of the glucosyltransferase activity in apoptotic cell death has also been debated since TcdA and TcdB co-localize with mitochondria even before modification of GTPases. This accumulation leads to mitochondrial damage followed by subsequent hyperpolarization of the mitochondria by TcdB, release of cytochrome *c*,

decrease of cellular adenosine triphosphate (ATP), and generation of reactive oxygen species (ROS) induced by TcdA (He et al. 2000).

An increasing number of studies have reported nonapoptotic cell death pathways triggered by TcdA and TcdB. Caco-2 treated with TcdB undergo rapid necrotic death through autoprocessing-independent and glucosyltransferase-independent mechanisms (Chumbler et al. 2012). In fact, necrosis is mediated by Rac1 activation in response to the presence of TcdB, which results in the recruitment of the cellular nicotinamide adenine dinucleotide phosphate (NADPH) complex and production of ROS by nonphagocytic cells (Farrow et al. 2013). This type of cell death is induced by high toxin concentrations (nanomolar), as cells treated within the picomolar range present the characteristic CPE whereas cells treated with nanomolar concentrations lose their membrane integrity (Chumbler et al. 2012). In line with this observation, monocytes and macrophages treated with nanomolar concentrations of TcdA and TcdB elicit a rapid loss of viability and present nonapoptotic cell death with release of lactate dehydrogenase (D'Auria et al. 2015). Additionally, nanomolar levels of TcdB have been reported to result in pyknotic cell death associated to chromatin condensation and H3 phosphorylation (Wohlan et al. 2014). Finally, ROS production induced by TcdB can trigger autophagy in colonic cell lines (Sun et al. 2015).

Altogether, these studies suggest that *C. difficile* cytotoxicity events are diverse and seem to depend on toxin concentration. Interestingly, the work of Li et al. demonstrates cleavage of pro-caspase 3 in Vero cells treated with nanomolar concentrations of wild-type TcdB and of toxin that lacks the cysteine protease activity (Li et al. 2013), suggesting that variations in cytotoxic effects could be related to the sensitivities of intoxication in distinct cell lines.

Cell Cycle Arrest

Cell cycle arrest has been associated to glucosylation of small GTPases by LCTs. TcdA and TcdB intoxication leads to formation of bi-nucleated cells due to inhibition of contractile ring formation in cytokinesis inducing cell cycle arrest in G1-phase; this event is dependent on Rho glucosylation (Huelsenbeck et al. 2009). Studies have reported p38-dependent activation of p53 in cells treated with TcdA and delay in histone phosphorylation and Cdk1/cyclin B activation in HeLa cells intoxicated with TcdB. Activation of p38 and p53 results in cell arrest in G2-M transition and activation of p21, leading to apoptosis. Cell cycle arrest in G2-M transition has also been related to the loss of the actin cytoskeleton induced upon intoxication with TcdA, but this arrest is not sufficient to induce cytotoxicity of colonic cells (Gerhard et al. 2008).

Immune Activation

Both TcdA and TcdB are potent enterotoxins that induce pro-inflammatory activation of epithelial and innate immune cells. Since Rho proteins play an important role

in the regulation of immune pathways, the relationship between Rho GTPase glucosylation and immune response has been addressed. This statement is supported by studies that show the overall inflammatory reaction induced by LCTs could be related to a common mechanism such as the glucosyltransferase activity. Furthermore, a growing body of literature has contributed to the current understanding of host immunity against *C. difficile*, and as described below not all toxin-mediated events are associated with the enzymatic activities of these proteins.

TcdA and TcdB trigger secretion of interleukin-8 (IL-8) by epithelial colonic cells including HT-29 and T84. Toxin-induced IL-8 from intestinal epithelial cells precedes Rho glucosylation (He et al. 2002). The activation of the nuclear factor-kappa B (NF-kB), one of the transcription factors involved in the induction of IL-8 production, occurs before Rho glucosylation can be detected and appears to be a consequence of mitochondrial alteration and production of ROS. TcdB treatment in epithelial cells induces the activation of extracellular-signal-regulated kinase 1/2 (ERK1/2) through the activation of the epidermal growth factor receptor (Na et al. 2005). In addition to IL-8, other inflammatory mediators can be released from intestinal epithelial cells following exposure to *C. difficile* toxins. TcdA can induce rapid release of chemokine monocyte chemoattractant protein 1 (MCP-1) by colonic cells, and interleukin-6 (IL-6) is also released by human intestinal epithelial cells treated with TcdA or TcdB (Ng et al. 2003).

Macrophages release high amounts of IL-8, interleukin 1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α) upon exposure to TcdA and TcdB (Sun and Hirota 2015). In human monocytes, IL-8 release is dependent on activation of the mitogen-activated protein kinases (MAPKs) and on the release of NF-kB through calcium and calmodulin activation mechanisms. Interestingly, accumulation of TcdA within the cell could trigger this event as IL-8 is only induced upon toxin cellular uptake. IL-1β production is associated with the sustained activation of p38 MAPK and the transient activation of ERK1/2. IL-1β release by THP-1 cells is due to the activation of the apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD)-inflammasome complex, which mediates caspase 1 cleavage and the subsequent maturation of IL-1β (Ng et al. 2003). IL-1β secretion from bone marrow derived macrophages is dependent of the glucosyltransferase activity of TcdB, as RhoA modification by this toxin induces Pryrin inflammasome activity (Xu et al. 2014). The glucoyltransferase activity of TcdA and TcdB is thought to also be essential in the release of TNF-α by macrophages, but rearrangement of the actin cytoskeleton does not increase the production of this cytokine (Quesada-Gómez et al. 2016). Furthermore, TNF-α release is induced by both TcdA and TcdB in a dose-dependent manner; nevertheless, TcdB is more potent than TcdA in stimulating TNF-α secretion (Sun et al. 2009).

In vitro, the release of IL-8 by monocytes intoxicated with TcdA and TcdB increases neutrophil migration and upregulation of adhesion molecules. In addition, neutrophils can be directly activated by toxins and activation by TcdB has been described to occur via the formyl peptide receptor. This receptor seems to recognize the glucosyltransferase domain of TcdB when found as a fragment, and as a result intracellular Ca^{2+} and ROS production increase (Goy et al. 2015). Dendritic cells are

important antigen-presenting cells in intestinal milieu. TcdA enhances dendritic cell maturation through the coordinated participation of the p38 MAPK, IkB kinase (IKK), and the NF-kB pathways. Whereas the majority of *C. difficile*-induced effects on cultured murine bone-marrow-derived dendritic cells seem to be toxin independent, toxin dependent mechanisms have been reported to regulate IL-1β secretion through the apoptosis-associated speck-like protein containing a CARD (ASC)-inflammasome complex (Jafari et al. 2013). Inflammasome activation also contributes to interleukin 23 (IL-23) production; nevertheless, TcdA or TcdB cannot directly drive IL-23 secretion. Hence, it has been proposed that toxin-induced IL-1β enhances IL-23 by inflammasome activation.

In addition to the intestinal epithelial and immune cells, mast cells can be directly activated by TcdA and TcdB, resulting in degranulation of inflammatory mediators like histamine and leukotrienes (Meyer et al. 2007). Cytoskeleton rearrangement in human mast cell line-1 (HMC-1) cells induced by TcdB is associated with degranulation events, and the expression of genes encoding for IL-8, the transcription factor c-Jun, and the heme oxygenase-1 is upregulated upon treatment with this toxin (Gerhard et al. 2011). TcdB activates the p38 MAPK and ERK1/2 signaling pathways, contributing to prostaglandin production and enhancing degranulation via autocrine signaling (Meyer et al. 2007). In agreement with the variations of potencies of TcdA and TcdB, the latter toxin is more potent at inducing HMC-1 cell activation.

Role of Toxins in the Pathophysiology of *C. difficile*-Associated Diseases

Clinical Effects of Purified TcdA and TcdB in Disease Models

Purified TcdA and TcdB have been shown to reproduce the pathophysiological events observed in CDI in different disease models. For instance, in the rabbit and mouse ileal loop model, the inflammatory response observed in CDI can be elicited by both toxins; however, most of these studies have focused on TcdA which seems to have a more potent enterotoxic activity than TcdB (Carter et al. 2012). TcdA acts on intestinal epithelial cells causing cellular rounding, detachment, apoptosis, and the secretion of pro-inflammatory cytokines, including the strong neutrophil chemoattractant IL-8 (Genth et al. 2008). In this regard, TcdA produces an activation of the inflammatory cascade via further release of IL-1β, interleukin 12 (IL-12), interleukin 18 (IL-18), interferon gamma (IFN-γ), TNF-α, macrophage inflammatory protein-1 alpha (MIP-1α), and macrophage inflammatory protein-2 (MIP-2). IFN-γ-deficient mice challenged with TcdA had less severe enteritis compared to wild-type mice, with a decreased fluid secretion, cellular edema and damage, and diminished myeloperoxidase production (indicating low neutrophil recruitment) (Ishida et al. 2004). Among the cellular responses induced by this toxin, the

infiltration of neutrophils is one of the most relevant being at the same time one of the pathological hallmarks of CDI. It has been demonstrated that antibody-mediated blocking of neutrophil migration in rabbits significantly reduces disease severity following challenge with TcdA. In addition, neutropenic rats showed a less severe CDI (Qiu et al. 1999). Moreover, the α-defensins human neutrophil protein (HNP)-1, HNP-3, and Paneth cell–derived enteric human defensin 5 have been reported to interact with high affinity with TcdB (but not with TcdA) to inhibit its activity (Giesemann et al. 2008). On the other hand, TcdB induces necrosis via NADPH oxidase-activation and oxidative responses mediated by inducible production of hydrogen peroxide and ROS (Farrow et al. 2013). This type of necrosis has been characterized by ATP depletion, loss of membrane integrity, and calpain/cathepsin activation. Since ROS are known to cause organ damage and are also implicated in the pathogenesis of ischemia and ulcerative colitis, it is possible that these responses contribute to gut damage. Quantitation of TcdB in human diarrheic stool specimens determine median concentrations of less than 10 pM (Song et al. 2015). Since the local concentration of TcdB in the proximity of the colonization site of clostridia is approximately 30-fold higher than in stool, the toxin would reach a concentration sufficient for ROS induction (Frädrich et al. 2016). Hence, antioxidative enzymes are important for pathogenic *C. difficile*; for example, the glutamate dehydrogenase (GDH), an essential metabolic enzyme, is crucial for the normal growth of *C. difficile* toxigenic strains. Consequently, the presence of extracellular GDH may protect *C. difficile* against ROS. Therefore, ROS-mediated cell death induced by TcdB contributes to pathogenesis of CDI.

Relevance of the Binary Toxin in CDI

CDT shares similarities with *C. perfringens*-iota toxin and possesses an ADP–ribosyltransferase activity that covalently modifies cell actin. However, CDT is not cytotoxic against Vero cells and the purified toxin inoculated into mice does not induce adverse effects. Ex vivo studies on cultured cells have indicated that CDT treatment increases the adherence of *C. difficile* to epithelial cells through the formation of protrusions, suggesting a role for this toxin in colonization (Schwan et al. 2009). Regardless of its role in CDI pathogenesis, it is clear that CDT is not a major virulence factor of *C. difficile*. Despite this, some epidemiological studies have associated this toxin with severe disease. In one study, the 30-day fatality rates of CDI patients infected with binary or nonbinary toxin-producing strain of *C. difficile* were determined. Patients infected with CDT-positive strains were more likely to present a severe outcome than patients infected with CDT-negative strains (Bacci et al. 2011). Additional studies that determine the concentration of CDT in stool, the relationship of CDT expression to TcdA and TcdB, and exhaustive analyses of CDI severity and mortality caused by CDT-producing strains are required to understand the precise role of CDT on the pathogenesis of this disease.

Integrated Role of TcdA and TcdB on the Pathogenesis of CDI

CDI is characterized by a loss of mucosal barrier function, secretory diarrhea, and colonic inflammation induced by the combined action TcdA and TcdB. These toxins cause infiltration of neutrophils, mast cells, and macrophages and the release of pro-inflammatory cytokines (Genth et al. 2008). Both TcdA and TcdB exert a pro-inflammatory activity, and thus the toxemia may induce a reaction that contributes to the severity of the disease by inducing the release of inflammatory mediators from intestinal epithelial and immune cells.

Furthermore, the toxins activate enteric neurons through an unknown mechanism inducing release of substance P, a process that may have an impact on the pathogenesis of CDI. In addition, some studies have suggested that TcdA and TcdB are associated with systemic disease since both toxins were detected in the serum at cytotoxic concentrations (Steele et al. 2012). Both toxins are cytotoxic to a variety of cultured cells, explaining the damage inflicted on tissues from various organs.

Even if the exact sequence of events mediated by TcdA and TcdB and leading to the development of CDI is not fully understood, a functional model depicting the interplay between both toxins is proposed (Fig. 1). Under this scenario, TcdA would first bind to receptors located in the surface of epithelial intestinal cells. This would allow the entrance of TcdA and the subsequent inactivation of small GTPases leading to the opening of tight junctions. The latter event would be enhanced by the neutrophil migration induced by TcdA-induced secretion of the chemoattractant cytokine IL-8. The opening of tight junctions would allow TcdB to access receptors on intestinal epithelial cells located at the baso-lateral surface resulting in an enhanced cytotoxicity exerted on these target cells. The combination of dead

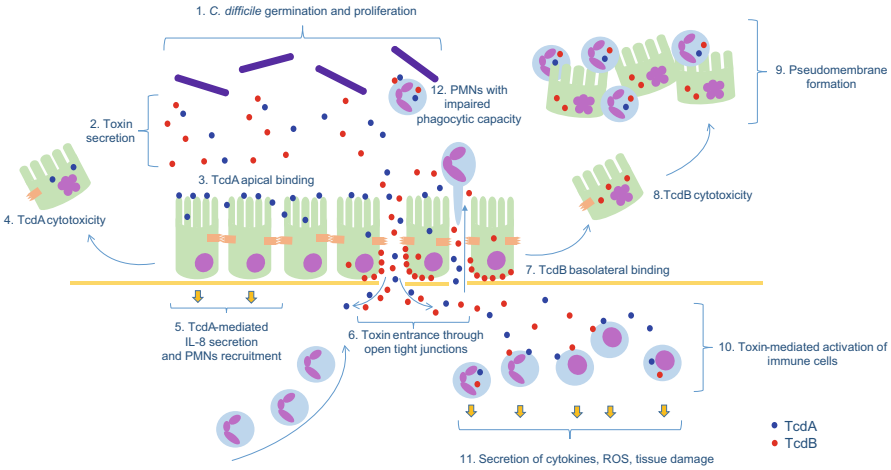

Fig. 1 Role of TcdA and TcdB in the pathogenesis of *C. difficile* infections. Model proposed for the pathophysiological events mediated by *C. difficile* toxins leading to the induction of antibiotic-associated diarrhea and pseudomembranous colitis

intestinal cells together with the infiltrate of immune cells would then give rise to the typical pseudomembrane observed in complicated cases of CDI. Contributing to the pathogenesis of the disease, both toxins would increase the inflammatory reaction by inducing activation of different sets of immune cells through glucosyltransferase-dependent or glucosyltransferase-independent mechanisms with the subsequent release of several cytokines and the associated tissue damage.

This model assumes the participation and relevance of both toxins in the pathogenesis of CDI; however, experimental observations raise doubts for this assumption. For instance, in humans, CDI is caused by strains that produce both toxins (TcdA+/TcdB+) and strains that produce only toxin B (TcdA-/TcdB+). The latter group of strains causes enterotoxic and cytotoxic effects, suggesting that TcdB is also an enterotoxin and likely sufficient to cause disease independently of TcdA (Carter et al. 2012). Interestingly, TcdA-negative strains usually encode for variant TcdBs that have a different panel of substrate than TcdBs from TcdA-positive strains suggesting that alternatively variant TcdBs might exert the role of both TcdA and TcdB (Quesada-Gómez et al. 2016).

On the other hand, recent studies have indicated that TcdB is the primary virulence factor in CDI as supported by *C. difficile* genetic manipulation studies reporting that TcdA-/TcdB+ mutants are as virulent as wild-type strains in three different animal models, whereas TcdA+/TcdB- are highly attenuated. Furthermore, transcriptomic analysis in the mouse model of *C. difficile* revealed that TcdB from the epidemic NAP1/027 strains is the major factor inducing host innate immune and pro-inflammatory responses (Carter et al. 2015).

Evolution and Origin of the *Clostridium difficile* Pathogenicity Locus

C. difficile is a highly variable bacterial species at the genomic level. This variability is evidenced by the relatively small size of its core genome reported to be as small as 30% and the relatively high phylogenetic distance between the clades defined by multilocus sequence typing (MLST) for this bacterium (Scaria et al. 2010). The pathogenicity locus (PaLoc), containing the genes encoding for TcdA and TcdB, does not escape from this variability and its phylogeny parallels to a large extent that encountered for the whole genome (Dingle et al. 2011). Due to the recent emergence of highly virulent strains and the obvious impact that variations in toxin sequences have on their pathogenicity, it is relevant to understand the evolution of the PaLoc.

Most prokaryotic genomes include clusters of genes that likely originated in other species. These regions, in the broad sense known as genomic islands (GIs), confer adaptive phenotypes related to increased metabolic capacity, symbiosis, and virulence, among other functions. GIs are of pivotal importance in pathogens, as they often encode resistance to antibiotics, phage infections, and heavy metals as well as other adaptations that increase pathogen fitness and transmissibility (Dhillon et al. 2013).

A growing body of literature has provided evidence of the existence of numerous GIs that can transfer by conjugation (Bellanger et al. 2014). Whereas some of them can catalyze their excision, transfer, and integration autonomously, others require the conjugation machinery of helper elements to transfer or the recombination machinery of the recipient to integrate. These noncanonical mobile genetic elements (MGEs) evolve by acquisition, deletion, or exchange of modules, thus they consist of a mosaic of elements that had integrated in tandem and/or within another element and had undergone subsequent reorganizations (Bellanger et al. 2014).

One example of a GI implicated in virulence is the PaLoc of *C. difficile*, as it includes the genes of toxins that play an essential role in the pathogenesis of CDI. All clinically relevant strains of this pathogen include PaLoc variants and, contrariwise, strains of *C. difficile* lacking a PaLoc have not been linked to disease categorically (Knight et al. 2015). A highly conserved 75/115 base pair (bp) noncoding region commonly replaces the PaLoc in this group of strains (Braun et al. 1996). Besides the PaLoc, almost one fourth of the *C. difficile* isolates known to date have an additional toxin locus in a separate region of the chromosome (CdtLoc) that includes both components of a binary toxin (*cdtA* and *cdtB*) along with a regulatory gene (*cdtR*). The rest of the isolates have a 68 bp sequence instead of the CdtLoc (Gerding et al. 2014).

In addition to *tcdA* and *tcdB*, which encode the LCTs, not necessary a typical PaLoc of 19.6 Kb contains genes for a ribonucleic acid (RNA) polymerase sigma factor named TcdR that positively regulates toxin expression. TcdR is a protein whose structure and function resemble a phage holin (TcdE), a potential negative regulator of toxin expression (TcdC), and a putative N-acetylmuramoyl-L-alanine amidase (CD630_06620). Some strains lack *tcdA* and others show deletions and premature stop codons in *tcdC* and polymorphisms in the receptor binding domain of *tcdA* or in *TcdB* (Knight et al. 2015). These variants have been associated with more severe clinical presentations of CDI. PaLoc variants can be detected indirectly through toxinotyping, a method that combines polymerase chain reaction (PCR) amplification of PaLoc fragments with endonuclease restriction of the resulting amplicons (Rupnik et al. 1998) or directly through next generation sequencing (Knight et al. 2015).

Although the PaLoc lacks a recombinase gene, does not contain an obvious *oriT*, and is almost without exception found at the same chromosomal position, phylogenomic analyses have revealed that the PaLoc has a complex evolutionary history (Dingle et al. 2014). Indeed, several lines of evidence have confirmed that the PaLoc is a MGE. First of all, the PaLoc exhibits an irregular distribution, with nontoxigenic and toxigenic strains sharing the same multilocus sequence type (ST) (Dingle et al. 2014). In this regard, it has been noted that the imperfect direct repeats that flank the PaLoc of toxigenic strains are present in the chromosome of a nontoxigenic strain (Knight et al. 2015). Second, some PaLoc show deletions such as those caused by failed transposition attempts (Elliott et al. 2014). Third and most categorical, Brower et al. succeeded in transferring variably sized deoxyribonucleic acid (DNA) fragments containing the PaLoc from a toxigenic *C. difficile* strain to various types of *C. difficile* nontoxigenic strains by a conjugation-like mechanism

that likely involves conjugative transposons and homologous recombination (Knight et al. 2015) as has already been seen in *Vibrio cholerae* (Knight et al. 2015) and *Bacteroides* sp. (Knight et al. 2015).

In agreement with its mobile nature, multiple, independent PaLoc acquisitions and losses events seem to have occurred among the six clades in which the *C. difficile* population structure is divided (Knight et al. 2015). As suggested by the large diversity of toxigenic genotypes included in the clade, strains from Clade 1 were likely the first to obtain a PaLoc. By contrast, the PaLoc distribution in Clade 4, which includes for the most part nontoxigenic strains, suggests recent acquisition. On the other hand, the absence of a perfect PaLoc integration site in genomes from the most divergent Clade C-I is a plausible explanation for the nontoxigenic status of most of its strains (Dingle et al. 2014). Some acquisitions are clade-specific, suggesting that these events happened after the clades diverged and expanded.

Although the PaLoc of most *C. difficile* isolates share a common genetic organization, this GI is in constant evolution and some deviations from this archetype exist. The most familiar variant is the nonsense mutation and 1.2 kb deletion at the 3′ end of the *tcdA* gene that distinguishes TcdA+TcdB+ strains from Clade 4 (RT017/toxinotype VIII) (Eichel-Streiber et al. 1999). Other atypical PaLoc organizations include: (i) a 1.1-kb insertion of putative MGE fragments in Clade 2 strain 8864 (Rupnik et al. 1998); (ii) two distinct PaLoc variants in Clade 5, one of which lacks *tcdR*, *tcdB*, *tcdE*, part of *tcdA*, and a region immediately upstream of the PaLoc and another one containing a 7.1 kb sequence showing ca. 90% identity to sequences from nontoxigenic Clade C-I strains at the PaLoc integration site (Dingle et al. 2014); (iii) a 9-kb insertion between *tcdE* and *tcdA*, including 8 kb of a transposable element related to Tn*6218*, in strains from five STs from Clade 3; and (iv) an IStron insertion within *tcdA* in various isolates (Braun et al. 2002).

To further ratify that the PaLoc is evolving, Monot et al. recently found new PaLoc integration sites and PaLoc types through comparative genomics of three atypical clinical strains (Monot et al. 2015). One new integration site is the intergenic region of the genes CD630_07750 (*spoVAE*) and CD630_07760. The other integration site was not completely elucidated, but the corresponding PaLoc was unexpectedly inserted next to a complete CdtLoc. In this work, the authors describe for the first time a strain that only synthesizes TcdA (TcdA+TcdB-) as well as toxigenic strains (TcdA−TcdB+CDT+) from Clade C-I, a group that was believed to only embrace nontoxigenic strains. These findings challenge current molecular and immunoassays diagnostic procedures for CDI, suggest that CDI figures may be understimated, and predict that new toxigenic strains may continue to emerge. Based on a *cdu1* tree, the TcdA+TcdB− isolate is a member of Clade 5, but according to a *cdd3* tree, it belongs to a different group. Its PaLoc is composed of *tcdR*, *tcdA*, and a putative coding sequence of 216 bp in between whose product is related to a prophage protein involved in endolysin and bacteriocin secretion (BhlA/UviB). The PaLoc of both toxigenic Clade C-I isolates, on the other hand, included *tcdR*, *tcdB*, *tcdE*, and, next to *tcdE*, a gene encoding an endolysin with N-acetylmuramoyl-L-alanine amidase activity (*cwlH*). In these two isolates, *tcdA* and *tcdC* were completely absent. Altogether, these findings are compatible with a

scenario in which two mono-toxin PaLoc were merged through multiple independent acquisitions to generate a stable bi-toxin PaLoc. After the fusion, the holin was conserved and the endolysin was removed to avoid lethality due to extended cell lysis. The *tcdR* gene of the contemporary bi-toxin PaLoc likely originated from the mono-toxin B PaLoc and the *tcdC* gene, which is not present in mono-toxin PaLoc strains, must have been provided by another event because *tcdR* and *tcdC* have evolved divergently. This model is supported by the partial identities of the *uviB* and *cwlH* genes to the region between *tcdE* and *tcdA* of *C. difficile* 630 (CD630). The work by Monot et al. is also fundamental since it confirms that all PaLoc known to date have toxins and the same array of accessory genes, namely a transcriptional regulator, a holin, and an amidase/endolysin.

The universal presence of holins and endolysis within the PaLoc strongly suggests a viral origin for this GI. This notion is by no means unexpected, as various virulence factors of Gram-positive bacterial pathogens, including *Clostridium novyi* (alpha toxin), *Clostridium botulinum* (neurotoxin C1), *Staphylococcus aureus* (enterotoxins), and *Streptococcus pyogenes* (toxins A and C), are included in bacteriophage genomes. *C. difficile* is not exempted from this kind of interkingdom interaction for its sporulation, and hence its transmissibility requires timely excision of the $skin_{cd}$ element; a prophage-like MGE that disrupts the sporulation-specific sigma factor *sigK* (Haraldsen and Sonenshein 2003). Two additional arguments strengthen the viral origin hypothesis of the PaLoc. First, lysogenization of some *C. difficile* temperate phages influence the expression of PaLoc toxins (Goh 2005), and second, several *C. difficile* phage carry homologs of the accessory gene regulatory (Agr) system (Hargreaves et al. 2013) that synchronize cell density, colonization, and toxin expression in this species (Martin et al. 2013).

Finally, the *C. difficile* PaLoc may have arisen through interspecies recombination. In this regard, some strains of *C. novyi*, *C. perfringens*, and *C. sordellii* have *tcdA*- and *tcdB*-related toxin genes and few *C. sordelli* strains carry PaLoc variants on plasmids whose gene content and organization is similar to those of *C. difficile*. In fact, the *C. sordelli* PaLoc also includes a transcriptional regulator, a holin-like protein and, in some cases, truncated analogues of *tcdA* (Monot et al. 2015).

Concluding Remarks

The mechanism of action of *C. difficile* toxins has been known for more than 20 years and all the steps leading to the cellular intoxication induced by TcdA and TcdB are fairly well described. Despite this knowledge, several questions on the precise role of small GTPases modification on the pathogenesis of CDI remain to be solved. For instance, there are several consequences of the intoxication by TcdA and TcdB that are claimed to be independent of the glucosyltransferase activity. Furthermore, the precise role of both toxins in the physiopathology of CDI is still under debate, even if recent papers have concluded that TcdB is the major virulence factor of *C. difficile*. Several strains of *C. difficile* with increased virulence have emerged in the last decade and interestingly these strains harbor toxins with variations at the

sequence level or PaLoc denoting constant evolution. These variant toxins seem to play a role in the increased virulence of these emerging isolates as they have been shown to be more efficiently translocated to the cytosol of the target cells. Recent evidence indicates that in addition, these variant toxins might have an extended substrate panel of small GTPases whose implications for virulence are still to be explored. Finally, but not less importantly, the plethora of target cells in vivo during actual infections are not fully understood as well as the differential effects that small GTPases modifications might have on those intoxicated cells. Under this scenario, the field of research on *C. difficile* cytotoxins is still a vibrant one and major scientific contributions addressing the questions posed above will help attain a deeper understanding of diseases inflicted by *C. difficile*, leading to better designed strategies to prevent, treat, and cure this important nosocomial disease.

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Tetanus and Botulinum Neurotoxins

10

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Abstract

Tetanus and botulinum neurotoxins are clostridial toxins that cause tetanus and botulism, respectively. Tetanus neurotoxin binds specifically to peripheral motoneuron nerve terminals at the neuromuscular junction and is endocytosed within vesicles, which transport the toxin retroaxonally to the spinal cord. Here, it enters the inhibitory interneurons that ensure the balanced contraction of opposing skeletal muscle, and the metalloprotease domain of the toxin inactivates VAMP/synaptobrevin, a protein essential for neurotransmitter release. The synapse of the inhibitory circuit at the spinal cord is blocked resulting in a characteristic spastic paralysis. The botulinum neurotoxins are produced in dozens of different isoforms that can be grouped into seven distinct serotypes. They bind to neurospecific receptors enriched in the presynaptic membrane of cholinergic nerve terminals and are then mainly internalized inside the synaptic vesicles, wherefrom their metalloprotease domain translocates in the cytosol and cleaves one of the three SNARE proteins that form the core of the nanomachine, which mediates the neuroexocytosis with ensuing flaccid paralysis. In spite of the opposing clinical symptoms, botulinum and tetanus neurotoxins intoxicate neuronal cells in the same way and have similar functional and structural organizations. Common features and molecular diversities between tetanus and botulinum neurotoxin mechanism of action will be discussed in this present chapter.

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Contents

Introduction

Tetanus neurotoxin (TeNT) and botulinum neurotoxin (BoNT) were identified as the sole cause of tetanus and botulism, respectively, over a century ago, after the discovery of the anaerobic and spore-forming bacteria of the genus *Clostridium* (Rossetto et al. 2014). For this reason, they belong to the family of clostridial neurotoxins (CNTs), which comprise one tetanus neurotoxin and several related botulinum neurotoxins. In fact, BoNTs are historically classified in seven different serotypes (BoNT/A to BoNT/G) based on their immunological properties. However, recently, thanks to the development of next-generation sequencing, many toxin variants named subtypes have been identified within each serotype (distinguished using an alpha-numeric code BoNT/A1, BoNT/A2, etc.), and many more are expected to be reported soon (Montecucco and Rasotto 2015; Smith et al. 2015). These neurotoxins are the most potent bacterial toxins yet known. The 50% lethal doses for susceptible mammals, including humans, range from 0.1 to 1 ng/kg of body weight. For this reason and due to the lack of immunization protection in the population, botulinum neurotoxins are included as Category A bioweapon select agents by the US Centers for Disease Control and Prevention. At the same time, thanks to scientific and clinical research, the BoNTs have been developed as therapeutics for the treatment of many human disorders characterized by hyperexcitability of peripheral nerve terminals and hypersecretory syndromes (Hallett et al. 2013). On the other hand, the unique tetanus neurotoxin or its derivatives are used by neuroscientists as a molecular tool to study mechanisms of exo-/endocytosis in central neurons, and its potential as a vehicle of small molecules at the central nervous system (CNS) for diagnostic and/or treatment purposes is studied in different laboratories. In addition, the identification of many different

BoNT variants and the recent advances in the production of recombinant and engineered BoNT and TeNT neurotoxins and derivatives increase their potential as therapeutics and/or as research probes.

Genetic Characteristics of TeNT and BoNTs

Tetanus toxin is produced by a uniform group of bacteria belonging to the *Clostridium tetani* species. The complete genome sequence of a toxigenic *C. tetani* strain has been determined (Bruggemann et al. 2003). It consists of a 2,799,250 bp chromosome encoding 2372 putative genes and of a 74,082 bp plasmid containing 61 genes. The TeNT-encoding gene and seven putative regulatory genes are localized on this plasmid, and its presence or absence renders a *C. tetani* strain toxinogenic or not toxinogenic. In contrast, botulinum neurotoxins are produced by different *C. botulinum* strains, which belong to four phylogenetically distinct groups, and by *C. butyricum* and *C. barati*. BoNTs are produced together with nontoxic accessory proteins (NAPs) to form progenitor toxin complexes (PTCs) of various sizes, whereas tetanus toxin does not form any complex. The BoNT and NAP genes are clustered in a DNA segment called the *bont* locus, which has different genomic localization (chromosome, plasmid, phage) in the various *C. botulinum* types and subtypes. NAPs include a nontoxic non-hemagglutinin component (NTNHA), which forms with the neurotoxins a hand-in-hand-shaped heterodimer, and several hemagglutinin components (HAs) or OrfX proteins (Popoff and Marvaud 1999). The crystallographic structures of PTCs of some toxin serotypes have been recently defined (Benefield et al. 2013; Lee et al. 2013) and suggest for NTNHA a protective role of the neurotoxin primarily from the many proteases and protein-modifying agents that are present in decaying biological materials where BoNT is produced and secondarily from the harsh environment of the gastrointestinal tract (for review, see Rossetto et al. 2014). Conversely, HA proteins of PTCs present multiple carbohydrate-binding sites which are likely to act as adhesins binding the intestinal mucus layer and the polarized intestinal epithelial cells of the intestinal wall through which BoNTs enter into the lymphatic circulation and then in the blood circulation (for detailed discussions, please see Simpson 2013; Fujinaga et al. 2013). In the PTC, the proteins are not covalently linked, but their association occurs in the bacterial cultures and in naturally contaminated food. The complex is stable at acidic pH, but dissociates at pH $\geq$ 7 (Eisele et al. 2011).

Molecular Architecture of TeNT and BoNTs

Despite the high number of variants, all BoNTs and TeNT are structurally similar and consist of a light chain (L, 50 kDa) and a heavy chain (H, 100 kDa) linked by a unique disulfide bond. The complete crystallographic structure of BoNT/A1, BoNT/B1, and BoNT/E1 reveals the presence of different domains (Lacy et al. 1998; Swaminathan and Eswaramoorthy 2000; Kumaran et al. 2009):

- The L chain is a zinc metalloprotease that specifically cleaves the three Soluble NSF Attachment Protein Receptor (SNARE) proteins necessary for neurotransmitter exocytosis.
- The N-terminal domain of the heavy chain (HN) assists the translocation of the L chain across the membrane of intraneuronal acidic vesicles into the cytosol.
- The C-terminal domain of the heavy chain (HC) is responsible for presynaptic binding and endocytosis, and consists of two subdomains (HC-N and HC-C) with different folding and membrane binding properties.

The crystal structure of the whole TeNT has not been determined yet, but those of its C-terminal domain (Umland et al. 1997) and of the L chain (Breidenbach and Brunger 2005) reveal an overall similarity to the structure of BoNTs. This multimodular structure of clostridial neurotoxins has been shaped by the evolution to fulfill the process of nerve terminal intoxication that can be divided into five individual steps: (i) binding to nerve terminals, (ii) internalization within an endocytic compartment, (iii) translocation of the L chain across the vesicle membrane, (iv) release of the L chain in the cytosol by reduction of the interchain disulfide bond, and (v) cleavage of SNAREs with ensuing blockade of the neuroexocytosis and nerve terminal paralysis (Fig. 1).

Binding of Tetanus and Botulinum Neurotoxins to the Presynaptic Membrane

Once in the extracellular body fluids, CNTs bind with an exquisite specificity to peripheral nerve terminals, mainly cholinergic, the surfaces of which are only a small proportion of the exposed total cell surface area. The C-terminal part of the HC domain (HC-C) mediates the interaction of CNTs with unmyelinated nerve terminals ensuring a rapid and strong interaction via a double receptor mechanism (Montecucco 1986) in which polysialogangliosides (PSGs) recruit TeNT and BoNT to specific areas of the plasma membrane enriched in protein receptors. TeNT binds to polysialogangliosides GT1b and GD1b, whereas BoNTs bind to different polysialogangliosides depending on the subtype. Experiments of intoxication of knockout mice lacking the b-series gangliosides showed that these gangliosides are the major essential substance for TeNT activity, whereas the a-series gangliosides account for retained toxicity of BoNT in these knockout mice (Kitamura et al. 2005). Interestingly, TeNT, BoNT/C and BoNT/D, and a mosaic toxin BoNT/DC harbor two PSG binding sites in their HC-C, whereas the other BoNTs possess only one PSG binding site defined by a conserved motif sequence (for a review, see Rummel 2013). The high-affinity binding and subsequent uptake of BoNT/A, BoNT/B, BoNT/DC, BoNT/E, BoNT/F, and BoNT/G into the presynaptic nerve terminal of the NMJ are achieved by the subsequent binding to lumenal domains of synaptic vesicle (SV) proteins (see lower panels of Fig. 1). BoNT/A and BoNT/E bind two different lumenal loops of the synaptic vesicle protein SV2, whereas its suggested role in BoNT/D and BoNT/F high-affinity binding is still to

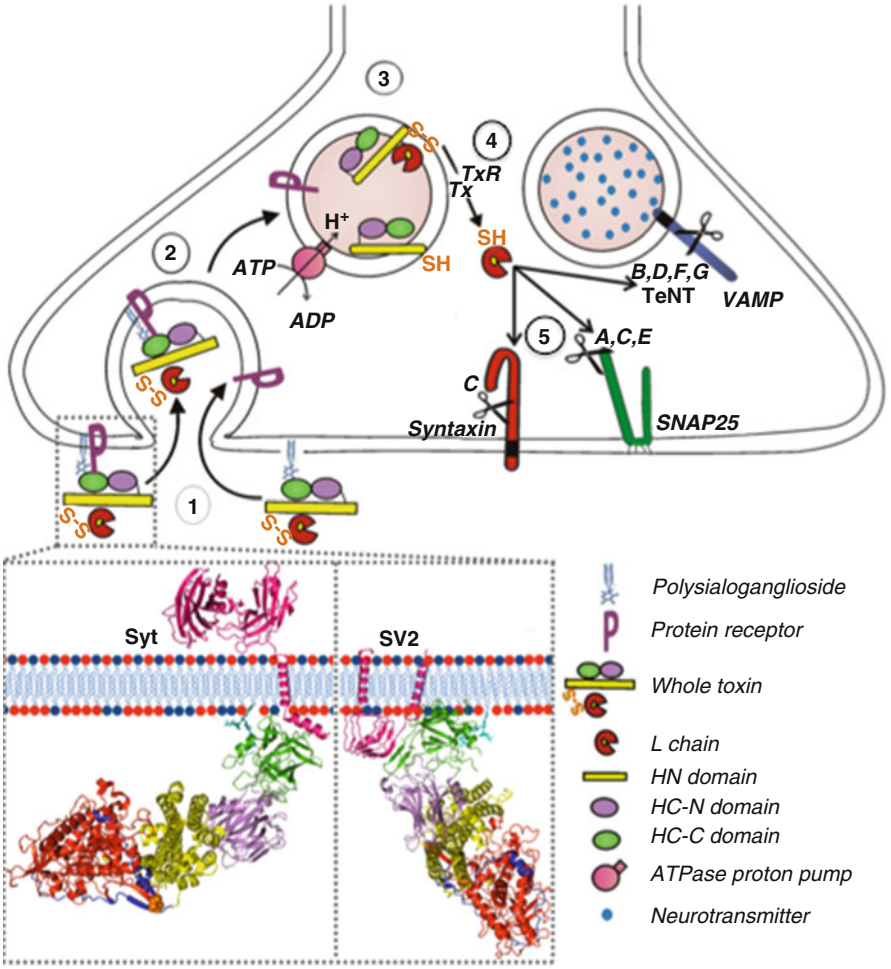

Fig. 1 The nerve terminal intoxication by clostridial neurotoxins is a multistep process. The first step in intoxication involves the binding of the carboxy-terminal end of the HC domain (the HC-C domain in *green*) to a polysialoganglioside receptor that is present on the presynaptic membrane, followed by binding to a protein receptor P (either synaptotagmin (Syt) or SV2) (step 1). The crystal structure of BoNT/B bound to Syt and PSG is shown on the lower left-hand side, and the crystal structure of BoNT/A bound to PSG and to SV2 is shown on the *lower right-hand side*. The toxin is then internalized inside synaptic vesicles (step 2) as it exploits the vesicular ATPase proton pump, which drives the reuptake of neurotransmitter. Owing to the acidification of the vesicle, the toxin becomes protonated, which results in translocation of the L chain across the synaptic vesicle membrane (step 3) into the cytosol. The thioredoxin reductase–thioredoxin system (TrxR–Trx) reduces the interchain disulfide bond (S–S, shown in *orange*) with ensuing release of the L chain in the cytosol (step 4). The L chain of TeNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G cleaves VAMP, the L chain of BoNT/A and BoNT/E cleaves SNAP-25, and the L chain of BoNT/C cleaves both SNAP-25 and syntaxin (step 5), all of which result in the inhibition of neurotransmitter release at the inhibitory interneurons of the spinal cord for TeNT and at the motor neurons of the NMJ for BoNTs

be conclusively demonstrated. BoNT/B, BoNT/DC, and BoNT/G bind a short alpha-helical segment of the lumenal domain of the synaptic vesicle protein synaptotagmin (Binz and Rummel 2009). The mode of binding of BoNT/C is less well understood. A coreceptor for BoNT/C has yet to be identified, and it has been proposed that BoNT/C uses PSG and a glycoprotein as receptors (Rossetto et al. 2014).

Binding and uptake of BoNTs are dependent on synaptic activity (Hughes and Whaler 1962). At variance, high-frequency stimulation does not increase binding of TeNT to the NMJ (Schmitt et al. 1981). It was recently shown that TeNT, in addition to polysialogangliosides, interacts with extracellular matrix proteins nidogen-1 and nidogen-2 enriched in the basal lamina within the synaptic space of the NMJ and this pathway targets it to the axonal retrograde route toward the spinal cord. TeNT is able to engage with SV2 and can enter SV recycling at the NMJ if no nidogen is available or if TeNT is applied at high doses (Bercsenyi et al. 2014).

Both ganglioside and protein receptor binding sites of CNTs are located in the HC-C subdomain. The N-terminal part of HC (HC-N) is conserved among CNTs and folds similarly to sugar-binding proteins of the lectin family, but its specific function is not known though some evidence indicate that it may improve BoNT adhesion to the neuron surface by interacting with anionic lipids (Muraro et al. 2009).

Toxin Internalization

After the high-affinity binding, BoNT and TeNT follow distinct internalization pathways, which account for the opposite clinical symptoms of botulism and tetanus, respectively. However, many pieces of recent evidence show that BoNT and TeNT share molecular hallmarks of internalization mechanisms. At the NMJ, TeNT follows a clathrin-dependent uptake pathway entering specialized transport carriers, which are spared from acidification and are directed toward the motoneuron soma located in the spinal cord (Bohnert and Schiavo 2005). TeNT then transcytoses into adjacent inhibitory interneurons where it enters via synaptic vesicles that are acidified, thus permitting the delivery of the L chain into the cytosol where it blocks inhibitory neurotransmission causing spastic paralysis (Matteoli et al. 1996; Yeh et al. 2010).

The BoNT binding to the lumenal domain of SV membrane proteins and their synaptic activity-dependent uptake strongly suggest that most of them are endocytosed at nerve terminals inside these organelles. Indeed, after intramuscular injection, BoNT/A1 is rapidly taken up and found in the average number of one to two molecules of toxin inside the lumen of the SV within the neuromuscular junction (Colasante et al. 2013). Also in cultured primary neurons, part of such internalization is clathrin mediated, and most of the toxin is detected inside the SV (Harper et al. 2011; Rossetto et al. 2014). The trafficking of BoNTs, after the initial step of entry inside the SV, may follow different pathways which may result in different entry times into the cytosol and would explain the “rapid” entry of BoNT/A and BoNT/E and the slower one of BoNT/B determined in primary cultures of neurons (Keller et al. 2004; Sun et al. 2012). Although BoNT trafficking has mainly been

restricted to distal synapses and the role of the synaptic vesicle cycle in BoNT/A1 internalization is undisputed, many lines of evidence suggest that activity-independent, "alternative" pathways also contribute to BoNT/A1 internalization. In fact, blockade of synaptic vesicle exocytosis in cultured motoneurons with BoNT/D does not completely prevent BoNT/A1 internalization (Restani et al. 2012). Further studies are required to determine whether BoNT/A1 exploits an extracellular matrix-dependent route which might direct the neurotoxin to an endosomal compartment shared with endogenous trophic factors and with TeNT (Schmieg et al. 2015). From these endosomes, BoNT/A1 would enter axonal carriers of retrograde axonal transport. Indeed, recent data have shown that TeNT and BoNT/A1 are transported, at least partially, by the same class of axonal organelles in compartmented motor neuron cultures and travel in axons along microtubules. These retrogradely moving BoNT/A1-positive carriers do not undergo acidification, which is critical for retaining these neurotoxins into the vesicle lumen and avoiding translocation of the L chain into the cytosol (Restani et al. 2012). Recently, compelling evidence of BoNT/A1 retrotransport to the central nervous system (CNS) was provided by using as readout the production of BoNT/A1-truncated SNAP-25 within CNS neurons after peripheral injection of the toxin, using antibodies very specific for the novel epitope generated by the BoNT/A1 cleavage of SNAP-25. Taken together, these evidence demonstrate that peripherally applied BoNT/A1 can directly affect central circuits since BoNT trafficking is not restricted to distal synapses, but involves long-range retrograde or anterograde transport (see Mazzocchio and Caleo 2015 for complete literature on central effects of BoNTs).

Toxin Translocation

Once BoNTs and TeNT are inside the acidic lumen of the SV in the nerve terminal of the α-motoneurons at NMJ or of the inhibitory interneurons at the spinal cord, respectively, the catalytic L chains translocate in the cytosol to exploit their metalloproteolytic activity toward SNARE proteins. As discussed above, the CNTs may traffic among various acidic compartments, but only the SV and late endosomes have a lumen sufficiently acidic to support translocation, which occurs below pH 6.0 (Pirazzini et al. 2011). The HN domain composed of a loop interacting with the L chain through a disulfide bridge and a central body containing two very long α-helices plays a crucial role in the translocation of the toxic moiety across the vesicle membrane. The molecular aspects of BoNT and TeNT translocation across the vesicle membrane into the cytosol have been only partially elucidated, but studies carried out in the past decade have provided considerable insight and have led to the proposal of a molecular model for this process (Montal, 2010; Fischer and Montal 2013; Rossetto et al. 2014; Pirazzini et al. 2016; Araye et al. 2016). The vesicular ATPase pumps protons into the SV, and, as a consequence of the ensuing low pH, conserved carboxylates located on the same BoNT surface that contains the interchain S–S bond become protonated. This surface becomes positively charged and eventually will collapse onto the negatively charged membrane surface. The low

pH above the SV lumenal membrane surface induces a concerted structural change of the L domain, of the HN domain, and of membrane lipids leading to insertion of both the L and the HN domain into the membrane. An ion translocation channel is thus formed that chaperones the passage of the partially unfolded L from the lumenal side to the cytosolic side of the vesicle membrane (Montal 2010). The transmembrane translocation of both BoNTs and TeNT at 37 °C is very rapid (i.e., it occurs in minutes) and therefore does not contribute significantly to the lag phase existing between toxin exposure and blockade of neurotransmitter release with ensuing neuroparalysis (Pirazzini et al. 2013b). Interestingly, membrane translocation of TeNT is much less efficient at 28 °C, and it is prevented at 20 °C, a finding that can contribute to explain why cold-blooded animals like reptiles and amphibians are poorly sensitive to TeNT (Pirazzini et al. 2013b). L remains attached to the HN until the interchain disulfide bond is reduced, a step that occurs at the end of membrane translocation (Montal 2010; Fischer and Montal 2013; Rossetto et al. 2014; Pirazzini et al. 2016).

Disulfide Bond Reduction

The lumen of the SV is oxidant, and low pH prevents the reduction of disulfide bond, which is required for the initiation of productive translocation. At variance, the cytosol has a reducing potential owing to the presence of several reducing molecules, and the maintenance of an appropriate redox balance is particularly important for the activity of key proteins. Cells possess several redox systems. It was recently found that thioredoxin reductase1–thioredoxin1 (TrxR1–Trx1) is responsible for the reduction of disulfide bonds in BoNTs and TeNT (Pirazzini et al. 2013a), and that both TrxR and Trx are bound to the cytosolic surface of the SV. Since the reduction of the interchain disulfide bond within nerve terminal cytosol is crucial for the toxic activity of all BoNTs and TeNT, it was used as the rationale for the development of mechanism-based pan-inhibitors of BoNTs and TeNT. Indeed, inhibitors of the TrxR–Trx redox system prevent the display of the SNARE-specific metalloprotease activity of the L chain of all serotypes of BoNTs and of TeNT in cultured neurons. More importantly, these inhibitors largely prevent the BoNT-induced paralysis in mice in vivo, regardless of the serotype involved (Pirazzini et al. 2013a; Zanetti et al. 2015).

SNARE Protein Cleavage

Once released in the cytosol of nerve terminals, the L chain of TeNT and BoNTs begins to cleave one of the three SNARE proteins: synaptosomal-associated protein of 25 kDa (SNAP-25) (BoNT/A, BoNT/C, and BoNT/E), vesicle-associated membrane protein (VAMP also known as synaptobrevin) (TeNT, BoNT/B, BoNT/D, BoNT/F, and BoNT/G), or syntaxin (BoNT/C) (Rossetto et al. 2014). These proteins are cleaved at single peptide bonds within their cytosolic domains, and all subtypes

share the same cleavage site with the exception of BoNT/F5 and a chimeric toxin BoNT/FA, which cleave a different peptide bond of VAMP with respect to BoNT/F1 (Kalb et al. 2012; Kalb et al. 2015). VAMP, SNAP-25, and syntaxin form a heterotrimeric coil-coiled complex, termed the SNARE complex, which represents the core of the neuroexocytosis nanomachine (Sutton et al. 1998). The proteolysis of one SNARE protein results in a nonfunctional complex with ensuing blockade of neurotransmission (Pantano and Montecucco 2014). The exquisite target specificity of clostridial neurotoxins is due to multiple interactions of the L chain with its substrate, including the cleavage site as well as exosites located along the sequence both before and after the hydrolyzed peptide bond (Rossetto et al. 1994; Chen 2014). Sequence analysis of SNAP-25, VAMP2, and syntaxin showed that these three SNARE proteins shared common motifs composed of hydrophobic and negatively charged residues. There are two copies of motifs in VAMP2 and syntaxin, and four copies in SNAP-25. Peptides derived from SNARE motifs could impair the ability of BoNTs and TeNT to inhibit acetylcholine (ACh) release, suggesting that TeNT and BoNTs recognize their substrates through multiple interactions which determine the exquisite substrate specificity and cleavage sites of the BoNT L chains (Rossetto et al. 1994). This also explains why long peptide substrates are needed to test the proteolytic activity of the L chain in vitro and the current lack of specific, and strong, small molecule inhibitors of their metalloprotease activity (Rossetto et al. 2014). It is noteworthy that TeNT and BoNT/B, although with different substrate recognition requirements, cleave the same target at the same peptide bond proving an incontrovertible proof that the opposite symptoms of tetanus and botulism are not due to a different intracellular target molecule, but to different neuronal targets: the inhibitory interneurons of the spinal cord for tetanus neurotoxin and the peripheral nerve terminals at the NMJ for BoNT/B.

Duration of Intoxication

Although the physiological properties induced by the cleavage of either VAMP, SNAP-25, or syntaxin are not equivalent at the neuromuscular junctions, all the clostridial neurotoxins cause a blockade of the regulated neurotransmission, which varies in duration according to each neurotoxin type. The L chain continues to cleave its SNARE target as long as it remains intact in the nerve cytosol, but this activity does not cause neuronal cell death in the intoxicated animal, though the animal may die by respiratory failure. Accordingly, if the patient survives this deficit by mechanical ventilation and appropriate pharmacological treatments, then he/she will recover completely from botulism (Johnson and Montecucco 2008). This is due to the eventual inactivation of the L chain, the removal of the cleaved SNARE, and their replacement by protein synthesis. The duration of the paralysis varies extensively depending on the (1) type of clostridial neurotoxin, (2) dose, (3) animal species, (4) mode of administration, and (5) type of nerve terminal. The order of duration of action in cultured rat cerebellum neurons is BoNT/A1 (>30 days) > BoNT/C (>25 days) > BoNT/B (~10 days) > BoNT/F (few days) > BoNT/E (~1 day)

(Foran et al. 2003; Keller et al. 1999). This order of duration correlates well with the duration of peripheral neuroparalysis in human and mice: BoNT/A ∼ BoNT/C > BoNT/B ∼ BoNT/D, BoNT/F and /G > BoNT/E (Eleopra et al. 2004; Morbiato et al. 2007). The main factor governing duration of action is the L chain lifetime within nerve terminals. This issue has not been investigated for TeNT. There is evidence that BoNT/E is ubiquitylated and rapidly degraded in cells by the proteasome system, whereas BoNT/A L chain appears to be refractory to the degradation possibly because it recruits de-ubiquitinases (Shoemaker and Oyler 2013). An N-terminal sequence and a C-terminal di-leucine motif present in the BoNT/A L chain and not in the BoNT/E L chain have been claimed to mediate the retention of the BoNT/A L chain to the plasma membrane and its persistence via recruitment of septins (Vagin et al. 2014). Although these findings support the view that duration of the paralysis induced by the BoNTs reflects the lifetime of the L chain of a BoNT inside the neuronal cytosol, other factors should be considered: in contrast to BoNT/E-truncated SNAP-25, BoNT/A and BoNT/C remove only few residues from the C-terminus, and their cleaved SNAP-25 forms nonfunctional SNARE complexes. Such unproductive SNARE complex prevents synaptic vesicle fusion and may contribute significantly to the long duration of action of BoNT/A and BoNT/C (Megighian et al. 2013; Pantano and Montecucco 2014). BoNT/A1 has been shown to cleave SNAP-25 for as long as 1 year in neurons in culture (Whitemarsh et al. 2014).

The duration of neuroparalysis induced by botulinum neurotoxins depends also on the animal species and on the type of intoxicated nerve terminals. In humans, the duration of action is about three times longer than in mice (i.e., BoNT/A 3–4 months in humans and 1 month in mice), and skeletal muscles recover about three times faster than autonomic cholinergic nerve terminals (in humans 3–4 months versus ∼1 year for BoNT/A1). Single intramuscular injection of BoNT/A1 elicits within 3–5 days a motor axon terminal sprouting. Occasionally, this also occurs at the first node of Ranvier, and the sprouts increase in length as well in complexity for about 40–50 days (Juzans et al. 1996). Nerve terminal sprouting was also observed in muscles injected with BoNT/C (Morbiato et al. 2007), with BoNT/D (Comella et al. 1993), and with BoNT/F, whereas no sprouts were detected following BoNT/E injection probably due to the short duration of neuroparalysis induced by this serotype (Meunier et al. 2003). The glial component of the NMJ, i.e., the perisynaptic Schwann cells, plays a crucial role by inducing and guiding sprouts along muscle fibers (Son and Thompson 1995). The perisynaptic Schwann cells sense synaptic activity at the NMJ by an unknown mechanism, and BoNT/A-induced blockade of acetylcholine (ACh) release was shown to cause Schwann cell proliferation and modification of gene expression profile (Georgiou et al. 1994). New functional synaptic contacts with the muscle fiber are formed, although synapses are immature but efficient enough in ACh release to cause a clustering of postsynaptic nicotinic receptors in the muscle fiber facing the growing sprouts. The late phase of synaptic remodeling is characterized by the retraction and regression of the redundant sprouts and by the functional recovery of the original parent NMJ (Comella et al. 1993). Taken together, these observations show that the duration of paralysis of the NMJ

induced by BoNT is also determined by the response of the peripheral Schwann cells and muscle fiber to the blockade of neuroexocytosis.

Uses

TeNT has not yet been used as a therapeutic, but it has a series of biological properties, such as the capability to employ neuronal retrograde transport machinery, that make it an interesting tool either as a neuroanatomical and functional tracer to investigate neuroanatomy and neuronal physiology, or administered directly by protein injection or by nonviral naked DNA methods, to carry therapeutic biological activity into the CNS (Toivonen et al. 2010). The use of the C-terminal domain of TeNT as a carrier to the CNS was first proposed by Bizzini and coworkers (1981), and several molecules, including reporter genes and potential therapeutic molecules, have been successfully transported into neurons by coupling them with TeNT-HC.

Recently, the HC-binding domain of TeNT was used to monitor the axonal transport in vivo in the sciatic nerve of mice with amyotrophic lateral sclerosis (ALS) at a presymptomatic stage, demonstrating that the appearance of retrograde transport deficit is an early and key pathogenic event in ALS (Bilsland et al. 2010).

TeNT is a strong antigen and even aged people may retain a significant anti-tetanus antibody titer. Future modifications of TeNT-derivative molecules to lower its immunogenic properties, as well as development of TeNT-based therapeutic interventions, are likely to further enhance its efficacy and applicability as a molecular neurotrophic courier.

Although there are not yet any clinical applications of drug delivery using a BoNT-based vehicle, various cargos have been proposed for delivery by suitable nontoxic BoNT-based vehicles for treatment of diseased neuromuscular junctions or to enhance motor neuron function. These include anti-neurotoxin therapies, anti-neurotropic viral treatments, neuronal enzyme replacement, ion channel modulators, neurotrophic factor receptor modulators, and protein replacement for hereditary or autoimmune presynaptic disorders (for a review, see Chaddock 2013). Consistent with the well-established action of BoNTs on ACh exocytosis, much more relevant is the use of BoNTs, in particular BoNT/A1 and BoNT/B1, as therapeutics for the treatment of human disorders characterized by hyperactivity of cholinergic nerve terminals innervating muscle fibers or glands. Since the pioneering studies of Dr. Scott (Scott et al. 1973) who treated strabismus by injecting BoNT/A into the orbicularis muscles, many dozens of thousands of papers have addressed a large number of indications in the field of neurology for treatment of dystonias and spasticity, urology, otorhinolaryngology, gastroenterology, and aesthetic medicine (Foster 2014). Recently great interest arose from the analgesic effect of BoNT injection. The myorelaxing and atrophic effect associated with an ill-known analgesic effect of BoNT brings relief from pain, and BoNT/A1 has been used in different pain syndromes including chronic migraine. It is now well documented that the analgesic effects of BoNT/A1 are related not only to its well-established paralytic effect but also to an effect on the nociceptor system (Wheeler and Smith, 2013). The

modular architecture of BoNTs and the established ability to produce by recombinant protein expression engineered neurotoxins with enhanced specificity for nociceptive nerve terminals will expand the therapeutic utility of BoNT for pain treatment (Masuyer et al. 2014). In the same context, the duration of BoNT activity assumes a paramount significance with respect to their therapeutic use, and long-lasting BoNTs require fewer injections and lower doses, limiting the possibility of immunization. Accordingly, there is a growing area of research that aims at changing binding specificity, affinity, and duration of BoNT action in order to obtain tailor-made therapeutic agents and more sophisticated tools to be used in cell biology studies (Chen 2014; Masuyer et al. 2014).

Conclusions and Future Directions

TeNT and BoNTs are two classes of clostridial neurotoxins, which, despite the opposite clinical symptoms of tetanus and botulism, have evolved as specific inhibitors of the neuroexocytosis nanomachine. Beside a very high genetic and proteomic heterogeneity, their molecular structure is very similar and has been shaped in order to exploit fundamental features and/or events of neuron physiology. The recent understanding of their modular structure and of their molecular mechanisms of neuron intoxication, together with advances in the techniques for production of recombinant proteins, has fostered researchers to modify these marvelous nanomachines in order to improve their properties in terms of cell targeting and duration of action. Moreover, recently, thanks to the development of next-generation sequencing, besides a unique tetanus toxin, many botulinum toxin variants have been identified. The biological significance of such a large and growing number of BoNTs has not been explained, but most likely it is related to the different modalities of growth, transmission, and toxin production of neurotoxigenic Clostridia causing animal botulism. It is likely that novel BoNTs with improved and/or different therapeutic targets/properties in terms of potency and duration of action will be discovered in the near future and will constitute a potential gold mine to be exploited for new clinical applications.

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Part IV

Escherichia Toxins

Escherichia coli Shiga Toxin

11

Samuel M. Chekabab and Josée Harel

Abstract

The main virulence factor of enterohemorrhagic *Escherichia coli* (EHEC) is the Shiga toxin (Stx) family with several toxin subtypes, encoded by diverse λ-like bacteriophages (Stx phages). These phages contribute to the dissemination of *stx* genes and the emergence of new EHEC strains and also to the regulation of Stx production. Thereby, Stx phages are considered the master factor for the virulence and emergence of EHEC strains.

To date, the puzzle pieces of the regulatory mechanism of Stx phages are still being accumulated. So that the whole view of Stx phage regulation may be deciphered, it is considered necessary that multiple organic and inorganic factors from the host and/or environmental reservoirs are taken into account and incorporated into the genetic dynamism and diversity of EHEC. This chapter presents the current knowledge about Stx and the regulation of Stx phage.

Keywords

Escherichia coli • STEC • Shiga toxin • Stx phage • Regulation

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Contents

Introduction

EHEC, a subset group of Stx-producing *E. coli* (STEC), are pathogenic strains, with *E. coli* O157:H7 being the prototypic strain. EHEC infection leads to the development of various disorders ranging from watery or bloody diarrhea to life-threatening diseases such as hemolytic and uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (Riley et al. 1983). Two cardinal virulence factors of EHEC are the type 3 secretion system (T3SS) and Stx. Encoded by the *locus of enterocyte effacement* (LEE), T3SS is involved in the formation of *attaching and effacing* (A/E) lesions on the colonic epithelium through the injection of specific effectors into epithelial cells. Interactions of bacterial effectors with eukaryotic signal transduction pathways lead to host cytoskeleton reorganization that is characterized by two key markers of A/E lesions: an effacement of microvilli and formation of pedestals beneath adherent EHEC (Schmidt 2010). The other critical virulence factor of EHEC, Stx, is encoded by genes located on lysogenic lambdoid phages. Stx is an A1:B5 toxin that binds specifically to a receptor at the surface of intestinal and glomerular endothelial cells. Stx blocks translation in intoxicated cells resulting in cell death by apoptosis and renal dysfunction (Griffin et al. 1988; Karmali et al. 1983).

The virulence of *E. coli* 0157:H7 can partially be attributed to its ability to establish infection at low doses in humans. The majority of EHEC infections present with hemorrhagic colitis: 91% of patients give a history of bloody diarrhea at some point during their illness. Significant morbidity and mortality secondary to infection are attributed to the development of HUS. The transmission of STEC may occur through contaminated foods, such as ground beef, ingestion of contaminated water, or direct contact with infected persons (e.g., in childcare settings) or animals or their environments. Numerous outbreaks as well as sporadic cases of EHEC infections and HUS have been documented worldwide with the largest outbreaks in industrialized countries. Modern industrialized large-scale food production has the potential to serve as a widespread vector in cases of food contamination.

Along with *E. coli* O157, other non-O157 STEC serogroups are emerging as leading causes of infection. According to the frequency of HUS, the *European Food*

Safety Authority reported that major serotypes or serogroups of concern are *E. coli* O157:H7, O26, O103, O145, O111, and O91 (EFSA 2009). Recent developments in next-generation sequencing now make it possible for routine whole genome sequencing of bacterial pathogens and to use single nucleotide polymorphisms (SNPs) to characterize isolates (Franz et al. 2014; Laing et al. 2008; Strachan et al. 2015). In 2011, Germany experienced the largest outbreak with a STEC strain ever recorded (Muniesa et al. 2012). The genome sequences of that pathogen revealed a clonal outbreak with enteroaggregative *E. coli* (EAEC) that has acquired a λ-like prophage carrying the genes for the Stx (Mellmann et al. 2011).

Bacteriophages encoding virulence factors have become essential drivers of bacterial diversity, evolution, pathogenesis, and general emergence of new pathogens. With their ability to transform a commensal bacterium to pathogen or simply adding new virulence factors, they are considered important mediators for host-pathogen interactions (Fortier and Sekulovic 2013; Zhang et al. 2007). Because the only known mechanism of Stx release is by lysing the bacterium combined with the reasons that DNA-damaging antibiotics lead to increased risk of HUS, there is a need for a more complete understanding of the regulation of *stx* genes and the implication of the carrying phage. A comprehensive overview of the regulatory processes of Stx phage and new insights of potential factors regulating *stx* genes is presented.

Shiga Toxins; Genes, Proteins, and the Mechanism of Toxicity

STEC encode two antigenically distinct Stx variants: Stx1 and Stx2. Different nomenclatures are used for these toxins. Verotoxins are those that are lethal for Vero cells in culture, but they are mostly called Shiga toxins because they are structurally related to the *Shigella dysenteriae* toxin, of which Stx1 displays 98% sequence homology, while Stx2 shares ~55% amino acid identity (O'Brien et al. 1982). In addition to *Shigella* and *E. coli,* Stx variants could be expressed by *Enterobacter*, *Citrobacter*, *Acinetobacter*, *Campylobacter*, and *Hamiltonella* bacterial species, suggesting that *stx* genes may be broadly distributed among bacteria.

Stx2 variant is the most commonly produced by STEC O157:H7 clinical isolates associated with severe human disease in Europe and Canada (Mora et al. 2007). Stx2 is more potent than Stx1. In mice, for example, Stx2 has been found to be approximately 400 times more toxic (as quantified by LD_{50} in mice) than Stx1, resulting in significantly increased renal damage, endothelial toxicity, and greater mortality (Jacewicz et al. 1999; Tesh et al. 1993).

Stxs are 70-kDa heterodimer A1:B5-type toxins that comprise a 33-kDa catalytic A-subunit bound non-covalently to a pentamer of 7.7-kDa B-subunits (Fraser et al. 2004). Expressed Stxs are either released upon bacterial cell lysis or translocated to STEC periplasm, and then secreted into the extracellular milieu where the five B-subunits form a structure that binds the globotriaosylceramide (Gb3) receptor on the surface of eukaryotic cells (Tarr et al. 2005). STEC damage to intestinal epithelium permits Stx access into systemic circulation before reaching the kidneys (Mukherjee et al. 2002). The well-described Stx mechanism of action

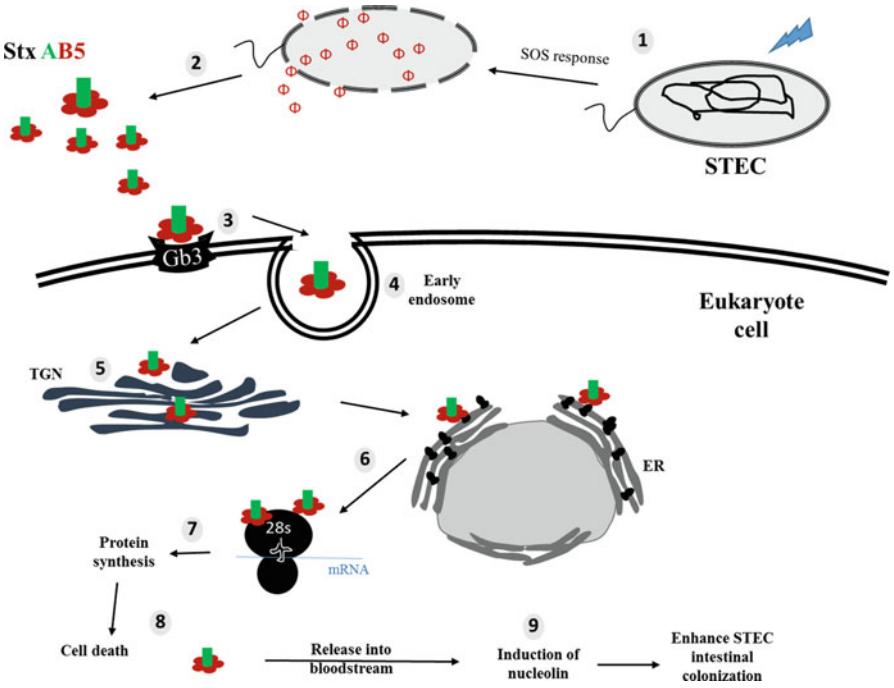

Fig. 1 The mechanism of action of Stx. The SOS response of STEC switches the Stx phage to lytic phase causing bacterium lysis (*1*). Subsequent phage release and high production of Stx (*2*) allow specific binding of Stx B-subunit pentamer to the globotriaosylceramide (Gb3) receptor on the surface of intestinal Paneth cells and the kidney epithelial cells (*3*). Stx holotoxin is then internalized but resists to the endocytic degradation pathway (*4*) and undergoes a retrograde transport from the trans-Golgi network (TGN) (*5*) to the lumen of the endoplasmic reticulum (ER) (*6*), where the RNA N-glycosidase activity of Stx A-subunit depurinates the 28S rRNA of the 60S ribosomal subunit (*7*). This arrests the protein synthesis and causes cell death (*8*). Subsequently, Stx released into the bloodstream and could reach other targets. Stx has several downstream effects including the induction of host cell protein nucleolin (*9*), which indirectly promotes STEC intestinal colonization

(Fig. 1) is protein synthesis inhibition and apoptosis of the intestinal Paneth cells and kidney epithelial cells that express Gb3 receptor (Mallard et al. 1998; Obrig 2010). Specific binding of B-subunit pentamer to Gb3 of targeted cells allows internalization of the A-subunit, bypassing the endocytic pathway and undergoing retrograde transport from the Golgi stacks to the lumen of the endoplasmic reticulum. N-glycosidase activity of the A-subunit depurinates the 28S eukaryotic rRNA, which stops protein synthesis. Subsequent to cell death, Stx toxin release into the bloodstream allows it to reach many targets and cause more damage to the host. For example, Stx was shown to induce the host cell protein nucleolin (Robinson et al. 2006), which has been described as a host receptor for EHEC adhesin, the intimin (Sinclair and O'Brien 2002). Thereby, free Stx promotes indirectly STEC intestinal colonization (Robinson et al. 2006).

Each Stx variant is encoded by an ~1,230-pb operon that contains an ORF for the subunit genes A and B, separated by intergenic 12–15 nucleotides. The complete operon is transcribed from a proximal promoter upstream to the A-subunit gene. However, each gene is preceded by a putative RBS sequence (Calderwood et al. 1987). The stoichiometry of the resulting holotoxin suggests differential transcriptional and/or posttranscriptional regulations of the A- and B-subunits, allowing multiplicity of the B polypeptides. Indeed, a model was proposed for overproduction of B polypeptide governed by an independent and more efficient RBS translational regulation coupled with *stxB* transcription from a putative independent promoter (Habib and Jackson 1993). This would contribute to the 1A:5B stoichiometry of the Stx holotoxin.

Shiga Toxin Phages

In *E. coli*, Stx operons are exclusively associated with active or cryptic prophages (Stx phage). These bacteriophages belong to the group of lambdoid phages integrated into the bacterial chromosome, a linear double-stranded DNA with lengths ranging from ~40 to ~70 kb (Schmidt 2001).

In the STEC O157:H7 EDL933 strain, Stx1 and Stx2 are encoded by two separate toxin-converting bacteriophages in the chromosome, 933 J and 933 W, respectively (O'Brien et al. 1984). Since their original isolation and analysis from STEC, many other Stx-encoding phages have been isolated and their sequences at least partially characterized. It has been shown that certain EHEC strains are more likely to be associated with human disease and "supershedding" animals. The study by Xu and colleagues (Xu et al. 2012) indicated that these EHEC strains are more likely to contain two related Stx phages, rather than one, and that the intercalating bacteriophages take control of the bacterial T3SS that is essential for ruminant colonization (Chase-Topping et al. 2007). More recently it was revealed by whole genome sequencing that Shiga toxin-encoding phages relate to the evolutionary core genome of this pathogen (Strachan et al. 2015). Interestingly, similar to many classical EHEC strains, the STEC O104:H4 outbreak isolate produces the Shiga toxin 2a (Stx2a) carried by a λ-like phage similar to the Stx2 phage 933 W (Beutin et al. 2012).

By definition, all phages carrying a *stx* gene are considered Stx phages. To date, 32 different Stx phages have been sequenced and recorded in GenBank. Among them, 13 Stx2 phages and 5 Stx1 phages are identified from different clades of the O157:H7 serotype (Kruger and Lucchesi 2015). The overall construction of both Stx1 and Stx2 phages is similar and their genes display some similarity, whereas other Stx1 phages contain different sets of homologous genes. Most of the analyzed Stx phages have gene organization and clusters with related functions similar to those of λ-phages including recombination, early regulation, replication, late regulation, lysis, and head and tail structural gene regions. However, the key genes involved in infection and propagation of Stx phages include *int*, *N*, *cI*, *cro*, *cII*, *Q*, *O*, *P*, *stx*, capsid, and tail structural genes and packaging are significantly diverse among 11 analyzed Stx phages (Smith et al. 2012). This supports the mosaicism hypothesis

of constant recombination events between Stx phages and their STEC host that drives their evolution.

Although integration sites for the identified Stx phages are variable, Stx1 and Stx2 phages are preferentially, but not exclusively, integrated, respectively, at sites *wrbA* and *yehV* (Besser et al. 2007; Bonanno et al. 2015). The Stx2a phage subtypes harbored by O104:H4 STEC strains were found to be integrated into the *wrbA* locus (Beutin et al. 2012). Other chromosomal sites were identified. The diverse locations may impact the induction of Stx phages and STEC virulence. However, the reasons for such integration in a specific chromosomal site of a particular host strain or group of strains are still poorly understood with likely dependence on the host strain (Serra-Moreno et al. 2007). There is also a need for a more complete understanding whether, when the preferred site is unavailable, the phage integrates or not into a secondary insertion site.

Being located within the late phage genes, the expression of *stxAB* operon is primarily under control of the phage cycle. Indeed, most of the studies agree that production of large quantities of Stx occurs during prophage induction. Like a classic λ-phage, the Stx phage remains quiescent because the *cI* repressor binds to the operator sites *OR* and *OL*, and inhibits the activity of the early promoters *PR* and *PL*, until a lytic phase when Stx phage takes advantage of the bacterial SOS response (Waldor and Friedman 2005). The SOS response is driven by the activity of the bacterial RecA protein, which leads to self-cleavage of the *cI* repressor. This allows transcription from phage promoters, including the *pR′* promoter that controls transcription of the *stxAB* operon. SOS response occurs generally in bacterial cells accumulating altered DNA and allows bacteria to stop DNA replication (Sutton et al. 2000). RecA is basally and constantly present in bacterial cells. RecA binds to damaged DNA and becomes an active protease that induces LexA auto-cleavage. LexA is a constitutive repressor of SOS regulon genes including *sulA* and *uvr* genes. SluA slowering of cell division allows for DNA reparation by the excinucleases UvrABC (Janion 2001). Very few *E. coli* ORFs (1%) are considered SOS regulon genes with a conserved promoter containing a LexA-binding sequence termed the SOS box (Fernandez De Henestrosa et al. 2000). In such a response, the SOS is a global cellular process under control of several physical and biological input factors that are considered indirect regulators of RecA-dependent Stx phages. Other factors (discussed in the next section) may have direct and RecA-independent effects on Stx phages and/or *stx* expression (Table 1).

Recently, Ogura and colleagues (Ogura et al. 2015) showed that Stx2 production levels in *E. coli* O157:H7 correlated with Stx2a phage subtypes that are classified according to the phage replication proteins. Stx2a phage subtypes of EHEC O157 may be critical factors for determining Stx2 production levels. Differences in the efficiency of phage induction among the subtypes appear to be specified by the types of replication proteins. This may explain the differences in Stx2 production levels among host strains.

Understanding the regulation of Stx phage and Stx production can potentially lead to the development of effective drugs inhibiting propagation of phages, and the production and release of toxins.

Table 1 Factors influencing Stx1 and Stx2 levels

Factors	Regulators	[a]Effects on Stx levels	References
SOS response	RecA	↗	(Waldor and Friedman 2005)
Antibiotics	RecA	↗	(Kimmitt et al. 1999)
Reactive oxygen species			
NO	NsrR	↘	(Vareille et al. 2007)
	NorR, NorV	↗	(Shimizu et al. 2012)
H2O2	RecA	↗	(Los et al. 2010)
Nutrients and essential elements			
Iron depletion	Fur	↗	(Calderwood and Mekalanos 1987)
Phosphate depletion	PhoB	↗	(Chekabab et al. 2014a, b)
Growth conditions			
Temperature	H-NS	↘	(Muhldorfer et al. 1996)
Stationary phase	QseB, QseF	↗	(Hughes et al. 2009)

[a]Effect on Stx1 and/or Stx2

Factors and Agents Regulating Stx Phages and *stx* Gene Expression

Antibiotics

Despite efficient diagnosis, researchers struggle to find an effective treatment for patients infected with STEC. The use of antibiotics is controversial and largely non-recommended. They may worsen symptoms and increase the risk of HUS. This concerns the antibiotics such as quinolones and mitomycin C that impact DNA integrity and replication, causing prophage induction and massive production of Stx that is then released with bacterial lysis (Los et al. 2010). Beta-lactam antibiotics that disturb the bacterial cell wall are also associated with the induction of Stx phage leading to the same toxic result (Wong et al. 2000). However, other antibiotics including azithromycin, doxycycline, phosphomycin, gentamicin, ceftriaxone, and rifampin are associated with either decreased or unchanged Stx production (McGannon et al. 2010). These antibiotics impact cell-wall or RNA translation without inducing Stx phage. The RecA-independent effects of some antibiotics on Stx production remain obscure. For instance, antibiotic treatment against STEC infection is still cautious. In vivo investigations will probably determine whether Stx-reducing antibiotics are potential therapy for STEC-infected patients.

During the O104:H4 epidemic, the *German Society of Infection* recommended the use of carbapenems for suspected invasive disease due to O104:H4. Although

resistant to penicillins and cephalosporins, the epidemic O104:H4 strains were still susceptible to carbapenems (Bielaszewska et al. 2011; Muniesa et al. 2012). Rifampin was suggested for safe eradication of EHEC from the intestinal tract.

Reactive Oxygen Species (ROS)

While STEC is colonizing the intestine, enterocytes detect pathogen-associated molecular patterns (PAMPs) and recruit neutrophils and macrophages to counteract infections. At the site of infection, these cells produce cytokines that induce the production of ROS such as nitric oxide (NO) and H_2O_2 which are cytotoxic to STEC. Simultaneously, STEC attempts to avoid the host immune system first by injecting T3SS effectors that inhibit NO synthesis by enterocytes and modulate the host nuclear factor-kappa B (NF-kB) inflammatory signaling (Gareau et al. 2011; Veraille 2008). Macrophages, for example, produce superoxide which reacts with NO and generates highly toxic peroxynitrite that is a potent bactericide (De Groote et al. 1995). However, it has been shown that STEC can survive and multiply within macrophages, and Stx enhances the resistance to the uptake and killing by macrophages (Poirier et al. 2008). To resist such nitrosative stress, STEC has evolved different biochemical NO reductase pathways through the detoxifying regulators NsrR and NorR. NsrR is a nitrite-sensitive repressor that blocks the SOS response of Stx2 phage (Vareille et al. 2007). Thereby, NsrR represses Stx production at the transcriptional level during stationary growth phase. This effect is because Stx phage is maintained in a lysogenic state; the inhibitory effect of NO was evident when STEC was grown in the presence of mitomycin C. However, the regulator NorR induces an O_2-sensitive NO reductase NorV that increases Stx2 expression while protecting STEC against NO stress under anaerobic conditions (Shimizu et al. 2012). This suggested that NorV represses the decrease of Stx2 production in response to NO through NsrR regulation. However, no evidence of either direct or indirect SOS-independent effects of NO stress on Stx exists yet.

The potent bactericidal agent H_2O_2 is known to damage DNA and to induce an *E. coli* SOS response (Goerlich et al. 1989). It is mainly produced by neutrophils, the first immune cell responder to bacterial infections (Springer 1994). However, STEC are also Ag43-expressing *E. coli*. Ag43 is a self-associating protein contributing to bacterial uptake and survival within human neutrophils (Fexby et al. 2007; Restieri et al. 2007). Moreover, STEC exposure to H_2O_2 or human neutrophils resulted in increased Stx production and phage titers (Wagner et al. 2001). A study by Los and colleagues (Los et al. 2010) corroborated findings that H_2O_2 increases Stx production.

ROS oxidative stress (NO and H_2O_2) generated during in vivo infections by either host immune cells or commensal bacteria might modulate Stx production and contribute to STEC pathogenesis.

Environmental, Gut, and Bacterial Growth Conditions

There is limited knowledge about specific conditions for induction of Stx phages and *stx* expression in the STEC-colonized human intestine. Signals from the host may arise within the intestine, for example, changes in metabolite concentration may allow bacteria to monitor infection and to alter their cytotoxicity. Many studies have investigated the impact of different lab growth parameters. Growth conditions such as pH range 6.5–8.5, osmolarity, oxygen tension, acetates, or carbon sources do not affect *stx* expression (Muhldorfer et al. 1996). However, other in vitro parameters may impact Stx phage induction or Stx production such as nutrient stress, vitamin availability, iron levels, and growth phase and temperature.

As a food- and waterborne pathogen, STEC copes with different nutritive stresses in their transmission routes to human, including phosphate (Pi) limitation. The primary adaptive response to Pi limitation or starvation, induction of the Pho regulon, is also implicated in different *E. coli* virulence phenotypes including STEC [reviewed elsewhere (Chekabab et al. 2014a; Crepin et al. 2011)]. During Pi limitation, the histidine kinase PhoR phosphorylates the response regulator (RR) PhoB. This in turn activates Pho regulon expression by binding to specific Pho box sequences located within the promoters of genes belonging to Pho regulon genes. A recent study (Chekabab et al. 2014b) demonstrated that many genes of the lysogenic phage 933 W were upregulated in STEC response to Pi-limiting conditions including *stx2* and *cro* while the CI repressor gene was repressed. PhoB indirectly inhibits transcription of the repressor gene *cI*, which in return releases the constitutive transcription of *cro* that should induce a lytic phage cycle. Prophage induction ultimately results in initiating Q-modified transcription at PR′ that transcends the tR′ terminator, leading to expression of downstream genes that include *stx2* and those encoding lysis functions. Another scenario could be direct PhoB binding onto sequences upstream of the Stx2 promoter that activates *stx2* transcription.

In Pi-limited conditions, the Stx2 phage induction appears to be RecA and LexA independent. RecA-independent induction also occurs in phages infecting *E. coli*. In phage λ, induction does not always imply repressor cleavage by RecA. In some cases, the mechanism involves RcsA, a regulator of colanic acid synthesis, and DsrA, a small regulatory RNA that, among other functions, prevents the degradation of RcsA (Rozanov et al. 1998). For example, the magnesium chelator EDTA affects the membrane structure of bacteria and triggers Stx phage by RecA-independent mechanisms (Imamovic and Muniesa 2012).

It has been shown that the Pho regulon of STEC is activated in the intestinal environment of gnotobiotic piglets (Pieper et al. 2013). The signal that leads to induction of the Pho regulon is likely to be Pi limitation. However, we cannot be certain, as PhoB can be regulated by numerous signals in addition to Pi concentration. Moreover, it was shown that both PhoB protein and Stx are critical for STEC fate within amoebae (Chekabab et al. 2012, 2013). This could be linked to an adaptive response of STEC to low Pi after exiting the mammalian host. STEC has

been found in poor nutrient aquatic environments where it might coexist with protozoan predators that would promote its survival outside the mammalian gut.

The predominant bacterial species in human gut belong to the phylum *Bacteroidetes*. It has been shown that *Bacteroides thetaiotaomicron* inhibits *stx2* gene expression (de Sablet et al. 2009). A mutant of *B. thetaiotaomicron* with impaired production of a specific transporter of vitamin B12 was recently found to lose the ability to inhibit the production of Stx2, and that vitamin B12 supplementation restores Stx2 synthesis (Cordonnier et al. 2016). This suggests that levels of digestive molecules such as vitamin B12, and by extension, activities of commensal bacterial species producing and/or consuming vitamin B12, may modulate Stx production by STEC.

Growing temperature has a moderate effect on Stx production, and this leads to the global DNA-binding regulator H-NS (Muhldorfer et al. 1996). In the absence of H-NS, Stx2 synthesis becomes temperature dependent, with higher levels at 30 °C and lower levels at 42 °C compared to the WT.

Iron levels control Stx1 production through the Fur regulator, while no effect has been observed on Stx2 (Calderwood and Mekalanos 1987). The promoter of *stx1* has a conserved Fur DNA-binding box, and its expression is inversely correlated to iron levels (Calderwood and Mekalanos 1987; O'Brien et al. 1982).

STEC entrance in stationary phase coincides with the highest bacterial toxicity and might influence the expression of *stx* (Bergholz et al. 2007; Konowalchuk et al. 1978). During the stationary phase, bacteria undergo adaptive responses controlled by cell-cell communication known as quorum sensing (QS) behavior. As an *E. coli*, the STEC two-component system QseBC plays a major QS role. QseC is a sensor histidine kinase that senses both bacterial-derived molecules (autoinducers) and mammalian host stress response hormones, i.e., adrenaline (Hughes et al. 2009). QseC then phosphorylates the response regulator QseB or a non-cognate RR QseF; both are transcriptional regulators that increase *stx2* expression in RecA-dependent manner (Hughes et al. 2009). The importance of global regulators in the modulation of *stx2* gene expression is exemplified by the findings that the RNA chaperone Hfq decreased *stx2* expression, and consequently could play an important role in the development of HUS upon EHEC infection (Kendall et al. 2011).

Conclusion and Future Directions

The debate about the use of antimicrobial agents for the treatment of an STEC infection has led to the rise of various alternative treatment approaches (Rahal et al. 2015). These have ranged from the use of natural products to the development of novel regimens that reexamine the use of antimicrobials. Various agents that mimic Stx receptors and bind them have been developed, thus reducing their availability to cellular process. SYNSORB Pk, a synthetic Stx receptor analog consisting of a Pk trisaccharide bound to Chromosorb® P, a multipurpose sorbent medium, was shown to have an abrogative effect on Stx in vitro. However, this agent

was ineffective in clinical trials (Trachtman et al. 2003). Cell-permeable agents that can bind Stx2 and potentially interfere with its intracellular trafficking have also been reported (Rahal et al. 2015). Preparations of antibodies that can bind Stx and neutralize their effects have also been reported (Delmas et al. 2014; Lapeyraque et al. 2011). As such, eculizumab, a humanized monoclonal antibody against complement component 5 (C5), was shown in small clinical studies to have beneficial effects on recovery from STEC-associated HUS including cases during the 2011 *E. coli* O104:H4 outbreak. Nevertheless, the main challenge with Stx-binding and Stx-blocking agents has been the timing of administration that needs to occur before the Stx effect is further amplified by a cascade of events involving the thrombotic, inflammatory, and complement systems. Some reports have indicated that inclusion of eculizumab in the treatment of STEC O104:H4-induced HUS results in no additional benefits (Kielstein et al. 2012; Menne et al. 2012).

Novel anti-virulence strategies include QS inhibitors, Stx receptor analogs, and pyocins, the bacteriocins produced by *Pseudomonas* spp. By their using different mechanisms of action from classic antibiotics, these therapies are thought to cause less selective pressure for drug resistance than classic antibiotics. Although several alternative anti-Stx strategies have been described in the literature, only one drug reached a phase 1 clinical trial. To bring newly discovered drugs emanating from basic science studies to the clinic, translation research is necessary.

Cross-References

- ▶ Role of *Clostridium difficile* Toxins in Antibiotic-Associated Diarrhea and Pseudomembranous Colitis
- ▶ Translocation of Toxins by Gram-Negative Pathogens Using the Type III Secretion System

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Escherichia coli STb Enterotoxin: A Multifaceted Molecule

12

J. Daniel Dubreuil

Abstract

Enterotoxigenic *Escherichia coli* (ETEC) produce heat-labile and heat-stable toxins. These toxins are active on the epithelial cells of the small intestine of man and animals, affecting the fluid homeostasis. One of the heat-stable toxins enterotoxin b (STb) is responsible for secretory diarrhea in animals and more specifically in pigs. Recent studies have provided information on the nature of its receptor and the structure-function of the toxin. The toxin was shown to form oligomers and this process seems indispensable for toxicity expression. As observed with in vitro models and cells in culture, diverse activities were determined for STb and some or all of these may account for the secretion resulting from the activity of this toxin. The next step will be to determine quantitatively how important each of these activities is on fluid secretion. The relative importance of STb compared to other ETEC toxins has been clarified in recent years. As STb is now recognized as a major player in post-weaning diarrhea in pigs, the focus has been on ways to prevent its toxicity. For example, vaccines composed of multivalent toxin molecules were designed, and preliminary results indicate that it could represent a reliable way to protect from ETEC intoxication. As described for many other bacterial toxins, in the near future, STb could be utilized as a tool to decipher cellular physiological process.

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Contents

Introduction

STb is one of the enterotoxins synthesized by *E. coli*. The discovery of this molecule dates back from 1978 (Burgess et al. 1978) when it was established that a heat-stable toxin, different from STa, was present in culture supernatant causing fluid accumulation in pig's small intestine. Comparison of the various toxins synthesized by enterotoxigenic *E. coli* (ETEC) led to the conclusion that LT and STa were provoking secretion using different signaling pathways and a third toxin with a distinctive mechanism of action was also produced by ETEC. Experimental challenges with engineered bacterial strains clearly showed the importance of STb in swine diarrhea (Zhang et al. 2006). The advent of herd vaccination against ETEC confirmed the role of STb as a diarrheagenic toxin (Melkebeek et al. 2013). Recently, the dogma pertaining to electrolytes and water secretion observed after cellular intoxication was revisited. Clearly, some enterotoxins could use diverse mechanisms and modify cellular structures in order to provoke secretory diarrhea. Various strategies are now explored to protect against the deleterious effects of enterotoxins. In view of the determined importance of STb in pig diarrhea, vaccination against this toxin has been considered. This chapter summarizes the knowledge gained on STb toxin in recent years and also leads to future avenues.

Enterotoxigenic *Escherichia coli*

E. coli is an inhabitant of the gut of various animals. The majority of *E. coli* strains live as commensals, but some possess genes coding for fimbriae, toxins, and other virulence factors rendering them a hostile party for the animal host.

E. coli producing enterotoxins acting on the small intestine are called ETEC. These bacteria can elaborate one or more toxins including LT (heat-labile toxin), STa (heat-stable toxin a), STb (heat-stable toxin b), and/or EAST1 (enteroaggregative heat-stable toxin 1). Over the years, LT and STa have been studied in detail, whereas STb and EAST1 would benefit from more attention. Up to now, EAST1 is regarded as analogous to STa as they share the same second messenger (Menard and Dubreuil 2002). The mechanism of action for STb differs from LT and STa/EAST1 as STb induces diarrhea without activating adenylate or guanylate cyclases (Hitotsubashi et al. 1992b).

Importance of STb in Man and Animals

Specific ETEC strains are responsible for severe diarrheal disease in humans and in the young of a variety of animal species (Dubreuil 2008). ETEC pathogenesis comprises two steps: (1) adherence to the small intestinal epithelial cells by means of colonization factors (CF); and (2) production of one or more enterotoxins that disrupt fluid homeostasis and causes secretory diarrhea (Fig. 1).

STb toxin is mostly associated with porcine ETEC, but it has also been sporadically reported in human isolates (Lortie et al. 1991; Handl and Flock 1992; Okamoto et al. 1993). Isolates implicated in diarrhea in pigs produce various combinations of LT, STa, STb, and EAST1 toxins (Dubreuil 2012). *E. coli* producing solely STb toxin although described in numerous papers were, for the majority, later shown to produce other toxins as well (Fairbrother, personal communication). This dilemma unfolded as PCR techniques could be used to rapidly establish the toxinogenic profile of bacterial strains. In nature, it thus appears that STb-only strains are rarer than previously believed.

Chapman et al. (2006) comparing the virulence genes of 75 *E. coli* strains isolated from healthy and diarrheic pigs clearly confirmed STb as an important virulence gene (Chapman et al. 2006). STb gene was associated with 25% of neonatal diarrhea and with 81.3% post-weaning diarrhea cases. No commensal strains tested were associated with STb. Berberov et al. (2004) had shown that EAST1, LT and STb toxins could be concurrently expressed by porcine ETEC strains (Berberov et al. 2004). The effect of STb was additive with LT and EAST1. STb contributed to the secretion observed, as 60% of piglets inoculated with LT-negative mutants developed diarrhea. STb and EAST1 expressed by ETEC strains had considerable effects on water and electrolyte losses similar to those cause by LT. Zhang et al. (2006) also reported that LT- and STb-positive strains caused diarrhea in piglets (Zhang et al. 2006). Experimental infection with an ETEC engineered strain

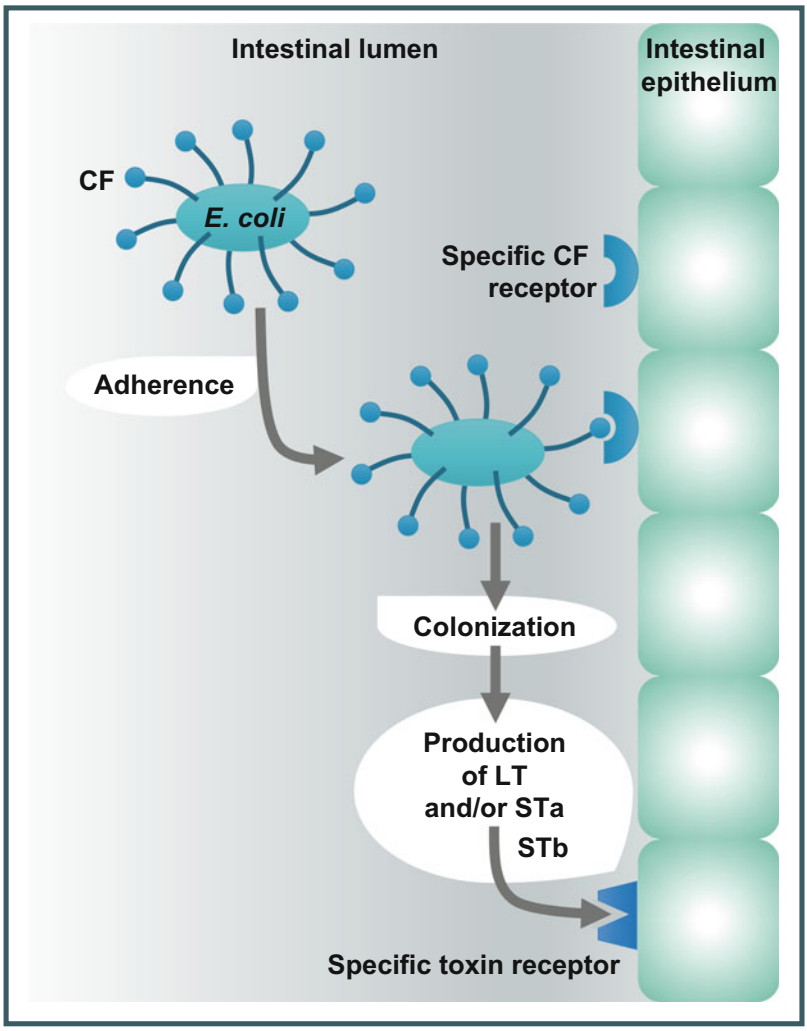

Fig. 1 ETEC pathogenesis. First, adherence of the pathogen to intestinal cells is done through production of colonization factors (CF) either fimbrial or non-fimbrial that interact with specific receptors present on the cell surface. This step permits in situ colonization. Second, the adhered bacteria produce one or more toxins that are active on intestinal epithelial cells leading to secretion

expressing STb as the only toxin caused clinical diarrhea in more than half of gnotobiotic piglets, suggesting that STb is an important virulence factor in porcine diarrhea. Overall, the study indicated that both LT and STb contributed to dehydration in challenged pigs. Nevertheless, the effect of LT was substantially greater than that of STb. Together these studies proved that STb is by itself able to provoke secretion in piglets and is an important contributor to diarrhea.

The contribution of different enterotoxins produced by an $F4^+$ ETEC strain (O149, $F4ac^+$, LT^+, STa^+, STb^+) to the induction of small intestinal secretion and early innate immune responses was investigated in weaned piglets using isogenic deletion mutants (Loos et al. 2012). Net fluid absorption of pig's small intestinal mucosa was measured 4 h after infection, allowing correlation of enterotoxin secretion with gene regulation using microarray analysis. A dominant role for STb in small intestinal secretion early after post-weaning, as well as in the induced innate immune response through differential regulation of immune modulators, was observed. Contrarily to previous studies, the data did not suggest a leading role for LT and STa in the induction of secretion. However, the presence of LT and STa together could compensate for the lack of STb.

As discussed before, studies using isogenic deletion mutants and a gnotobiotic piglet (2 weeks old) infection model had highlighted the importance of LT as a virulence factor, compared to STb (Zhang et al. 2006). On the other hand, Loos et al. (2012) suggested a major role for STb in the early secretory response in 5-week-old piglets. The discrepancy observed could be explained by a difference in age of the piglets used, confirming that this parameter is a crucial element to take into account when evaluating toxicity of STb. These results are in agreement with the fact that the presence of STb is more often associated with ETEC isolated from post-weaning diarrhea than from neonatal diarrhea (Moon et al. 1986; Chapman et al. 2006).

An inverse relationship between the presence of STb toxin-induced fluid accumulation and adherence of ETEC in pig ligated jejunal loop was reported (Erume et al. 2013). The results supported the hypothesis that enterotoxin-induced fluid accumulation flushes the progeny microorganisms into the intestinal lumen, thereby increasing the possible fecal shedding and transmission of the pathogen to new animal hosts.

STb Gene

STb is encoded by the *estB* gene found on heterologous plasmids carrying other properties, including other enterotoxins, colonization factors, antibiotic resistances, and transfer functions. The *estB* gene is highly prevalent in ETEC strains isolated from pigs with post-weaning diarrhea (Zhang et al. 2006; Vidotto et al. 2009; Mohlatlole et al. 2013). Interestingly, a recent study conducted in Argentina on ETEC subclinical infection in pigs revealed that numerous potentially virulent ETEC genotypes cohabit and circulate in swine populations without manifestation of neonatal or post-weaning diarrhea (Moredo et al. 2015). This study indicated a prevalence of 97.5% for *estB* gene. For comparison purposes, the frequency of *estB* in ETEC isolates from suckling pigs with diarrhea in China is 20% (Liu et al. 2014).

Regulation of *estB* is under environmental control. As shown, STb production varies with the composition of the culture medium. The quantity of STb produced also varies with the ETEC isolate. It was reported that the culture of STb-positive

isolates is less in brain-heart infusion (BHI) and tryptic soy broth (TSB), than in Luria broth. The repressive effect of glucose on STb production was also described (Busque et al. 1995). A study by Erume et al. (2010) indicated that production of STb was higher when either STb clones, or porcine ETEC isolates, were grown in BHI supplemented with 2% casamino acids (CA) compared to TSB, indicating that CA could potentially have a positive effect on toxin production (Erume et al. 2010). Addition of lincomycin to BHI-CA at optimal concentration was shown to increase LT production; however, it caused a significant decrease in STb levels. Polymyxin B used in an attempt to increase production and/or release of STb toxin, as has been demonstrated for LT and cholera toxin (CT), when added to the medium did not improve the yield of STb. Addition of both lincomycin and polymyxin B abolished detectable STb production altogether.

STb Toxin

STb peptide is synthesized as a 71-amino-acid precursor comprising a 23-amino-acid signal sequence. Mature STb toxin comprises 48 amino acids (Mr of 5,200 Da) containing four cysteine residues involved in disulfide bridges formation and required for toxicity expression (Dubreuil 2008) (Fig. 2). STb has an isoelectric point of 9.6, is insoluble in methanol, and loses biological activity following ß-mercaptoethanol treatment. It is highly sensitive to proteases, particularly

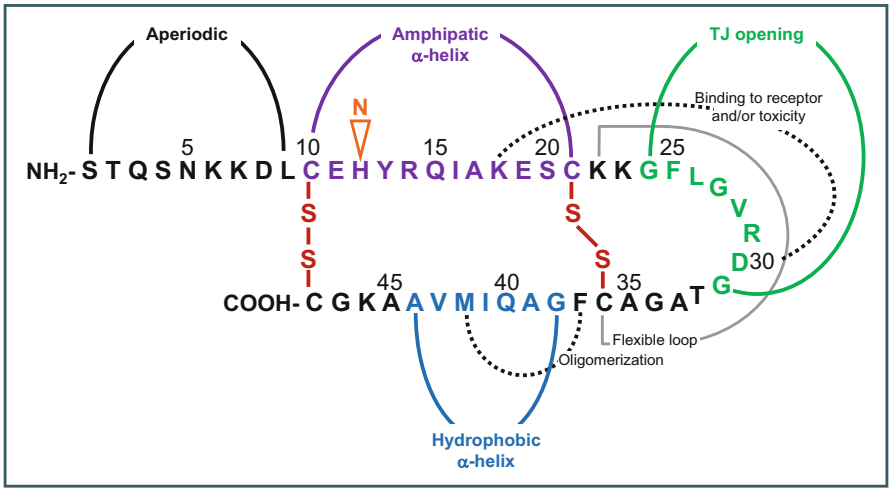

Fig. 2 Structure of STb toxin showing the toxic and oligomerization domains and the site responsible for tight junction opening. The *arrow* indicates the amino acid change observed in the variant

trypsin-like enzymes. These biochemical characteristics render the purification of STb from culture supernatant a hazardous task. STb bears no homology to STa and EAST1 enterotoxin at the nucleotide or amino acid levels.

Receptor

The chemical nature of STb receptor was elucidated using chemical and enzymatic treatments of pig jejunum tissue. A glycosphingolipid sulfatide ($SO_4$3-galactosyl-ceramide), a molecule present in high number in the plasma membrane of intestinal epithelial cells, was identified as the functional receptor (Rousset et al. 1998). Using pig jejunal tissue and mass spectrometric analysis, hydroxylated sulfatides with a ceramide moiety of 16 carbons were identified as the major species present (Beausoleil et al. 2002). Surface plasmon resonance analyses indicated a dissociation constant of 2.4 ± 0.6 nM for the STb-sulfatide interaction (Goncalves et al. 2008).

3D Structure

Using nuclear magnetic resonance, the 3D structure of STb was determined. It indicated the molecule is helical between residues 10–22 and residues 38 and 44 of the mature molecule (Sukumar et al. 1995) (Fig. 2). The α-helix from residue 10 to 22 is amphipathic exposing many polar amino acids. The flexible loop between residues 22 and 36 contains many charged amino acids and four glycines. The α-helix between residues 36 and 44 comprises many hydrophobic amino acids. Circular dichroism studies showed that reduction of disulfides bridges results in a dramatic loss of structure correlating with a loss of function, as previously determined. The peptide spanning from Cys10 to Cys48 was shown to have full biological activity, and the first seven amino acids at the NH_2-terminus are not involved in either the structure or toxicity of STb.

Toxic Domain and Function

Numerous studies were conducted to resolve the diverse domains of STb toxin. Hydrophobic and electrostatic interactions were shown to be important for STb attachment to its receptor, sulfatide. For example, residues in the flexible loop K22, K23, and R29 when mutated independently (alanine scanning strategy) reduced the binding to sulfatide (Labrie et al. 2001a). Concurrently, a diminished toxicity was observed. Mutating glycines in the loop (G24, G27, G31, and G34) also decreased the attachment to sulfatide and toxicity. The toxic domain of STb was established to be comprised between K18 and D30 (Fig. 2). For all mutants in this sequence, a reduction in the binding to sulfatide resulted in a comparable or greater

reduction in toxicity. This observation certainly implies that binding to the receptor is a prerequisite to secretion.

STb associates as oligomers in the form of hexamers and heptamers (Labrie et al. 2001b). It was inferred that hydrophobic residues could play a role in the process. Using site-directed mutagenesis, F39, I41, and M42 were shown to be indispensable for oligomerization of STb as independently mutating these amino acids prevented oligomer formation. Oligomerization was not observed when β-mercaptoethanol was present, indicating that STb 3D structure is required. In other words, disulfide bonds must be formed to observe toxin oligomerization. Mutations of these three residues also resulted in a marked decrease in toxicity.

The data obtained to this day indicate that binding of STb to sulfatide is possible through interaction with basic residues present in or close to the flexible loop. On the other hand, the hydrophobic amino acids in the α-helix are responsible for the organization of the STb molecules as oligomers. As mutation of hydrophobic residues in the COOH-region showed abrogated toxicity, it suggests that formation of oligomers must be a requirement for toxicity expression. This step most probably precedes or follows the interaction with its receptor and eventual cellular internalization.

Variant

Over the years, the amino acid sequence for STb toxin was found to be highly conserved between ETEC isolates studied. Nevertheless, a variant was discovered with a point mutation at position 12 (His to Asp) (Taillon et al. 2008). This variant was observed in 25 of 100 Canadian pig ETEC isolates randomly selected. The same variant was reported in Hungaria (Nagy and Fekete 2005), indicating that it might be common and widely distributed. This variant molecule when tested in vitro was binding to the same receptor molecule and could internalize in cells in culture, showing similar properties to the wild-type toxin (Taillon et al. 2012). No other variant was reported for STb toxin.

Internalization

STb is internalized in rat jejunal epithelial cells (Labrie et al. 2002). This step is required for toxicity expression as STb mutants not able to penetrate the cell membrane were found to be biologically inactive. Using a cellular model, confocal microscopy and cell markers, mitochondria hyperpolarization was observed in NIH-3T3 cells early after intoxication (Goncalves and Dubreuil 2009). This event was coupled to the observation of STb-labeled molecules colocalizing with mitochondria, confirming STb internalization. This was not the case for a reduced and alkylated form of the toxin.

Mechanism of Action

Overall, the mechanism of action of STb toxin deduced from experimental data can be summarized by stating that STb binds through its galactose sulfate moiety to an acidic glycosphingolipid, sulfatide, a molecule widely distributed on intestinal epithelial cells (Rousset et al. 1998) (Fig. 3). Once inside the cell, a GTP-binding regulatory protein is stimulated resulting in a Ca^{++} level increase activating calmodulin-dependent protein kinase II (CAMKII) (Dreyfus et al. 1993). Activation of protein kinase C (PKC) phosphorylates the Cystic fibrosis transmembrane regulator (CFTR) (Fujii et al. 1997). PKC also inhibits Na^{+} uptake by acting on an unidentified Na^{+} channel. CAMKII opens a calcium-activated chloride channel (CaCC) and could be involved in phosphorylation of CFTR. The increased calcium

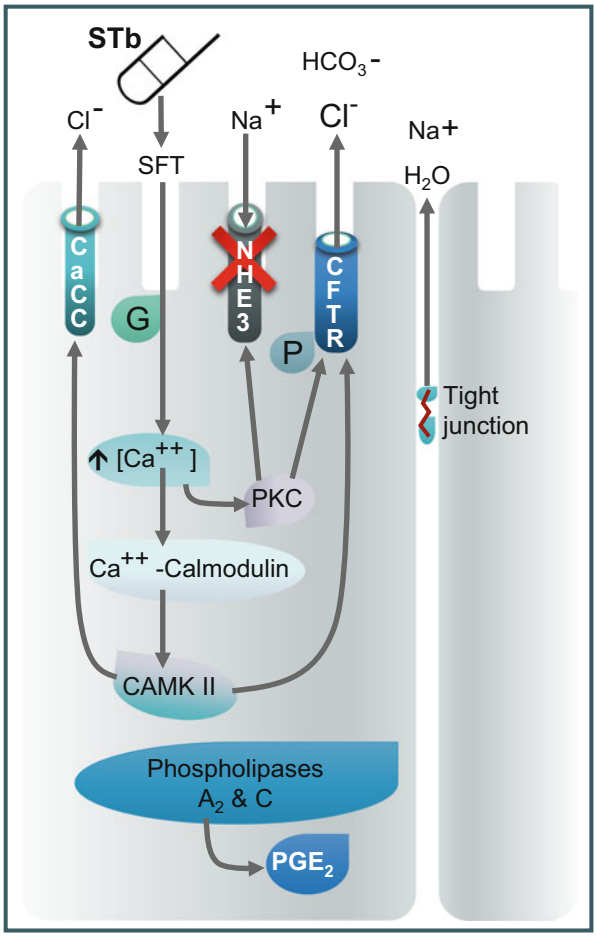

Fig. 3 Mechanism of action proposed for STb induction of secretory diarrhea. STb toxin binds to sulfatide on the epithelial cell and is then internalized. Action of STb on a G protein results in a Ca^{++} increase that activates a calmodulin-dependent protein kinase II (CAMKII). This kinase phosphorylates Cystic fibrosis transmembrane regulator (CFTR) resulting in Cl^{-} and $HCO3^{-}$ secretion. Elevated Ca^{++} also activates protein kinase C (PKC) which activates CFTR as well as inhibits Na^{+} uptake through an unidentified Na^{+} channel. CAMKII also open a calcium-activated chloride channel (CaCC). The initial elevation of Ca^{++} level inside the cell influences the activities of phospholipases A2 and C leading to the formation of PGE2 from membrane lipids. STb also affects cell tight junctions leading to a loss of barrier function

levels also influence the activities of phospholipases A_2 and C leading to the release of arachidonic acid from membrane phospholipids and formation of prostaglandin E2 (PGE_2) and 5-hydroxytrytamine (5-HT) or serotonin) from enterochromaffin cells (Hitotsubashi et al. 1992a; Harville and Dreyfus 1995; Peterson and Whipp 1995). Both compounds mediate the transport of H_2O and electrolytes out of the intestinal cells by a yet unknown mechanism.

Secretion/Dogma

Activation of ion channels and ensuing water passage due to osmotic pressure in the luminal space of the intestine has been held responsible for secretory diarrhea induced by enterotoxins produced by ETEC and other bacterial pathogens such as *V. cholerae* cholera toxin (Dubreuil 2012). This concept is nowadays widely accepted by the majority of researchers. This dogma although questioned, and even when alternative explanations are provided, still remains the basic explanation for secretory diarrhea. Nevertheless, recent studies provided evidence that beside activation of ion channels, particularly Cl^- channels, some ETEC enterotoxins can act on cell structures or components to contribute to the fluid losses observed (Lucas et al. 2005; Lucas 2010).

Alternative Mechanisms

Cellular Alterations

Throughout the years, it appeared that for STb some cellular alterations were observed that could in fact be held responsible, at least partly, for secretion. For example, although STb is classified as a cytotonic toxin meaning it does not affect cellular structures, a study reported cellular alterations following STb treatment of pig jejunal loops (Whipp et al. 1985). The villous length was affected but the crypt depth was not. In the same way, another study indicated that STb induced microscopic cell structural alterations in the intestinal mucosa including shorter villi and an increased occurrence of disrupted epithelium at the villous tips consistent with a reduced absorptive capacity (Whipp et al. 1986). Taken together, these cellular changes could be related to a loss of intestinal absorptive capacity, suggesting that STb could impair absorption as well as induce net secretion. At the same time, no mechanism explaining such histological damages was put forward.

Using confocal microscopy, toxin internalization was observed and confirmed in NIH-3T3 cells as observed earlier in a rat model (Goncalves and Dubreuil 2009). FITC-labeled STb matched with mitochondria and early after cell intoxication, mitochondria hyperpolarization was noted. Later, histological alterations such as membrane budding, granular cytoplasm, and enlarged nucleus suggested apoptotic-

like morphological changes. To pursue this path, Syed and Dubreuil (2012) using human (HRT-18) and animal (IEC-18) cells, respectively, from the colon and ileum, revealed activation of caspase-9, an initiator of mitochondria-mediated apoptosis, and caspase-3, an effector of caspase-9 following STb intoxication (Syed and Dubreuil 2012). Condensation and fragmentation of nuclei as well as DNA fragmentation resulted from the action of STb toxin. At least in these cell lines, STb induced a mitochondria-mediated caspase-dependent apoptotic pathway that could account for the previously observed cellular alterations.

Permeabilization

STb was shown to render permeable piglet jejunal brush border membrane vesicles (Goncalves et al. 2007). Nonspecific pores, allowing the passage of anions and cations, were formed by active wild-type toxin, while poorly active STb mutants were not able to form pores. Gonçalves and Dubreuil (2009) observed that STb could permeabilize the plasma membrane of NIH-3T3 cells (mouse fibroblasts), with the cellular esterases remaining active, indicating that the intoxicated cells were still alive over the experiment time period (Goncalves and Dubreuil 2009).

Secretion Mediators

Many cellular messengers were shown to be implicated in STb mechanism of action. For example, prostaglandins were involved in STb mechanism of action (Hitotsubashi et al. 1992b; Fujii et al. 1995; Dubreuil 1999). In particular, PGE2 and 5-HT are produced in response to STb, and these mediators play a role on the enteric nervous system acting on secretion (Harville and Dreyfus 1995). 5-HT has a role in contracting smooth muscles and increases the peristaltism (Hitotsubashi et al. 1992a; Guttman and Finlay 2008). These molecules are central to the mechanism of action of STb, as inhibiting their production resulted in a marked decrease in fluid secretion.

Tight Junction Opening

Tight junctions control the paracellular traffic of ions and water by forming a physiological barrier in epithelial cells. Recent experiments indicated that STb increased the paracellular permeability to high molecular weight marker proteins (Ngendahayo Mukiza and Dubreuil 2013). This was not observed with a nontoxic STb mutant (D30V).

In addition, it was determined that STb presents a consensus sequence with *V. cholerae* occludens toxin (Zot). An octapeptide (GRLCVQDG) found in Zot

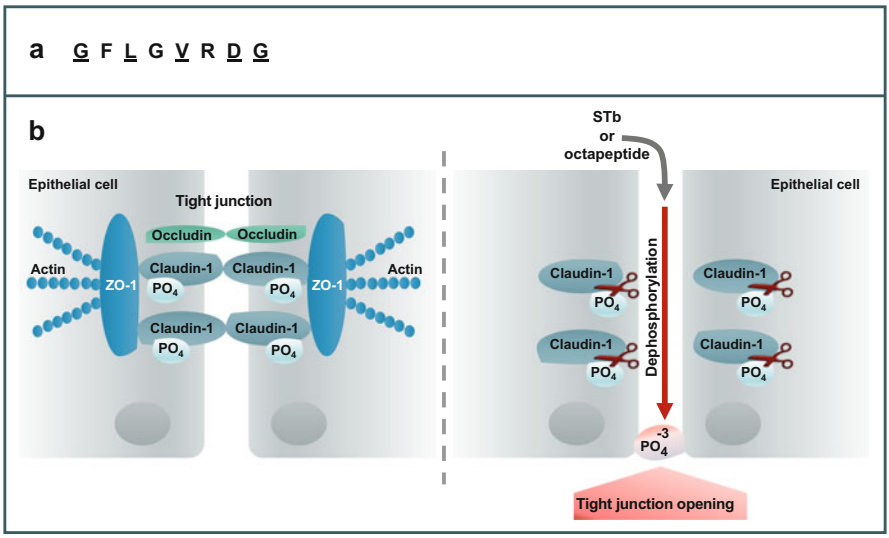

Fig. 4 STb toxin activity on tight junction. (**a**) *Underlined* is the conserved sequence between *V. cholerae* Zot and STb toxins. (**b**) Activity on claudin-1, a protein with a major role in maintaining tight junction integrity. STb dephosphorylates claudin-1, leading to its migration from membrane to the cytoplasm. Only some of the proteins present in tight junction are represented to alleviate the figure

was shown to affect tight junctions of Caco-2 cells (Schmidt et al. 2007). In the same way, using a synthetic peptide, the sequence GFLGVRDG found in STb toxic domain (Fig. 4a) induced epithelial barrier dysfunction in T84 cells (Ngendahayo Mukiza and Dubreuil 2013). Zot functions by mimicking a natural host protein referred as zonulin which regulates barrier function. The zonulin receptor is found predominantly in the small intestine, although colonic cell line Caco-2 also expresses detectable levels of this protein.

An investigation to understand the mechanism underlying the action of STb on cellular changes of tight junction resulting from STb action on T84 cells was undertaken. F-actin stress fibers were markedly altered following STb treatment. Redistribution or fragmentation of ZO-1, claudin-1, and occludin was concurrently noted. As claudin-1 is a major structural and functional tight junction protein responsible for epithelium integrity, further studies were performed to understand its modification following STb action (Nassour and Dubreuil 2014). Changes in claudin-1 were revealed using immunoblot and confocal microscopy. STb treatment of T84 cells provoked the redistribution of claudin-1 from the membrane to the cytoplasm. The rate of dislodgement was favored by addition of calcium to the culture medium. The loss of membrane claudin-1 was the result of dephosphorylation of this important tight junction constituent (Fig. 4b). The mechanisms by which these changes occur were not elucidated. By increasing intestinal permeability, STb might cause diarrhea due to the leakage of water and electrolytes into the lumen due to hydrostatic pressure.

ETEC Protection/Vaccines

Vaccination represents a good way to protect swine against ETEC infection (You et al. 2011). Owing to its low molecular weight, STb is a poorly immunogenic molecule, but antibodies can be produced against mature toxin provided numerous boosting injections are given. Therefore, STb toxin cannot be used as an antigen in vaccine development unless its immunogenicity is enhanced. A satisfactory serological response can be obtained following immunization with either fusion proteins or proteins chemically coupled to STb (Zhang and Francis 2010; You et al. 2011). Neutralizing antibodies can be obtained, but this occurs rarely when the native toxin is used as immunogen. Anti-STb antibodies can neutralize STb toxicity but are unable to neutralize STa or CT toxins (Hitotsubashi et al. 1992a).

ETEC remains an important cause of post-weaning diarrhea. These strains mostly express F4 or F18 fimbriae (Melkebeek et al. 2013; Chen et al. 2014). Preventing colonization of the small intestine is an effective way to control diarrhea (Fig. 1). Colonization can be prevented by local production of anti-F4- and/or anti-F18-specific secretory IgA. The oral route is the logical approach to obtain the desired immune response. For this reason, vaccines based on the predominant fimbriae associated with ETEC responsible for post-weaning diarrhea have been developed (Cox et al. 2002; Fairbrother et al. 2005; Santiago-Mateo et al. 2012). One live orally administered vaccine against F4-positive ETEC strains has been commercialized in Canada, and is available in numerous countries.

However, as epidemiological studies showed that most porcine ETEC strains harbor multiple enterotoxins but may lack colonization factors (fimbrial or non-fimbrial); hence, this strategy may not be successful (Liu et al. 2014). A more effective way to control ETEC would be to aim directly at the enterotoxins that present synergistic effect in enhancing pathogenicity. Effective vaccines need to be polyvalent, aiming at the enterotoxins in order to stimulate an immune response that would take into account all toxins, including LT, STa/EAST1, and STb. This might represent a more universal approach for preventing ETEC infections. For human vaccine development, an LT toxoid (LT_{R192G}) was created that maintained the molecule's immunogenicity (Zhang and Sack 2015).

Such an approach was used by Zhang and Francis (2010) where LT, a commonly used molecule as adjuvant in vaccine development, was fused to STb (Zhang and Francis 2010). LT toxoid (LT_{R192G}) derived from a porcine ETEC strain, as already described for a human LT toxin, was fused to a mature STb peptide. This LT_{R192G} fusion protein practically enhanced STb immunogenicity. In fact, in rabbits and pigs, anti-LT and anti-STb antibodies were detected following immunization. A challenge with an STb-positive ETEC strain demonstrated that all ten suckling piglets born from immunized gilts were healthy, whereas piglets born from nonimmunized gilts developed diarrhea. This result definitely showed that anti-STb antibodies developed were protective against STb toxin.

A genetically constructed trivalent enterotoxin fusion protein (STa-LTB-STb) was produced in an effort to develop a single toxoid containing three ETEC enterotoxins for vaccination (You et al. 2011). In this construct, the toxicity of STa

was reduced by mutagenesis of one cysteine, whereas STb retained the full biological toxicity. Mice immunized with the single toxoid elicited significant antibody responses to LTB, STa, and STb. The antisera could neutralize the bioactivity of LT and both heat-stable enterotoxins. Clearly, the trivalent fusion enterotoxin had the potential to serve as a useful toxin-based vaccine against ETEC-induced diarrheal disease.

Passive immunization could also represent an attractive way to control ETEC infections. In an effort to explore this avenue, You et al. (2014) used an LTB-STa-STb fusion protein to immunize laying hens (You et al. 2014). Chicken egg yolk immunoglobulins (IgY) produced against the three enterotoxins present in the construction were shown to be neutralizing in a suckling mouse model. Recently Liu et al. (2015) evaluated two live attenuated *E. coli* strains expressing LT_{R192G}-STa and LT_{R192G}-STb fusion immunogen in an immunization trial in BALB/c mice (Liu et al. 2015). The result showed both local and systemic immune responses against LT, STa, and STb after oral immunization with the toxoids. The induced antibodies had a neutralizing effect on LT, STa, and STb. The ultimate goal of the described studies was to develop a broadly protective vaccine against ETEC infection. Hence, the developed toxoids could be included in multivalent vaccines to provide broad-spectrum protection against diarrhea caused by ETEC expressing various fimbriae. Inclusion of immunogenic and nontoxic STa derivatives in these vaccines was specially evaluated (Taxt et al. 2010).

Future Avenues

From recent animal studies, STb toxin can now be defined as a major player, at least, in pig diarrhea (Loos et al. 2012; Erume et al. 2013). On the other hand, the acquired knowledge on STb toxin, on its receptor, and the mechanistic understanding could help design ways to control its deleterious effects in animals. As discussed, *E. coli* STb toxin was shown to be active in diverse ways in vitro and in cellular models. These activities still have to be demonstrated in animals to be confirmed as playing a significant role in diarrhea. As for example, in vitro apoptosis-induced cell death observed with HRT-18 and IEC-18 cell lines could explain the loss of villous cells in pig jejunum, yet, the apoptosis described will have to be confirmed in an animal model. Also, as channel activation is an important element involved in secretion, the alternative mechanisms observed in distinct studies performed with STb could nevertheless be involved in the observed fluid losses. For example, opening of tight junctions could represent an influential element for electrolytes and water secretion. More importantly, the quantitative significance of these alternative mechanisms will have to be determined in order to figure out their relative relevance and contribution in the process. It is also attractive to think that new information like STb activity as a nonspecific pore former and the role it plays on tight junctions could be used in the future, for example, for drug delivery. In the coming years, exciting

knowledge acquisition on STb toxin could translate in the development of tools to investigate and understand in more details cellular physiology, as well as preventive measures, to control in an effective way ETEC infections.

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Structure, Enterotoxicity, and Immunogenicity of Enterotoxigenic *Escherichia coli* Heat-Stable Type I Toxin (STa) and Derivatives

13

Qiangde Duan and Weiping Zhang

Abstract

Heat-stable toxin type I (STa) is one of the two major types of enterotoxins produced by enterotoxigenic *Escherichia coli* (ETEC) bacteria that cause children's diarrhea and travelers' diarrhea. STa toxicity and its antigenicity, when coupled to a carrier protein, are associated with unique protein structural characteristics. Changes of primary structure of STa molecule result in reduction or loss of enterotoxicity and alteration of host receptor binding activity as well as immunogenicity potency. This chapter will briefly review structure, enterotoxicity, and immunogenicity potency of STa toxin and derived STa mutants.

Keywords

STa • ETEC • STa mutants • STa toxicity • STa immunogenicity

Contents

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC) bacteria that produce heat-labile toxin (LT) and/or heat-stable toxin (ST) are a leading bacterial cause of diarrhea in children younger than 5 years, in developing countries (WHO 2006; Black et al. 2010; Kotloff et al. 2012, 2013). ETEC associated children's diarrhea results in 150,000–300,000 deaths of young children each year and long-term negative health impacts (WHO 2006; Black et al. 2010; Guerrant et al. 2002; Niehaus et al. 2002; Lorntz et al. 2006). STa-producing ETEC bacteria tend to be more virulent than LT-producing ETEC bacteria and are the third, the fourth, and the fifth leading cause of moderate to severe diarrhea in infants aged 0–11 months and children aged 12–23 months and aged 24–59 months, respectively (Kotloff et al. 2012, 2013). ETEC bacteria are also the most common cause of travellers' diarrhea to immunologically naïve travelers, including civil and military personnel deployed at ETEC endemic countries and regions (Sack et al. 1975, 2007; Sack 1978; Black 1990; Sanders et al. 2005; Hill and Beeching 2010).

There are two classes of heat-stable toxins produced by ETEC bacteria: the methanol soluble heat-stable toxin type I – STI or STa and the methanol insoluble heat-stable toxin type II – STII or STb (Burgess et al. 1978; Picken et al. 1983). Different from STb toxin which is produced by ETEC bacteria causing diarrhea in young pigs, STa is produced by ETEC bacteria that cause diarrhea in animals and humans. ETEC STa toxin can be further recognized as type Ia or porcine-type STa (STp, pSTa, or STaI) and type Ib or human-type STa (STh, hSTa, or STaII). These two types of STa toxin are similar in amino acid sequences and biological activities. The STp or pSTa is a peptide of 18 amino acids and is expressed by ETEC bacteria mainly associated with diarrhea in young animals (So and McCarthy 1980; Takao et al. 1983; Sekizaki et al. 1985; Lazure et al. 1983). Although pSTa is occasionally detected in ETEC strains isolated from human diarrheal patients, whether it directly contributes to human diarrheal disease has not been investigated. The STh or hSTa is a peptide of 19 amino acids and is associated with human diarrhea (Aimoto et al. 1982; Moseley et al. 1983a, b; Thompson and Giannella 1985). But a recombinant *E. coli* strain expressing a porcine-type fimbrial adhesin and hSTa can cause diarrhea in young pigs as an isogenic strain expressing pSTa toxin (Zhang and Zhang 2010). Additionally, the main peptide domain of pSTa and hSTa exhibits same protein structure. There is a right-hand spiral conformation with three beta-turns along the spiral backbone held tightly by three disulfide bonds, as revealed by crystallographic images of a synthetic fully toxic pSTa peptide pSTa(5–17) (a peptide carries amino acids from the 5th residue to the 17th residue of pSTa) and nuclear magnetic resonance (NMR)-determined topological structure of a synthetic hSTa peptide hSTa(6–18) (a peptide carries amino acids from the 6th residue to the 18th residue of hSTa) (Ozaki et al. 1991; Carpick and Gariepy 1991; Sato et al. 1994). Synthetic peptides pSTa(5–17) and hSTa(6–18) are homologues and exhibit the same enterotoxicity as native STa (Aimoto et al. 1983; Ikemura et al. 1984; Yoshimura et al. 1984).

Disease mechanism of STa toxin in causing diarrhea has been extensively investigated (review articles Gyles 1994; Sears and Kaper 1996; Nataro and Kaper 1998; Nair and Takeda 1998; Weiglmeier et al. 2010; Croxen et al. 2013; Kopic et al. 2014). Ingested ETEC bacteria in contaminated food or drink attach to host receptors at intestinal epithelial cells and proliferate in host small intestine. Colonized ETEC bacteria produce STa and/or LT enterotoxins, and deliver toxins to the outer membrane. Secreted STa toxin binds to transmembrane guanylate cyclase C (GC-C) receptor at the apical membrane of host intestinal epithelial cells (Giannella et al. 1983; Shimomura et al. 1986; Schulz et al. 1990) and activates guanylate cyclase pathway, leading to increase of intracellular cyclic-guanosine-3′,5′-monophosphate (cGMP) levels (Field et al. 1978; Hughes et al. 1978; Rao et al. 1980; Crane et al. 1992; Mezoff et al. 1992; Nair and Takeda 1998). Despite details in STa toxin causing fluid secretion not being fully understood and different secretion models being proposed (Lucas 2010), it is generally believed increase of intracellular cGMP levels initiates two pathways in intestinal epithelial cells (Vaandrager et al. 2000). These pathways trigger secretion of chloride into the intestinal lumen but inhibit absorption of sodium chloride by intestinal epithelial cells (Forte et al. 1993; Vaandrager et al. 1997a), leading to fluid hyper-secretion and watery diarrhea.

STa Toxin Structure

STa toxin, encoded by *estA* gene (So and McCarthy 1980), is initially biosynthesized as a 72-amino acid precursor (Rasheed et al. 1990; Okamoto and Takahara 1990; Yamanaka et al. 1993). STa precursor is composed of three segments: a pre-region, a pro-region, and a mature STa region. Cleaved by signal peptidase inside the inner membrane, STa precursor is truncated into a peptide of the first 19 amino acid residues known as the pre-region and a peptide of 53 amino acids carrying the pro-region, and the mature STa region named as pro-STa. This pre-region functions as a signal peptide to translocate the pro-STa peptide out from the cytoplasm to periplasm (Rasheed et al. 1990; Okamoto and Takahara 1990; Yamanaka et al. 1993; Yang et al. 1992), by a SecA-dependent export pathway (Pugsley 1993). Pro-STa peptide inside bacterial periplasm is further cleaved to the pro-region (20–53 or 20–54 amino acids) and the mature STa peptide (54–72 or 55–72 amino acids). This pro-region is demonstrated to translocate the mature STa peptide from the periplasm to outer membrane (Rasheed et al. 1990; Yang et al. 1992; Yamanaka et al. 1998). Whether proteolytic cleavage off the pro-region is essential for mature STa peptide secretion to the outer membrane remains controversial (Okamoto and Takahara 1990). It was shown that a 53-amino acid peptide which includes a putative pro-region is secreted as a mature heat-stable toxin by *Yersinia enterocolitica* (Yoshino et al. 1995). The pro-region is also suggested to play roles in resistance to protease and assistance of STa peptide folding (Okamoto and Takahara 1990).

Fig. 1 Amino acid sequences and disulfide bonds of enterotoxigenic *Escherichia coli* (ETEC) heat-stable toxins (hSTa, pSTa), STs produced by other bacteria, and analogues guanylin and uroguanylin. C-ST: STa of *Citrobacter freundii*; NAG-ST: ST of *Vibrio cholerae*; M-ST: ST of *Vibrio mimicus*; Y-STa: STa of *Yersinia enterocolitica*; K-ST-A1 and K-ST-B: STs of *Klebsiella pneumoniae*

The 18- or 19-amino acid biologically functional mature STa peptide carries six cysteine residues (Fig. 1). Mediated by bacterial thiol disulfide oxidoreductases DsbA (Yamanaka et al. 1994), these six cysteines form three disulfide bonds (Gariepy et al. 1986, 1987; Shimonishi et al. 1987): Cys6–Cys11, Cys7–Cys15, and Cys10–Cys18 in hSTa (or Cys5–Cys10, Cys6–Cys14, and Cys9–Cys17 in pSTa) (Fig. 1). Disruption of these disulfide bonds greatly reduces synthetic hSTa peptide (hSTa6–18) binding to intestinal cells (Gariepy et al. 1987) and also abolishes or significantly decreases STa enterotoxicity or activity in stimulating intracellular cyclic GMP levels in an intestinal cell line T-84 (Okamoto et al. 1987; Yamasaki et al. 1988; Svennerholm et al. 1988; Hirayama 1995; Batisson et al. 2000; Batisson and Der Vartanian 2000). Mutation of either Cys6 to alanine or Cys17 to alanine abolishes pSTa toxic activity, whereas mutation of Cys5 to alanine, Cys10 to serine, or switch of Gly16Cys17 to Cys16Gly17 greatly reduces pSTa enterotoxicity (Okamoto et al. 1987).

Thirteen amino acid residues, from Cys6 to Cys18 of hSTa – hSTa (6–18) or from Cys5 to Cys17 of pSTa – pSTa(5–17), are identified as the STa toxic domain or STa toxin core sequence (Gariepy et al. 1986, 1987; Yoshimura et al. 1985; Kubota et al. 1989; Ozaki et al. 1991). This domain is essential for STa binding to host receptor and full STa enterotoxic activity, and is highly conserved in hSTa and pSTa, as well as among heat-stable toxins produced by other bacteria including *Vibrio cholerae* (designated as NAG-ST) (Takao et al. 1983; Arita et al. 1986; Ogawa et al. 1990), *V. mimicus* (M-ST) (Arita et al. 1991), *Yersinia enterocolitica* (Y-STa) (Takao et al. 1985; Delor et al. 1990), *Citrobacter freundii* (C-ST) (Guarino et al. 1987a, 1989), and *Klebsiella pneumoniae* (Albano et al. 2000) (Fig. 1). These ST toxins have common biological activities and structural or antigenic characterization. Heat-stable toxins produced by other bacteria including *Enterobacter cloacae* (Klipstein and Engert 1976), *Aeromonas hydrophila* (Burke et al. 1983; Sha et al. 2002), and *Proteus* spp.

(Back et al. 1980) were also reported, but these heat-stable toxins are not homologous to ETEC STa (Sha et al. 2002) or their homology to STa is less well characterized.

STa Biological Activity

In vivo enterotoxicity of STa toxin was initially demonstrated in a suckling mouse assay (Dean et al. 1972). By injecting bacterial culture filtrates or STa toxin into stomachs of infant mice and then weighing intestine, versus body carcass, STa enterotoxicity can be detected. The STa suckling mouse assay remains the gold standard for in vivo measurement of STa enterotoxicity (Sack et al. 1975; Burgess et al. 1978; Dobrescu and Huygelen 1973; Jacks and Wu 1974; Giannella 1976; Mullan et al. 1978; Alderete and Robertson 1978; Frantz and Robertson 1981; Robertson et al. 1983; Rolfe and Levin 1994; Turvill et al. 1999). STa production or STa enterotoxicity can also be detected in pig, rabbit, calf, and even guinea-pig loop assays, by measuring accumulated fluid in ligated gut loops (Burgess et al. 1978; Robertson et al. 1983; Moon et al. 1970; Lariviere et al. 1972; Evans et al. 1973; Choudhry et al. 1991; Zhang et al. 2008). Later, in vitro methods were developed and include the Ussing chamber assay using rabbit or rat intestinal segments or colonic cell lines to measure change in short-circuit current (scc) after STa exposure (Forte et al. 1993; Vaandrager et al. 1997a; Young and Levin 1991; Cuthbert et al. 1994; Kuhn et al. 1994), radioimmunoassay with iodinated ST and anti-STa antisera (Frantz and Robertson 1981; Giannella et al. 1981), direct or competitive ELISA with anti-STa antisera and STa conjugates (Lockwood and Robertson 1984; Thompson et al. 1986; Ronnberg et al. 1984), and STa cGMP and T-84 cell assay (Guerrant et al. 1980; Rao et al. 1981; Waldman et al. 1984; Guarino et al. 1987b) have been applied to detect STa toxin production and to measure STa enterotoxicity.

STa toxin, after being secreted to the outer membrane by colonized ETEC bacteria, binds to specific affinity receptors located at the host intestinal epithelial cell apical membrane. STa is observed to enhance activity of enzyme guanylate cyclase and to elevate intracellular cGMP levels (Field et al. 1978; Hughes et al. 1978). Isotope-labeled STa toxin binds to a receptor at the apical membrane of intestinal enterocytes (Giannella et al. 1983; Gariepy and Schoolnik 1986; Kuno et al. 1986). These observations directly led to the identification of the STa specific receptor, a 1050-amino acid intestinal guanylyl cyclase C (GC-C) receptor (Schulz et al. 1990; Vaandrager et al. 1993a; de Sauvage et al. 1991; Basu et al. 2010). This heavily glycosylated membrane bound protein is almost exclusively expressed at intestinal epithelial cells, along the crypt villus axis but mostly at the tip of the villus in the small intestine, and the crypts in colon (Krause et al. 1994). GC-C is suggested to be the predominant receptor (Mann et al. 1997; Schulz et al. 1997), but may not be the sole receptor for STa or analogue guanylin (Hugues et al. 1991; Hakki et al. 1993), since GC-C knockout mice remain healthy, respond normally to other secretory signals, and show activity following binding by STa (Mann et al. 1997; Schulz et al. 1997; Carrithers et al. 2004).

STa binding to GC-C receptor activates conversion of GTP (guanosine triphosphate) to cyclic GMP, leading to elevation of intracellular cGMP levels in intestinal epithelial cells. Elevation of cGMP levels activates GMP-dependent protein kinase (PKG)

pathway (in small intestine) and protein kinase A (PKA, also known as AMP-dependent protein kinase) pathway (in colon) (Vaandrager et al. 2000) and PKG isoform II (PKGII)-dependent phosphorylation of the cystic fibrosis transmembrane conductance regulator (CFTR) (Chao et al. 1994; Vaandrager et al. 1997b, 1998). Activation of the PKG and PKA pathways and CFTR phosphorylation lead to increase in chloride and bicarbonate secretion but inhibition of sodium ion absorption in intestinal epithelial cells (Vaandrager et al. 1993b; Ostedgaard et al. 2001). Secretion of chloride and bicarbonate increases the intraluminal osmolality of the intestine, causing water to be secreted into the lumen and resulting in watery diarrhea.

While STa toxin inhibition of sodium absorption and promotion of water secretion is well accepted, STa causing secretion of chloride has been questioned (Lucas 2001, 2010). It is argued that STa acting through chloride secretion leads to water secretion is only proven in vitro but not in vivo (Lucas 2001). STa causes reduction in fluid absorption but is not necessarily an increase of fluid secretion (Lucas 2005, 2008; Nelson et al. 2007). Lucas (2001) suggested that STa exposure leads to robust and consistent intestine luminal acidification, likely a consequence of Na^+/H^+ exchange instead of Cl^- secretion, at brush border membrane, leading to reduction of water and fluid absorption (Lucas 2001). Thus a different model was proposed: inhibition of Na^+/H^+ exchanges is the central, and possibly only, event resulting from STa exposure and STa affecting Na^+/H^+ exchange at the intestinal intestine is sufficient to change net water movement (Lucas 2001). This new model, however, is under challenge because it is based on observation from a single group with a focus exclusively upon the pathophysiological process and neglects evidence that rectal electrode measurements have illustrated chloride secretion in vivo (Kopic et al. 2010; Murek et al. 2010).

STa Immunogenicity

The 18- or 19-amino acid STa is proven poorly immunogenic. Injection of crude extract of STa + ETEC bacteria or purified STa failed to raise anti-STa antisera (Burgess et al. 1978; Alderete and Robertson 1978; Evans et al. 1973). Evans et al. (1973) observed that rabbit antiserum derived from enterotoxins of ETEC prototype strain H10407 (CFA/I, LT, hSTa, pSTa) showed neutralizing activity against LT but failed in neutralizing STa toxin (Evans et al. 1973). Alderete and Robertson (1978) found that rabbit antiserum derived from purified STa toxin showed an even much reduced neutralization level against purified STa toxin, compared to rabbit antiserum derived from STa:BSA (bovine serum albumin) conjugates which itself exhibited a low neutralizing titer, concluding that STa was poorly immunogenic (Alderete and Robertson 1978). When serum samples of diarrheal children from Bangladesh were titrated, we were unable to detect anti-STa IgM, IgG, or IgA antibodies responses.

STa immunogenicity can be improved after being coupled to a strongly immunogenic carrier protein. By chemically conjugating purified STa toxin to BSA, Alderete and Robertson (1978) found STa immunogenicity was facilitated and induced anti-STa antiserum that showed increasing neutralizing activity against

STa toxin (Alderete and Robertson 1978). Subsequent studies verified that STa:BSA chemical conjugates enhanced anti-STa immunogenicity and induced anti-STa antibodies (Frantz and Robertson 1981; Robertson et al. 1983; Brandwein et al. 1985; Aref and Saeed 2012). Purified or synthetic STa was also chemically conjugated to other proteins including bovine immunoglobin G (Giannella et al. 1981; Moon et al. 1983), ETEC LT toxin (Klipstein et al. 1982) or the B subunit of LT (LT_B) (Klipstein et al. 1983a, b, 1984, 1985, 1986), ovalbumin or thimerosal-inactivated *Bordetella bronchiseptica* cells (Frantz et al. 1987), and biotin (Germani et al. 1992, 1994) to facilitate anti-STa immunogenicity, and resultant conjugates were shown to induce anti-STa antibodies.

STa can also be genetically fused to a strongly immunogenic protein as a recombinant fusion protein to induce anti-STa antibodies. STa mature peptide was fused to the A subunit of LT toxin (Sanchez et al. 1986), the B subunit of LT (Guzman-Verduzco and Kupersztoch 1987; Sanchez et al. 1988a; Clements 1990; Cardenas and Clements 1993), the B subunit of *Vibrio cholerae* cholera toxin (CT) (Sanchez et al. 1988b), a dimer of a synthetic IgG-binding fragment (ZZ) (Lowenadler et al. 1991), *E. coli* OmpC outer membrane protein (Saarilahti et al. 1989), or a subunit of bacterial fimbrial adhesins (Zhang and Zhang 2010; Batisson and Der Vartanian 2000; Pereira et al. 2001; Deng et al. 2015). These chimeric fusion proteins were demonstrated to induce antibody response specific to STa.

STa Mutant Structure and Enterotoxicity

STa mutants were constructed to characterize STa toxin structure and biological activities, and to be safe antigens in ETEC vaccine development. Early studies largely focused on examination of roles played by particular amino acid residues in STa structural features and relevance in biological activity. By substituting the Cys6 or Cys17 with an alanine, Okamoto et al. (1987) found pSTa completely lost its enterotoxic activity in an infant mouse suckling assay (Okamoto et al. 1987). They also noted that a substitution of Cys5 with alanine or Cys10 with serine, or a switch between Cys17 and Gly16, pSTa showed a large decrease of enterotoxic activity. These results led them to conclude that disulfide bonds of C6–C14 and C9–C17 are essential, whereas C5–C10 disulfide bond may not be essential, for pSTa structure and biological activity (Okamoto et al. 1987). That conclusion is supported by a different study in which substitution of Cys7 or Cys7 and Cys18 of a synthetic peptide hSTa(5–19) with alanine resulted in much reduced toxic activity in an infant mouse assay (Svennerholm et al. 1988). The less importance of C5–C10 disulfide bond in pSTa enterotoxicity is reflected by the structure and functions of guanylin and uroguanylin, analogues of STa. Guanylin and uroguanylin have only two disulfide bonds without the equivalent C5–C10 bond (Fig. 1), but just like STa they can fully bind to GC-C receptor and activate guanylate cyclase. Different from the above conclusion, Gariepy et al. (1987) suggested that disulfide bond C7–C15 (equivalent to C6–C14 of pSTa) is the most important bond, followed by the C6–C11 bond (equivalent to pSTa C5–C10), while the C10–C18 bridge (equivalent

to pSTa C9–C17) is the least important for biological activity of synthetic peptide hSTa(6–18) (Gariepy et al. 1987).

STa mutants with substitutions of a particular amino acid with various replacement residues inside the toxic domain display different enterotoxic activity. The pSTa mutants with the Asn11 substituted with His (histidine), Gln (glutamine), Lys (lysine), Arg (arginine), Asp (aspartic acid), or Tyr (tyrosine) varied greatly at reacting with a monoclonal antibody (Mab) against hSTa and polyclonal antibodies (competitive ELISA) against pSTa (suggesting variations of antigenic topology among STa mutants) and also reduction of enterotoxic activity in a suckling mouse assay (Okamoto et al. 1988). Mutants $pSTa_{N11K}$ (Asn11 replaced with Lys) and $pSTa_{N11R}$ (Asn11 substituted by Arg) lost stimulation of fluid accumulation in an infant mouse intestine, indicating loss of enterotoxicity. In contrast, mutants $pSTa_{N11H}$, $pSTa_{N11Q}$, $pSTa_{N11D}$, and $pSTa_{N11Y}$ were only shown with a different reduction of enterotoxicity (Okamoto et al. 1988). Furthermore, *E. coli* expressing the $pSTa_{N11D}$ or $pSTa_{N11K}$ were unable to stimulate fluid accumulation in mouse intestinal loops (Okamoto et al. 1992). Similar to mutation at the Asn11 of pSTa, when the Ala14 of peptide hSTa(6–19) or the Ala13 of peptide pSTa(5–17) was replaced with Gly (glycine), Ser (serine), Asp, Glu (glutamic acid), Gln, Val (valine), Ile (isoleucine), Leu (leucine), Phe (phenylalanine), Lys or Arg, or both Ala14 with Leu and Thr16 with Ala, Yamasaki et al. (1990) found that while the Gly ($hSTa_{A14G}$) and Ser ($hSTa_{A14S}$) mutants had toxicity reduced 65–75%, the other mutants reduced the toxicity over 16,000 times (Yamasaki et al. 1990). In addition, when Asn12, Pro13, or Ala14 of a hSTa synthetic peptide was substituted with different amino acids, resultant single-amino-acid mutated STa peptides also differed at enterotoxic activity (Yamasaki et al. 1988; Hirayama 1995).

STa mutants with mutations at different residues exhibit variance at enterotoxicity but also receptor binding activity. It was reported that peptide hSTa(6–18) with mutation at the Glu8, Leu9, Asn12, Pro13, Ala14, or Gly17 varied significantly at binding to rat cells and enterotoxic activity to suckling mice (Carpick and Gariepy 1991). Mutants $hSTa_{N12A}$ and $STa_{A14D\text{-}A}$ (alanine replaced with D-alanine) showed a significant decrease or loss of receptor binding activity as well as enterotoxicity, but peptide $hSTa_{G17A}$ was as toxic as the native peptide hSTa(6–18) and retained binding activity (but reduced) to rat cells. Among the other mutants, those mutated at the Glu8 (except $hSTa_{E8A}$) or Leu9 were less affected regarding enterotoxicity and receptor binding activity in general, compared to mutants derived from the Asn12, Pro13, or Ala14 (Carpick and Gariepy 1991).

Different STa mutant studies sometime yielded inconsistent results. For examples, one study indicated that the Gly14 mutant ($hSTa_{A14G}$) was shown with 90% reduction in toxicity and 94% reduction in receptor binding activity, suggesting the Ala14 residue is located at or near the receptor binding domain (Carpick and Gariepy 1991). That is supported by STa protein crystallographic data, which suggested the side chains of Pro12 and Ala13 of pSTa (equivalent to Pro13 and Ala14 of hSTa) in the hydrophobic beta-turn (the second beta-turn) may react directly with host epithelial receptor (Ozaki et al. 1991). This same Gly14 mutant peptide ($hSTa_{A14G}$),

however, was shown with only 65% toxicity reduction but similar binding activity of the wildtype peptide hSTa(6–19) in a different study (Yamasaki et al. 1990), referring mutation of Ala14 with glycine did not alter hSTa protein conformation at the receptor binding site. Whether the synthetic peptides used in these two studies had a similar level of uniformity, is unknown.

While the six cysteine residues and the Asn12, Pro13, and the Ala14 in the toxicity domain were extensively investigated, the Glu8, Leu9, and Thr16 were less examined for their role(s) in STa enterotoxicity. Peptide hSTa(6–18) with Gly17 mutated to alanine ($hSTa_{G17A}$) remained toxic as the native peptide and retained much of the binding activity, to rat cells (Carpick and Gariepy 1991). Whether peptides had the Gly17 replaced by other residues retain enterotoxicity and receptor binding activity, was not studied. It was observed recently that full-length hSTa with Glu8 replaced by Pro (proline), Phe, Ser, Gly, Arg, or Lys failed in stimulating intracellular cGMP in T-84 cells or induced fluid accumulation in piglet gut loops, whereas hSTa with Glu8 substituted by Ala (alanine) or Gln retained STa enterotoxicity (Liu et al. 2011a). It was also noticed that hSTa with Leu9 mutated to Val, Ile, Gln, Lys, Arg, Gly, or Ser had enterotoxicity significantly reduced or completely lost. In contrast, hSTa mutants with Thr16 replaced by Lys, Arg, Gly, or Ser, or Gly17 replaced by Ala, Lys, Gln, or Arg, were able to stimulate fluid accumulation (but at reduced levels) in piglet ligated gut loops (Liu et al. 2011a).

STa Toxoid Immunogenicity Potency

Recently, the focus of STa mutant study is shifted toward identification of STa toxoids, facilitation of STa toxoid immunogenicity potency, and application of STa toxoids in ETEC vaccine development. It was once suggested that STa toxoids along with LT toxoids should be explored as safe antigens to induce broad antitoxin immunity in ETEC vaccine development (Eaglestone and Hirst 1995). Indeed, early studies showed that native STa, when chemically conjugated or genetically fused to a strongly immunogenic carrier protein, was able to induce a neutralizing anti-STa antibodies, whereas short synthetic nontoxic STa peptides were proven unable to induce neutralizing anti-STa antibodies. That led to the conclusion that STa peptides without residual toxicity cannot induce a neutralizing antibody response (Svennerholm and Holmgren 1992, 1995), suggesting that STa enterotoxicity is associated with antigenicity potency. This dilemma of STa antigen retaining some enterotoxicity but antigens not being safe for ETEC vaccines yet they induce a neutralizing antibody response, but safe nontoxic STa antigens cannot induce neutralizing anti-STa antibodies, becomes a major roadblock in ETEC vaccine development (Zhang et al. 2010; Zhang and Sack 2012).

The tight association between STa enterotoxicity and antigenicity has been questioned (Zhang et al. 2010; Takeda et al. 1993; Taxt et al. 2016). Recent studies indicated that STa antigenicity can be independent from enterotoxicity. Nontoxic

full-length STa toxoids were demonstrated for the first time to induce neutralizing anti-STa antibodies, after being genetically fused to a monomeric LT toxoid (a single open reading frame consisting of one A subunit with the 192th arginine mutated to glycine and one B subunit) (Zhang et al. 2010; Liu et al. 2011b). Full-length STa mutants including $pSTa_{N11K}$, $pSTa_{P12F}$, $pSTa_{A13Q}$, and $hSTa_{P14Q}$ were found not toxic, demonstrated by culture supernatant of these mutant strains did not stimulate an increase of cGMP in T-84 cells, and recombinant bacteria were unable to induce fluid accumulation in piglet ligated gut loops. When each of these STa toxoids was genetically fused to the LT monomer, all four LT_{R192G}-$STa_{-toxoid}$ fusions induced anti-STa and anti-LT antibodies in the intramuscularly (IM) immunized rabbits or the intraperitoneally (IP) immunized mice. Moreover, rabbit or mouse serum and fecal suspension samples showed neutralizing activity against STa toxin (Zhang et al. 2010; Liu et al. 2011b).

Different STa toxoids in LT-STa toxoid fusions varied at anti-STa antigenicity (Liu et al. 2011a; Zhang et al. 2010). That led to identification of optimal STa toxoids which have enterotoxicity abolished but anti-STa antigenicity retained. These STa toxoids will be preferred to be fused or conjugated to a LT toxoid monomer or other carrier proteins for ETEC vaccine development. A mini-library of STa toxoid was thus initially constructed, and each toxoid was examined for stimulation of intracellular cGMP in T-84 cells (by culture supernatant from the same amount of bacteria, based on culture OD_{600}) or for fluid accumulation in ligated gut loops (by 2×10^9 CFU bacteria) and also reactivity to anti-STa antiserum (Liu et al. 2011a). Consequently, this STa toxoid library was expanded to include STa mutants derived from the 12th, the 13th, or the 14th residue (Table 1), and 14 STa toxoids were selected to be genetically fused to a double mutant LT monomer ($dmLT_{G192G/L211A}$, LT A subunit has the 192 arginine and the 211 leucine substituted with glycine and alanine, respectively) for dmLT-$STa_{-toxoid}$ fusions (Ruan et al. 2014). Since copy number of a STa toxoid in LT-STa toxoid fusions displayed dosage effect at anti-STa antigenicity (Zhang et al. 2013), three copies of each STa toxoid were genetically fused to the dmLT monomer. These 14 3x$STa_{-toxoid}$-dmLT toxoid fusions were verified in vitro nontoxic and then used to immunize mice in two independent laboratories. Among them, 3xSTa_{N12S}-dmLT (the toxoid fusion with three copies of STa toxoid STa_{N12S} fused at the N-terminus, the C-terminus, and between the A and the B subunits of dmLT) was shown to induce the greatest anti-STa IgG and IgA antibody responses in the intraperitoneally immunized mice. Moreover, antibodies derived from toxoid fusion 3xSTa_{N12S}-dmLT had the greatest neutralizing activity against STa toxin (Ruan et al. 2014).

This 3xSTa_{N12S}-dmLT toxoid fusion was further demonstrated to induce neutralizing anti-STa (and anti-LT) antibodies in subcutaneously (SC) immunized mice as well as intramuscularly immunized pigs. Mice subcutaneously immunized with this toxoid fusion developed great titers of anti-STa and anti-LT IgG antibodies. Mouse sera neutralized STa and CT (cholera toxin, a homologue of LT) and prevented these toxins from stimulation of intracellular cGMP and cAMP in T-84 cells. Additionally, pregnant sows intramuscularly immunized with this toxoid fusion developed robust anti-STa and anti-LT IgG in serum as well as IgG and IgA in colostrum samples.

Table 1 In vitro enterotoxicity of STa mutants, measured by stimulation of intracellular cyclic GMP concentrations (pmole/ml) in T-84 cells. Intracellular cGMP levels were measured with a cGMP EIA kit by following the manufacturer's protocol (Enzo Life Sciences, Farmingdale, NY, USA)

E. coli strains	STa[a]	N4Y[b]	E8I	E8Q	L9A	L9D	L9E	L9K	L9T	N12K	N12S
Enterotoxicity (%)	100	74.4	29.1	61	1.7	0.1	0.3	6	0.1	0.2	1.0
E. coli strains	P13A	A14L	A14Q	A14T	A14V	T16M	T16K	T16Q	T16R	T16S	(−)
Enterotoxicity (%)	0.2	0.3	0.3	0.2	0.4	16	0.2	34.5	26.7	94	0.1

[a]Native STa precursor gene (*est*A) was cloned into vector pUC19 at HindIII and BamHI sites and expressed in *E. coli* BL21 strain

[b]Mutants with a single amino acid substituted with a different residue; for an example, N4Y refers that the 4th amino acid asparagine (Asn) of hSTa was replaced with tyrosine (Tyr)

Piglets born to the immunized mothers were protected when orally challenged with a LT+/STa + ETEC strain (Rausch et al. 2016). Data from these preclinical studies may indicate that this 3xSTa_{N12S}-dmLT toxoid fusion may represent a good toxin antigen for ETEC vaccine development.

Conclusion

STa is a small-sized, but potent toxin produced by enterotoxigenic *E. coli* bacteria that cause children's and travelers' diarrhea. STa toxin binds to GC-C receptors at host small intestinal epithelial cells and disrupts fluid homeostasis by elevating intracellular cyclic GMP in host epithelial cells, inhibiting absorption of sodium ions. STa itself is poorly immunogenic but becomes immunogenic after being chemically conjugated, or genetically fused, to a strongly immunogenic carrier protein. STa can have enterotoxicity reduced or eliminated through site-directed mutation. Nontoxic STa toxoids after being genetically fused to a LT toxoid also become immunogenic and induce neutralizing anti-STa antibody responses. LT-STa toxoid fusion antigens may be potentially used as safe antigens for ETEC vaccine development.

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Part V

Helicobacter Toxins

Helicobacter pylori CagA: The Bacterial Oncoprotein

14

Paolo Ruggiero

Abstract

Helicobacter pylori infects the human stomach, causing atrophic gastritis, peptic ulcer, and gastric cancer. Among the virulence factors of *H. pylori*, the *cag* pathogenicity island (*cag* PAI) has been identified in the *H. pylori* genome, coding for several proteins that constitute a type IV secretion system (T4SS), whose main function is to inject bacterial factors into the host cell. In particular, CagA protein, encoded by cytotoxin-associated gene A (*cagA*) that is part of the *cag* PAI, is injected into the host cell in a T4SS-dependent manner. CagA, once into the cell, can be phosphorylated by host enzymes. Both phosphorylated and non-phosphorylated CagA initiate a series of intracellular events, which may dramatically interfere with cell morphology, motility, polarity, proliferation, and differentiation, leading to invasive phenotypes of host cells. Thereby, CagA has earned the definition of "bacterial oncoprotein." Epidemiological studies in humans, as well as studies in animals infected with CagA-positive *H. pylori* strains, or in transgenic mice expressing CagA, indicated a clear link between CagA and the development of precancerous lesions and eventually gastric cancer. Although, besides CagA, other *H. pylori* factors have been linked to gastric cancer development, CagA appears to be the major agent responsible for the *H. pylori*-related carcinogenicity. The development of malignancy is also linked to host factors, such as proinflammatory genetic background. It might be expected that treatments or vaccines targeting CagA, even in the case they only partially affected the *H. pylori* burden, would decrease the risk of malignant outcome of the infection.

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Helicobacter pylori • Peptic ulcer • Gastric cancer • CagA • Cag pathogenicity island

Contents

Introduction

Helicobacter pylori is a spiral-shaped, flagellated, microaerophilic, Gram-negative bacillus. It inhabits the human stomach with prevalence higher than 50% worldwide, varying according to the geographic area and generally increasing with the age of the subjects. *H. pylori* gastric colonization/infection is most commonly acquired at pediatric age, and it may persist for the entire life of the human host.

After the *H. pylori* presence in human stomach was reported for the first time (Marshall and Warren 1984), its relationship with gastritis and peptic ulcer in humans was evidenced (Marshall and Warren 1984; Goodwin et al. 1986). Initially the bacterium was named *Campylobacter pyloridis* and then *C. pylori*, before its definitive classification in the genus *Helicobacter*. The discovery of *H. pylori* and of its link to peptic ulcer led to award the 2005 Nobel Prize in Medicine to Barry Marshall and Robin Warren. It was subsequently found that *H. pylori*-infected subjects are at higher risk of gastric cancer (Correa et al. 1990; Forman et al. 1990): for this reason, and for the further evidences of the link between *H. pylori* infection and gastric cancer, the International Agency for Research on Cancer (IARC) has classified *H. pylori* as a group 1 carcinogen (IARC 1994, 2012). Moreover, *H. pylori* was found to be the causative effect of gastric mucosa-associated lymphoid tissue (MALT) lymphoma (Wotherspoon et al. 1991). *H. pylori* is the only bacterium known to cause cancer in humans.

To establish effective and long-lasting colonization of the gastric mucosa and to get nutrients from the host's tissue, *H. pylori* has developed several mechanisms that generate an inflammatory status, at the same time allowing the bacterium to evade or alter the host immune response; these mechanisms in the majority of the infected

population lead to asymptomatic gastritis only, but in some subjects, they may be the origin of the immune pathogenesis of gastric inflammation and mucosal disease. Indeed, the pathological outcome of *H. pylori* infection results not only from direct bacterial action but also from host response and susceptibility, as indicated by several studies that, for instance, showed cytokine gene polymorphisms to be associated to resistance/susceptibility to *H. pylori* and different outcomes of *H. pylori* infection. Thus, when individuals with proinflammatory genetic background are infected by *H. pylori* strains expressing particularly dangerous factors, the immune response may initiate chronic inflammation causing corpus gastritis and hypochlorhydria, which in turn may evolve to gastric atrophy, to gastric ulcer, and eventually to malignancy.

Symptomatic patients that are diagnosed with *H. pylori* infection are usually subjected to antibiotic-based treatment. Successful eradication of *H. pylori* results in regression of peptic ulcer and MALT lymphoma. The efficacy of the current standard triple therapy based on proton pump inhibitor and two antibiotics has dropped below 80%, mainly due to antibiotic resistance: thus, modifications of the therapy composition and regimen are being actively investigated.

Among several *H. pylori* vaccines that had shown good efficacy in animal models, only few underwent clinical trials, generally giving disappointing results. More recently, instead, the encouraging results of a randomized, double-blind, placebo-controlled, phase 3 pediatric trial with a recombinant urease-based oral vaccine have been reported (Zeng et al. 2015). Urease is a major protein of *H. pylori*: it exerts an activity essential for the survival of the bacterium in the gastric niche, catalizing the conversion of urea to carbon dioxide and ammonia, which in turn neutralizes the gastric juice acidity. The vaccine afforded 71.8% protective efficacy against *H. pylori* infection 1 year after vaccination and still 65% efficacy 3 years after vaccination, with adverse events not significantly different from those that occurred in the placebo group, both in terms of quantity and quality (Zeng et al. 2015).

Among the virulence factors of *H. pylori*, the first to earn the definition of toxin was the vacuolating toxin A (VacA), whose cytotoxic activity was observed in vitro, and its direct action on damaging the gastric epithelial mucosa evidenced in vivo (see also ▶ Chap. 15, "Interaction of *Helicobacter pylor*i VacA Toxin with Its Target Cells," by V. Ricci, in the present book).

Then, another *H. pylori* protein was identified and characterized, whose presence was closely associated with that of VacA, encoded by cytotoxin-associated gene A (*cagA*) and thus named CagA (Covacci et al. 1993; Tummuru et al. 1993); the studies on CagA evidenced its relationship with the most severe outcomes of *H. pylori* infection, including gastric cancer (Blaser et al. 1995). For the numerous detrimental activities exerted by CagA on host cells, it deserves the definition of toxin, and moreover in particular it has been referred to as "bacterial oncoprotein" (Hatakeyama 2003).

The present chapter will focus on CagA and especially its role in the development of gastric carcinoma and the related mechanisms elucidated so far.

The *cag* Pathogenicity Island and the Type IV Secretion System

After the discovery of CagA, the studies on its gene *cagA* revealed that it is encompassed by a chromosomal DNA insertion element of about 40 kb, which exhibits typical characteristics of pathogenicity islands and thus was named *cag* pathogenicity island (*cag* PAI); it was proposed to represent a secretion system for exportation of bacterial factors (Censini et al. 1996). Indeed, this *H. pylori* structure was found to belong to the type IV secretion system (T4SS) family, which is harbored by many Gram-negative pathogens that use it to translocate virulence factors into host cells.

H. pylori strains were classified in two types, type I and type II, according to the presence or the absence of *cag* PAI. Type I strains, having the *cag* PAI in their genome, were found to be associated with the more severe gastroduodenal disease. Not only the presence but also the intactness of *cag* PAI was found to be associated with the severity of histopathological changes in the gastric tissue of patients infected with *H. pylori* (Ahmadzadeh et al. 2015). Interestingly, high *cag* PAI diversity was observed, as coexistence of variants of the same strain with different *cag* PAI genotypes was detected in a significant proportion of patients infected by *cag* PAI-positive *H. pylori* (Matteo et al. 2007). The relevance of this diversity to the *H. pylori* infection strategy deserves further investigation: it may be part of the genetic diversification that allows *H. pylori* to persist during chronic colonization/infection.

The *cag* PAI consists of about 30 genes, which encode as many Cag family proteins that constitute the *H. pylori* T4SS, or play a role in its biogenesis or functions, or whose function has not been identified yet (Backert et al. 2015). The *H. pylori* T4SS consists of inner and outer membrane-spanning complexes and a surface-located pilus, composed of the Cag family proteins. The major function of T4SS is to translocate CagA into the host cell, where it can be phosphorylated by the host enzymes and initiates a series of events that contribute to the gastric disease development and eventually to malignancy: these specific aspects will be treated in the subsequent paragraph dedicated to CagA and its activities on host cells. *H. pylori* utilizes T4SS also to deliver bacterial peptidoglycan into the host cell; internalized peptidoglycan has been suggested to contribute to the activation of proinflammatory signaling cascade through interaction with the cytosolic pathogen recognition molecule nucleotide-binding oligomerization domain containing 1 (NOD1), thus initiating a NOD1-mediated host defense against *cag* PAI-positive *H. pylori* strains (Viala et al. 2004). The *cag* PAI is the major factor responsible for the induction of transcription factor NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) and IL-8 (interleukin-8) by *H. pylori.*

Among the Cag proteins other than CagA, CagL deserves particular attention. It is surface-exposed on the tip of the T4SS and contains an RGD (Arg-Gly-Asp) motif that is commonly found in many integrin ligands; mutations in RGD site, even at the level of single amino acid substitution, abolish the type IV secretion and the subsequent CagA translocation and phosphorylation (Kwok et al. 2007). The RGD motif is able to directly bind the α5β1 integrin, a cell adhesion receptor that is located

in the basolateral host cell membrane (Kwok et al. 2007), and also the αVβ6 integrin, another receptor for viruses that expose an RGDLXXL (Arg-Gly-Asp-Leu-x-x-Leu) motif (Barden and Niemann 2015). Another surface-exposed motif of CagL, comprising FEANE (Phe-Glu-Ala-Asn-Glu) sequence, acts as an enhancer of the interaction of CagL with integrins, most likely in the early stage of T4SS-integrin interaction (Conradi et al. 2012). Thus, CagL was proposed to act as a specialized adhesive that allows T4SS to enter in contact with the host cells, necessary for the subsequent CagA delivery. It was also proposed that CagL, besides its role of establishing the contact with the host cell surface, concomitantly activates host tyrosine kinases to favor CagA phosphorylation at the site of injection (Kwok et al. 2007). Other Cag proteins have been described to bind host cell integrins, namely, CagA, CagY, and CagI, which are able to bind β1 integrin in an RGD-independent manner: this interaction of T4SS with the host cell causes a conformational switch that is necessary to initiate effector protein translocation (Jiménez-Soto et al. 2009). CagH (localized in the inner membrane), CagI (periplasmic, surface-associated, and secreted), and the already cited CagL deserve to be mentioned for their involvement in pilus biogenesis, and in particular CagI and CagL are essential to pilus formation, while CagH is relevant to the regulation of the pilus elongation (Shaffer et al. 2011). Moreover, the five components of the membrane-spanning core complex of *H. pylori* T4SS, namely, CagM, CagT, Cag3 (also known as Cagδ), CagX, and CagY, have been shown to be required for T4SS activity (Frick-Cheng et al. 2016). CagD (a dimer localized in the cytosol, inner membrane, periplasm, surface-associated, and released) has been shown to be essential for CagA translocation, but not for pilus assembly (Cendron et al. 2009).

H. pylori CagA Structure and Activities on Host Cells

CagA Structure

CagA was initially identified as a high-molecular-mass *H. pylori* antigen associated with peptic ulcer disease, as evaluated by the serum antibody levels against this protein found in infected patients. The first attempts of molecular characterization of this protein (1) did not reveal any significant homology with other known proteins; (2) identified a motif of five amino acids, EPIYA (Glu-Pro-Ile-Tyr-Ala), present in several repeats of 102 bp; (3) showed that *cagA* gene is not present in all *H. pylori* strains and, when present, it is strictly associated with the production of VacA, the *H. pylori* cytotoxin; and (4) evidenced that CagA is an immunodominant antigen and indicated that seropositivity to CagA associates with the more severe gastrointestinal disease and gastric cancer (Covacci et al. 1993; Crabtree et al. 1993; Tummuru et al. 1993). The molecular mass of CagA ranges from 128 to 144 kDa, depending on the number of repeats that are present.

The crystal structure of a large N-terminal portion of CagA, of about 100 kDa, has been resolved, corresponding to amino acids 1–876 (Hayashi et al. 2012) or 1–884 (Kaplan-Türköz et al. 2012), indicating a structured N-terminal region and an

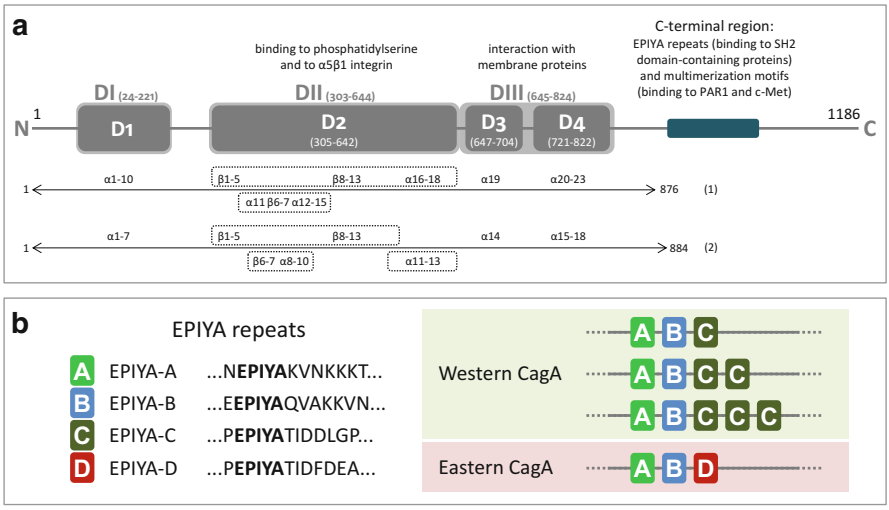

Fig. 1 CagA structure. (**a**) Structure indicating the protein domains (DI–DIII according to Hayashi et al. 2012; D1–D4 according to Kaplan-Türköz et al. (2012) with their main functions and the distribution of α-helices and β-strands according to (*1*) Hayashi et al. (2012) and (*2*) Kaplan-Türköz et al. (2012). The portions forming the subdomains of DII or D2 are surrounded by dashed boxes. (**b**) Families of EPIYA repeats characterizing Western and Eastern CagA (*left*) and scheme of the most frequent occurrences of the different repeats along the CagA sequence (*right*)

intrinsically disordered C-terminal region that directs versatile protein interactions (Fig. 1a).

According to the different reports, the N-terminal portion of CagA consists of three or four domains, DI, DII, and DIII (Hayashi et al. 2012) or D1, D2, D3 (Kaplan-Türköz et al. 2012) and D4, where DI corresponds to D1, DII to D2, and DIII to D3 plus D4.

According to Hayashi et al. (2012), DI consists of 10 α-helices, DII consists of 13 β-strands and 7 α-helices and is divided in two subdomains, and DIII comprises 5 α-helices. According to Kaplan-Türköz et al. (2012), D1 consists of the first 7 α-helices, D2 encompasses 13 β-strands and 6 α-helices (with helix 9 divided in 9a and 9b) and is divided in three subdomains, and D3 and D4 comprise 1 and 4 α-helices, respectively. The difference observed between DI and D1 in the number of α-helices is likely due to the fact that Kaplan-Türköz et al. reported poor quality of the electron density map in this area that prevented unambiguous model building.

D1 interacts only with D2 with a very small interaction surface, suggesting its mobility. Also, the other CagA domains contain loops that may confer flexibility, which may be necessary for the numerous interactions exerted by CagA with other molecules. D2 and D3/D4 together form a structural core of CagA made from 12 α-helices and a large β-sheet. D2 comprises 13 antiparallel β-strands, 11 of which form a single-layer β-sheet region, whose interactions with other tracts of the molecule suggest it is part of a rigid core of CagA. The single-layer β-sheet is

stabilized by two independent helical subdomains. The structure of D2 also revealed a basic amino acid cluster that mediates the interaction of CagA with host cell phosphatidylserine; moreover, it was shown that D2, and in particular the proximal part of the single-layer β-sheet, is involved in the β1 integrin binding (Hayashi et al. 2012; Kaplan-Türköz et al. 2012). D3/D4 structure, encompassing 5 α-helices (1 for D3 and 4 for D4), shows flexibility; D4 contains an N-terminal binding sequence that interacts with the disordered C-terminal binding sequence within the unstructured C-terminus of CagA: this intramolecular interaction induces a loop-like structure of the C-terminus.

The C-terminal portion of CagA contains the EPIYA segments and CagA multimerization (CM) sequence, which, respectively, act as binding sites for SH2 domain-containing protein tyrosine phosphatase (SHP2) and protease-activated receptor 1 (PAR1) (Higashi et al. 2002; Saadat et al. 2007): these sequences are exposed, thanks to the interaction of C-terminus with D3/D4.

The C-terminal CagA region shows sequence variability, in particular for the segment that can be found in several repeats, which includes the EPIYA motif, containing a tyrosine phosphorylation site. EPIYA motifs can be found in some variants, in particular with replacements for P, E, and A. Based on the flanking amino acids, four different EPIYA repeats have been identified, named EPIYA-A, EPIYA-B, EPIYA-C, and EPIYA-D (Fig. 1b). While A and B variants are well conserved among *H. pylori* strains, marked difference in geographical distribution has been found for C and D variants. Based on such a difference, two CagA families have been identified, the so-called Western CagA and East Asian CagA. The Western CagA contains EPIYA-A and EPIYA-B followed by up to three C segments, while the East Asian CagA contains A and B followed by one D segment (Hatakeyama 2003; Higashi et al. 2002; Xia et al. 2009). The large majority of *H. pylori* isolates can be distributed among one of the above-described patterns of EPIYA repeats; however, occasionally, one or more of the repeats may be found absent or duplicated. It has been shown that the activity of CagA is influenced not only by the number but also by the flanking sequences of tyrosine phosphorylation sites; thus, the existence of distinct patterns of EPIYA repeats may explain why gastric carcinoma has higher prevalence in East Asia than in Western countries.

Binding of *H. pylori* to Host Cell and CagA Translocation

H. pylori adheres to the host cells in the proximity of the apical-junctional complex, which represents for the cell a barrier, adhesion site, and pathways network to control cell polarity, proliferation, and differentiation processes. Besides several *H. pylori* outer membrane proteins (OMPs) that mediate interactions between the bacterium and the host cells, T4SS, as mentioned before, is able to directly contact the host cell through binding of bacterial CagL to the host cell α5β1 integrin. Binding of T4SS is followed by CagA translocation into the host cell. However, CagA itself is able to interact with both α5β1 integrin (an interaction that enhances CagA translocation)

and plasma membrane phosphatidylserine. Phosphatidylserine is usually part of the inner leaflet of the cellular plasma membrane, but it is externalized to the outer leaflet in response to the contact of *H. pylori* with the cell, a prerequisite for CagA internalization (Murata-Kamiya et al. 2010). This suggests an alternative mechanism by which CagA, already exported to the bacterial surface, contributes to its translocation. However, CagA cannot enter autonomously inside the host cell in the absence of an intact T4SS.

Intracellular Events Triggered by CagA Internalization

Once into the host cell, CagA localizes on the inner surface of the plasma membrane and subsequently may undergo tyrosine phosphorylation at the EPIYA motifs by the host cell tyrosine-protein kinase Src (c-Src) (Stein et al. 2002) or by the nonreceptor tyrosine kinase Abelson murine leukemia viral oncogene homolog 1 (c-Abl) (Poppe et al. 2007): both of them are proto-oncogenes. Once internalized, CagA, either phosphorylated (p-CagA) or non-phosphorylated, is able to initiate a series of intracellular events, described in the subsequent paragraphs, which may dramatically interfere with cell morphology, motility, polarity, proliferation, and differentiation (Backert et al. 2015; Kaplan-Türköz et al. 2012; Stein et al. 2013), leading to invasive phenotypes of host cells. Most of the studies that analyzed the various activities exerted by CagA to alter the signal transduction of the host cell have been made possible by the use of CagA isogenic mutant *H. pylori* strains; moreover, the role of phosphorylated CagA was elucidated by using *H. pylori* strains having CagA mutated at the EPIYA phosphorylation sites.

Interaction with SHP-2

Phosphorylated EPIYA CagA motifs serve as scaffolds to recruit SH2 domain-containing proteins and specifically the Src homology phosphatase 2 (SHP-2) (Higashi et al. 2002). Consequently to the binding to p-CagA, SHP-2 is activated and in turn triggers the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) cascade (Higashi et al. 2004). This activates NF-kB and eventually results in both proinflammatory signaling and abnormal cell proliferation. The complex constituted of p-CagA, and SHP-2 can also block the focal adhesion kinase (FAK) (Tsutsumi et al. 2006), initiating an abnormal morphological transformation. Recently, it has been shown that duplication of EPIYA-C from one to two or more increases SHP-2 binding of Western CagA by more than 100-fold (Nagase et al. 2015), confirming that the number of EPIYA repeats influences the possible malignant outcome of *H. pylori* infection. This finding has been also proven in the animal model of *H. pylori* infection of Mongolian gerbil (Ferreira Júnior et al. 2015). Moreover, it has been proposed that a single SHP-2 is capable of binding two p-CagA proteins (Higashi et al. 2002) and that such a CagA dimerization markedly stabilizes the complex consisting of p-CagA and SHP-2, thereby potentiating SHP-2 deregulation (Nagase et al. 2011).

Interaction with Csk

Through binding to C-terminal Src kinase (Csk), p-CagA can inhibit the c-Src activity, i.e., it can block the CagA phosphorylation, thus constituting an interesting self-modulation by CagA of its own activity. This particular mechanism would deserve further investigation, as its higher or lower efficiency might contribute to explain why the *H. pylori* infection with CagA-positive strains evolves to malignancy only in a relatively low number of subjects. Moreover, it has been reported that c-Src inactivation leads to tyrosine dephosphorylation of the actin-binding protein cortactin and concomitant cortactin redistribution to actin-rich cellular protrusions. c-Src inactivation and cortactin dephosphorylation are required for rearrangements of the actin cytoskeleton (Selbach et al. 2003).

Interaction with ASPP2

In the cytoplasm, p-CagA interacts with the apoptosis-stimulating protein p53-2 (ASPP2), causing relocalization of ASPP2 from cytoplasm to the inner surface of the plasma membrane (Buti et al. 2011). The interaction involves the N-terminal region of CagA and a proline-rich sequence of ASPP2 plus numerous ASPP2 regions distributed throughout the protein sequence (Nešić et al. 2014; Reingewertz et al. 2015). ASPP2 is normally activated by DNA damage or oncogenic stimuli to initiate the apoptotic pathway. Instead, the relocalization of ASPP2 upon interaction with p-CagA leads it to interact with p53, causing abnormal p53 degradation, which in turn determines the block of the apoptotic signaling that otherwise, in the absence of CagA, p53 would have induced. Compromised apoptosis would permit the survival of damaged cells; thus, it could favor cancer development. Moreover, the degradation of p53 can interfere with terminal cell differentiation.

Interaction with PAR1b/MARK2

H. pylori causes recruitment of the polarity-associated PAR1b/MARK2 serine/threonine kinase, a member of the partitioning-defective 1 (PAR1)/microtubule affinity-regulating kinase (MARK), from the cytosol to the plasma membrane. PAR1 family has been shown to possess a CagA-binding sequence. The interaction of CagA with PAR1b/MARK2, with the consequent inhibition of the latter, not only causes disruption of apical junctions and polarity defects but also prevents lumen formation and tubulogenesis, which are important hallmarks of epithelial differentiation. Given the role exerted by MARK kinase in phosphorylating microtubule-associated proteins, it may be hypothesized that CagA may inhibit PAR1-dependent microtubule-associated protein phosphorylation and thereby may elicit junctional and polarity defects through impaired microtubule-based transport (Saadat et al. 2007; Zeaiter et al. 2008).

Interaction with PRK2

Similar to PAR1b/MARK2, the serine/threonine kinase PRK2 has been shown to be recruited to the plasma membrane in presence of CagA, upon direct interaction between CagA and PRK2; however, such interaction appears to involve different

domains from those involved in the binding to PAR1b/MARK2 (Mishra et al. 2015). The interaction between CagA and PRK2 inhibits PRK2 kinase activity, which eventually may influence cytoskeletal rearrangements and translocation of β-catenin to the nucleus (see the paragraph "Interaction with E-Cadherin and β-Catenin") leading to disruption of cellular polarity, with consequent destabilization of cellular junctions and/or cell adhesion.

Interaction with c-Met

CagA has been reported to bind and activate the hepatocyte growth factor receptor (c-Met) (Churin et al. 2003), which is implicated in invasive growth of tumor cells. Binding of CagA to c-Met promotes cellular processes leading to a forceful motogenic response; this invasive phenotype can be suppressed in the presence of E-cadherin. However, the interaction of CagA with c-Met and the subsequent events are still controversial (Pachathundikandi et al. 2013). Binding of CagA to c-Met has been also proposed to influence the nuclear accumulation and transcriptional activity of β-catenin, as described in the following paragraph. Recently, in human- and mouse-derived gastric organoids, CagA was found to interact also with CD44, which acts as a co-receptor for c-Met; such interaction was found to play a functional role in *H. pylori*-induced epithelial cell proliferation that indeed was lost in infected organoids derived from CD44-deficient mouse stomachs (Bertaux-Skeirik et al. 2015).

Interaction with E-Cadherin and β-Catenin

E-Cadherin binds β-catenin, in the complex that anchors the cytoplasmic domain of E-cadherin to actin cytoskeleton, forming adherens junctions between epithelial cells. In normal cells, this regulates the epithelial barrier formation, the paracellular pathway, and the polarity. CagA, independently of its phosphorylation, is able to bind E-cadherin, thereby destabilizing the E-cadherin/β-catenin complex and causing cytoplasmic/nuclear accumulation of β-catenin, which in turn activates proinflammatory, proliferative, and anti-apoptotic signaling (Murata-Kamiya et al. 2007). Moreover, CagA directly associates with β-catenin and indirectly through the binding to mucin-1 (MUC1), whose cytoplasmic region is known to bind β-catenin; interestingly, the increase of MUC1 expression in the gastric mucosa was found to be able to counteract *H. pylori*-induced IL-8 production, likely by binding β-catenin and thereby impeding its accumulation (Guang et al. 2012). More recently, CagA-dependent, mediated by c-Met and/or PI3K/Akt (phosphatidylinositol-3-kinase), phosphorylation of β-catenin has been reported, which may contribute to nuclear accumulation and transcriptional activation of β-catenin and eventually lead to induction of cancer stem cell-like properties (Yong et al. 2016). The proposed mechanism includes from one side CagA binding to glycogen synthase kinase 3β (GSK-3β) and depletion of its activity, inhibiting β-catenin degradation; from the other side, the already mentioned CagA interaction with E-cadherin makes β-catenin available for phosphorylation, which is mediated by Akt upon interaction of CagA with c-Met. This phosphorylation increases nuclear accumulation and transcriptional activity of β-catenin, resulting in increased Wnt

(Wingless-related integration site)/β-catenin signaling. This activation upregulates the expression of octamer-binding transcription factor 4 (Oct-4) and Nanog; since these transcription factors have the role of maintaining the pluripotency and self-renewal of embryonic stem cells, their upregulation may promote the emergence of cancer stem cell-like properties in gastric cancer cells (Yong et al. 2016).

Interaction with Grb-2 and Activation of Ras-ERK Pathway

It has been reported that ERK can be activated by non-phosphorylated CagA and that such an activation may occur independently of both SHP-2 and c-Met, through a Ras-Raf-MEK-ERK-NF-kB signaling pathway, with consequent induction of IL-8 production. It has been hypothesized that the event initiating this signaling might be the binding of CagA to the adaptor protein growth factor receptor-bound protein 2 (Grb-2), which has been shown to activate Ras (Brandt et al. 2005).

Tumor Suppressor Gene Hypermethylation

Epigenetic changes are involved in the development of many cancers, and, in particular, aberrant hypermethylation of promoter region CpG islands of tumor suppressor gene is largely involved in carcinogenesis in the stomach. *H. pylori* infection has been shown to be associated to high levels of hypermethylation in gastric epithelium. It has been found that O^6-methylguanine DNA methyltransferase (MGMT) gene methylation, already known to be related to gastric carcinogenesis, is also significantly associated with infection with CagA-positive *H. pylori* strains (Sepulveda et al. 2010). This has been more recently confirmed by the observation that CagA downregulates the MGMT expression by inducing hypermethylation in its promoter region, suggesting that CagA might induce gastric carcinogenesis by causing hypermethylation of tumor suppressor genes, with the MGMT as a representative (Zhang et al. 2016). The mechanism involves CagA-enhanced interaction between 3-phosphoinositide-dependent protein kinase 1 (PDK1) and Akt, increasing Akt phosphorylation; p-AKT activates NF-kB, which then binds the DNA methyltransferase 1 (DNMT1) promoter and increases its expression. Finally, the upregulated DNMT1 promotes tumor suppressor genes hypermethylation (Zhang et al. 2016).

Evidence of the Role of *H. pylori* CagA in Gastric Cancer

The Evidence that *H. pylori* Infection Causes Gastric Cancer

The relationship between *H. pylori* infection and increased risk of gastric cancer was initially established by epidemiological investigation. Indeed, the first studies that assessed the prevalence of seropositivity to *H. pylori* in relation with the gastric cancer found significant correlation (Correa et al. 1990; Forman et al. 1990) and estimated for the subjects infected by *H. pylori* an increased risk of developing gastric cancer, as compared with the risk observed for noninfected subjects (odd ratio = 2.77) (Forman et al. 1990). As already stated in the "Introduction," the risk

of developing gastric cancer upon *H. pylori* infection may also increase depending on host factors, such as a proinflammatory genetic background. The link between *H. pylori* infection and gastric cancer was confirmed by several subsequent studies (IARC 2012). A recent meta-analysis that included 24 studies, corresponding to 715 incident gastric cancers among a total of 48,064 individuals, showed that patients who underwent eradication of *H. pylori* infection had a lower incidence of gastric cancer than those who did not receive eradication therapy; eradication provided significant benefit for asymptomatic infected individuals and for those after endoscopic resection of gastric cancers (Lee et al. 2016), confirming previous observations.

The availability of animal models of *H. pylori* infection made possible to obtain formal evidence that *H. pylori* infection causes gastric cancer. Atrophic changes were initially observed in a mouse model of *H. pylori* infection after long-term observation (Lee et al. 1993): since atrophic gastritis is considered to be a premalignant condition that favors the development of gastric cancer, further studies attempted to evaluate the possible malignant outcome of *H. pylori* infection in animal models. It was found that mice do not develop gastric cancer upon *H. pylori* infection alone, but coadministration of carcinogenic substances such as *N*-methyl-*N*-nitrosourea (MNU) or *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (MNNG) allowed observing significantly higher development of gastric cancer in the *H. pylori*-infected group as compared with control, noninfected mice (Han et al. 2002). While wild-type mice do not develop gastric cancer upon *H. pylori* infection, some transgenic mice do, such as those deficient for TGF-β, p27, or trefoil factor 2 (TFF2) or those overexpressing gastrin or IL-1β (reviewed in IARC 2012): these studies, besides confirming the relevance of *H. pylori* infection to gastric cancer development, provided insights in the role of the host factors targeted by the mutations. Recently, also trefoil factor 1 (TFF1) knockout mice demonstrated increases in chronic inflammation and frequency of invasive gastric adenocarcinoma upon *H. pylori* infection (Soutto et al. 2015): both TFF1 and TFF2 belong to the trefoil factor family secretory peptides, which exert protective and healing effects after mucosal damage. Also, mice knocked out for osteopontin, which is overexpressed in various types of cancer, showed decreased *H. pylori*-induced gastric carcinogenesis upon MNU administration, mainly due to suppression of a proinflammatory immune response (Lee et al. 2015).

A carcinogenicity study was performed in a rhesus monkey model of *H. pylori* infection in combination with the oral carcinogen N-ethyl-N-nitrosoguanidine (ENNG), which is similar to nitrosamines found in foods such as smoked fish and pickled vegetables: transcriptional analysis of biopsy specimens 5 years post-infection revealed striking changes in monkeys receiving both *H. pylori* and ENNG, showing a neoplasia-specific expression profile characterized by changes in multiple cancer-associated genes. Monkeys receiving *H. pylori* + ENNG developed gastritis, intestinal metaplasia, and neoplasia, while those receiving *H. pylori* alone developed gastritis only. Based on these results, a synergistic effect of *H. pylori* and the carcinogen in inducing gastric neoplasia in primates was proposed (Liu et al. 2009).

Another suitable animal model to study *H. pylori*-related carcinogenesis seems to be Mongolian gerbil. Indeed, an initial study demonstrated that gerbils spontaneously develop gastric adenocarcinoma upon long-term *H. pylori* infection, without the need of treatment with carcinogen substances (Watanabe et al. 1998). However, subsequent studies showed high variability of the gastric cancer development rates; thus, also in this model the additional use of MNU or MNNG was introduced, obtaining not only high and reproducible gastric cancer development rates but also the possibility of shortening the period of observation (Tokieda et al. 1999).

The Evidence that CagA Plays a Central Role in *H. pylori*-Related Gastric Cancer

The analysis of CagA seropositivity in a population including 103 *H. pylori*-infected subjects that developed gastric cancer, and 103 *H. pylori*-infected subjects that did not develop gastric cancer, revealed an association between the infection with CagA-positive strains and the increased risk of developing adenocarcinoma of the stomach (odd ratio = 1.9) (Blaser et al. 1995). Such an association was confirmed by several subsequent reports (IARC 2012). This observation stimulated the investigation on the role of CagA in determining the malignant outcome of the *H. pylori* infection in appropriate in vitro and in vivo models.

All of the CagA activities described in the previous paragraph "Intracellular events triggered by CagA internalization" demonstrated a crucial role of CagA, once delivered into the host cell, in triggering abnormal intracellular signaling that may eventually lead to invasive phenotype.

In the gastric carcinogenicity model of Mongolian gerbil, the essential role of T4SS in inducing the most severe disease was demonstrated, as the *cagE* isogenic mutant, in which the translocation of CagA is impaired, induced very mild histopathological changes as compared with both wild-type strain and *vacA* isogenic mutant (Ogura et al. 2000). However, another study in gerbils did not found different changes at the level of gastric epithelium between wild-type and an isogenic *cagA* mutant *H. pylori* strain (Peek et al. 2000), then focusing the attention on the role of the intact *cag* PAI and consequently of the functional T4SS rather than CagA only (Israel et al. 2001). A further study evidenced that both in gerbils and mice, upon infection with a wild-type *H. pylori* strain, but not with the *cagA* isogenic mutant, the levels of spermine oxidase (SMO) increased in gastric epithelial cells, with generation of oxidative stress and consequent H_2O_2 production, apoptosis, and DNA damage (Chaturvedi et al. 2011); notably, while it was found that *H. pylori* caused apoptosis in gastric epithelial cells, it was also observed that a substantial fraction of cells infected with CagA-positive strains were protected from apoptosis, thus at high risk for malignant transformation.

A formal demonstration of the role of CagA as a bacterial oncoprotein and of the importance of CagA tyrosine phosphorylation in the development of *H. pylori*-associated neoplasms was provided by transgenic mice expressing CagA. In fact,

transgenic mice expressing wild-type CagA, but not those expressing a CagA phosphorylation-resistant mutant, showed gastric epithelial hyperplasia, and some of the mice developed gastric polyps and adenocarcinomas of the stomach and small intestine (Ohnishi et al. 2008). A recent work, done with transgenic mice systemically expressing CagA and treated with a colitis inducer, revealed that CagA worsens the inflammation, whereas inflammation strengthens the oncogenic potential of CagA, thus evidencing that CagA and inflammation may reinforce each other in creating a downward spiral toward neoplastic transformation (Suzuki et al. 2015).

Conclusion and Future Directions

A large body of evidence indicates that the risk of gastric cancer or premalignant lesions is higher in subjects infected with CagA-positive *H. pylori* strains versus those infected with CagA-negative strains. CagA tyrosine phosphorylation plays a central role in CagA activity (Cover 2016), even though non-phosphorylated CagA is also able to trigger very dangerous intracellular signalings. It appears that the progression toward malignancy derives from the effects that CagA exerts on the host cells combined with an enhanced inflammatory response at the level of the gastric mucosa, which can reinforce each other (Cover 2016; Suzuki et al. 2015). It must be said that several other *H. pylori* factors have been proposed to be involved in increasing the risk of gastric cancer development: the already mentioned VacA and in particular some of its isoforms, several OMPs such as outer inflammatory protein A (OipA) and the adhesins BabA and SabA (blood group antigen-binding adhesion and sialic acid-binding adhesion, respectively), DupA (duodenal ulcer-promoting gene), etc. (Cover 2016); however, the most virulent alleles of the genes coding for these proteins are often associated with CagA-positive strains, an observation that reinforces the idea that CagA plays a major role in the malignant outcomes of *H. pylori* infection.

It has been suggested that, in spite of the association of *H. pylori* infection with gastric cancer, it was able to avoid negative selection in that it did not damage severely the premodern human societies (Atherton and Blaser 2009). Indeed, gastric cancer is developed by elderly people, scarcely affecting the population if the life expectancy is below 50 years and, very important from the point of view of evolutive pressure, not influencing the population at the age of reproduction; conversely, it represents a threat in the modern society, in which the life expectancy is much higher. On the other hand, it has been proposed that *H. pylori* provides some benefits on early life, in particular in reducing the risk of acid-related esophageal diseases and asthma (Atherton and Blaser 2009). However, these data appear weak or controversial, when considering large-controlled studies (Graham 2015; IARC 2012; Wang et al. 2013), most probably reflecting the diversity of infecting strain types, host genetic background, geographic areas, socioeconomic status, etc., differences already observed more in general to influence the outcome of *H. pylori* infection. The idea that *H. pylori* infection might provide some benefits to the host may deserve further scientific investigation, but at the same time, if misinterpreted, it might orient

the public opinion against the eradication of *H. pylori*, increasing the risk of peptic ulcer and gastric cancer. In this frame, a vaccine or a treatment effective against the disease rather than the infection could be the balanced solution. In other words, for instance, a treatment or a vaccine specifically targeting CagA might only partially influence the *H. pylori* burden in the stomach, but it could be expected to prevent the severe outcomes of the infection. To date, while the therapeutic treatment against *H. pylori* has been shown to be efficacious in counteracting peptic ulcer and MALT lymphoma, results from randomized studies have not had sufficient power to evaluate the effect of the impact of *H. pylori* eradication on gastric carcinoma risk (IARC 2012). This is conceivably due also to the fact that, at the stage in which the treatment is administered, it might be too late to induce regression of the mechanisms of carcinogenesis, that once initiated, may reach a point of no return and thus progress even in the absence of the bacteria. From this point of view, prophylactic vaccination appears more promising in terms of reduction of risk of gastric carcinoma.

Cross-References

► Interaction of *Helicobacter pylor*i VacA Toxin with Its Target Cells

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Interaction of *Helicobacter pylori* VacA Toxin with Its Target Cells

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Vittorio Ricci and Patrizia Sommi

Abstract

H. pylori infection is one of the most common bacterial infections worldwide and represents the greatest risk factor for gastric malignancy. The relevance of *H. pylori* for gastric cancer development is equivalent to that of tobacco smoking for lung cancer. Among the virulence factors of this bacterium, the protein toxin VacA plays a pivotal role in the overall strategy of *H. pylori* towards achieving persistent gastric colonization and significantly contributes to the pathogenesis of gastric cancer and peptic ulcer disease. VacA is classified as a pore-forming toxin. Because it exerts many pleiotropic effects on mammalian cells and tissues, VacA has been proposed as a paradigm for toxin multifunctionality. Nevertheless, most of its effects on host cells depend on its channel-forming activity in intracellular sites. Thus, VacA has been also envisaged as a new type of A–B toxins (in which the A subunit exhibits pore-forming instead of enzymatic activity) acting intracellularly as a cell-invasive chloride channel. This chapter focuses on the molecular mechanisms through which VacA binds to, is internalized by, and exerts multiple effects in its target cells of the human host.

Keywords

VacA toxin • *Helicobacter pylori* • Gastric cancer • Clathrin-independent endocytosis • Intracellular trafficking

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Contents

Introduction

The protein toxin VacA is one of the most important virulence factors of the Gram-negative bacterium *Helicobacter pylori* which colonizes the stomach of about half the global population, making it one of the most common bacterial infections worldwide (Cover and Blanke 2005; Boquet and Ricci 2012; Cover and Peek 2013; Salama et al. 2013).

H. pylori has been classified by the World Health Organization as a class I carcinogen, i.e., in the same category as asbestos and tobacco smoke (IARC 1994). *H. pylori* infection is the strongest known risk factor for gastric malignancy, with a relevance for gastric cancer development equivalent to that of tobacco smoking for lung cancer (Cover and Peek 2013; Ricci et al. 2015). Once acquired, if untreated, the infection becomes chronic and persists throughout life in spite of an immune response of the host (Romano et al. 2006; Salama et al. 2013). *H. pylori* infection systematically results in an active chronic gastritis that can remain clinically silent for several decades after initial infection or evolve into more severe diseases, such as peptic ulcer, gastric cancer, or lymphoma of the mucosa-associated lymphoid tissue (MALT) (Cover and Peek 2013; Ricci et al. 2011; Wroblewski and Peek 2013). Development of gastric cancer occurs only in a small percentage of *H. pylori*-infected subjects. *H. pylori*-related gastric carcinogenesis appears to result from a well-choreographed interaction between the pathogen and its host, which depends on strain-specific bacterial factors, host genotypic traits, and permissive environmental factors (Cover 2016; Cover and Peek 2013; Romano et al. 2006).

The production of a protein toxin by *H. pylori* was discovered by Leunk et al. (1988). These authors observed that a single heat-labile, protease-sensitive,

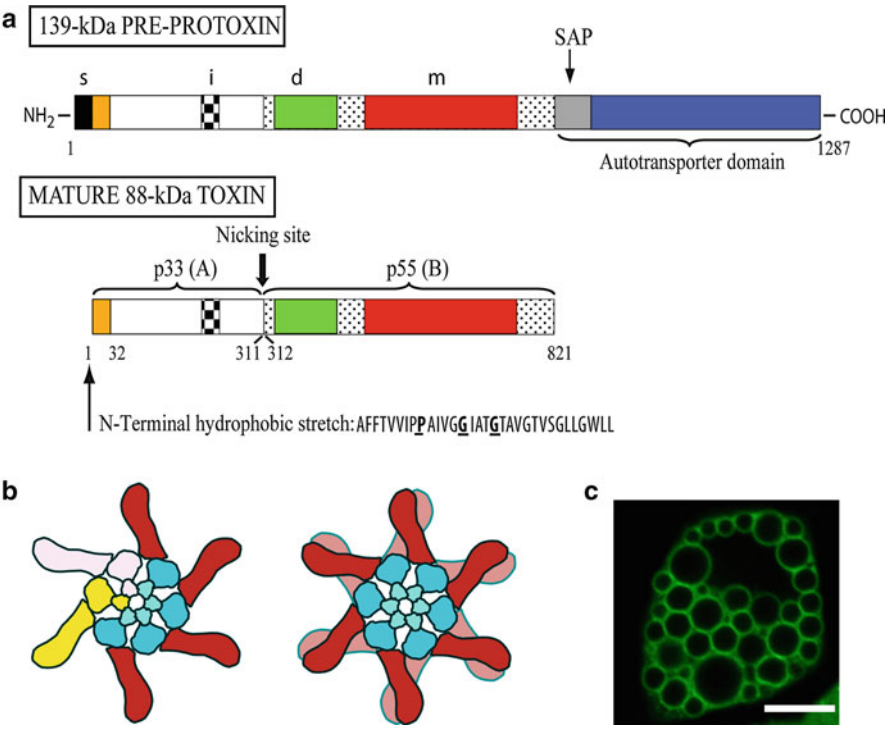

Fig. 1 (**a**) Schematic showing VacA primary structure and processing. The prototypical s1/m1/d1/m1 VacA toxin from the *H. pylori* 60190 strain (ATCC 49503), GenBank accession U05676, is here depicted. Polymorphic regions (s, i, d, and m) are also displayed. A s2-type mature VacA would contain a N-terminal 12-amino-acid hydrophilic extension which is not present in the s1-type toxin. Extracellularly, the mature 88-kDa toxin may be cleaved into an N-terminal 33-kDa and a C-terminal 55-kDa fragment (p33 and p55, respectively). The fragments remain noncovalently associated and may represent two distinct subunits (A and B, respectively) of the toxin. The N-terminus of p33 contains a unique 32-amino-acid hydrophobic stretch which (and especially residues P9, G14 and G18; here in bold underlined) is reported to play a pivotal role in VacA action. See text for details. SAP, secreted α peptide. (**b**) Structure-based model of VacA oligomerization. *Left panel*: monomeric VacA oligomerizes into hexamers supported by intermolecular interactions between the N-terminal portions of p33 in adjacent monomers, as well as contacts between p33 and an adjacent p55 arm. Red domains, p55; azure domains, p33. Two p88 monomers are colored yellow and light pink, respectively, to better show monomer interactions. *Right panel*: schematic of dodecamer formation (Reproduced and adapted from Chambers et al. (2013), with permission from Elsevier). (**c**) VacA-induced cytoplasmic vacuolation in HeLa cells incubated with broth culture filtrate from *H. pylori* 60190 strain for 16 h. Immunofluorescence assay for the small GTPase Rab7 demonstrates the origin of vacuoles from the late endosomal compartment. Bar: 10 μm

and ammonium-sulfate-precipitable factor present in bacterial broth culture supernatants is able to induce massive cytoplasmic vacuolation in cultured cells (Fig. 1). This factor was therefore termed "vacuolating toxin," VacA. Because VacA apparently exerts many pleiotropic effects on mammalian cells and tissues, it has been proposed as a paradigm for toxin multifunctionality (Cover and Blanke 2005).

VacA is currently classified as a pore-forming toxin, even though of nonconventional type. At difference with classical pore-forming toxins, VacA neither cause major alterations of the plasma membrane nor its cytotoxicity seems to primarily depend on pore-forming action at the plasma membrane level. In contrast, most of the toxin activities depend on its internalization and intracellular trafficking (Boquet and Ricci 2012; Cover and Blanke 2005). Although not all its biological effects seem to depend on its channel activity, it has been suggested that VacA could be envisaged as a "cell-invasive" chloride channel (Rassow and Meinecke 2012) or even a prototype of a new class of intracellularly-acting A–B toxins, in which the A subunit exhibits pore-forming instead of enzymatic activity (Boquet and Ricci 2012).

VacA Genetics and Synthesis

All *H. pylori* strains contain a single *vacA* gene in their chromosome. The *vacA* gene encodes an approximately 139-kDa pre-protoxin that undergoes both N- and C-terminal cleavage during the secretion process through an autotransporter pathway (type Va protein secretion system) (Fig. 1a) (Boquet and Ricci 2012; Cover and Blanke 2005; Ricci et al. 2015). This processing yields: (a) an N-terminal 3-kDa signal fragment (which directs the protein to the periplasm); (b) the mature 88-kDa toxin; (c) a small (10.5–12-kDa) secreted peptide with no known function (named SAP, for secreted α peptide, or p10) derived from the 15-kDa linker region of the autotransporter domain; and (d) a 33-kDa C-terminal β-barrel domain (which mediates transport of the passenger domain across the outer membrane) that remains localized with the bacteria (Fig. 1a).

The mature 88-kDa toxin is secreted as a free-soluble protein into the culture medium, but a significant fraction remains associated with the bacterial cell surface, spatially organized into distinct toxin-rich domains (Ilver et al. 2004). Surface-associated VacA can be then delivered to host cells through release of bacterial outer membrane vesicles, a mechanism exploited by several Gram-negative bacteria for virulence factor delivery to host cells or through a contact-dependent mechanism, which would prevent toxin dilution in the extracellular environment (Ilver et al. 2004; Ricci et al. 2011, 2015).

Extracellularly, the mature toxin may be further cleaved at the site of a hydrophilic surface-exposed, protease-sensitive loop into an N-terminal 33-kDa and a C-terminal 55-kDa fragment/domain (named p33 and p55, respectively) that remain noncovalently associated (Fig. 1). VacA was thus proposed to be a classical A–B toxin which acts through enzymatic activity on a cytosolic target (Boquet and Ricci 2012; Cover and Blanke 2005). However, no evidence of such an enzymatic action of the toxin has been provided. The deletion of the proteolytic site required for such a cleavage does not influence VacA vacuolating activity, thus suggesting that the cleavage in two subunits is not essential for toxin action (Boquet and Ricci 2012; Cover and Blanke 2005).

Although nearly all *H. pylori* strains secrete a VacA product, there is considerable interstrain variation in vacuolating activity of the secreted product. Apparently,

the most important reason of such a variability is the high genetic structural diversity of the *vacA* gene that exhibits different polymorphic rearrangements, which in turn results in secreted products with different amino acid sequences (Atherton 2006; Cover and Blanke 2005). It is now widely accepted that the *vacA* genotype is a key determinant of toxin activity and exhibits a high clinical relevance.

Allelic Diversity and its Clinical Significance

Four main families of *vacA* alleles are currently known (reviewed in Cover and Blanke 2005; Ricci et al. 2015). Although recombination occurs quite commonly in *H. pylori*, such families of *vacA* sequences remain relatively intact. This fact strongly suggests that such sequences have favorable functional properties and represent a selective advantage for the bacterium.

The initial studies found that two main polymorphic regions are located near the 5′terminus (the s-region) and in the mid-region (the m-region) of the gene, respectively (Atherton et al. 1995; Cover and Blanke 2005).

The *vacA* s-region encodes the signal peptide, which may have two main forms, s1 and s2. Type s1 and s2 VacA proteins differ in the site at which their N-terminal signal sequence is cleaved. The s2 VacA contains a N-terminal 12-amino-acid hydrophilic extension which is absent in the s1 toxin (reviewed in Cover and Blanke 2005). Only s1-type VacA proteins induce vacuolation in cultured cells (Cover and Blanke 2005). The s1 allele has been further differentiated into s1a, s1b, and s1c subtypes, and certain subtypes have a geographically-restricted distribution (Cover and Blanke 2005; Ricci et al. 2015).

The *vacA* m-region encodes an approximately 800 bp region in the p55 domain of the toxin. Two main alleles have been identified, m1 and m2 (Atherton et al. 1995; Cover and Blanke 2005; Pagliaccia et al. 1998; Ricci et al. 2015). Polymorphism in the m-region has been associated to differences in cell tropism by VacA proteins. The m1 toxin is able to cause vacuolation in a larger number of cell lines in comparison to m2 VacA (Pagliaccia et al. 1998). The fact that m2 VacA binds poorly to HeLa cells suggests that the observed differences in cell tropism between m1 and m2 types are accounted for by the existence of distinct cellular receptors for m1– and m2 – type toxins, respectively. The m-region has been further differentiated in subtypes (i.e., m1a, m1b, m2a, and m2b), the m2b allele being predominantly observed in East Asian strains (Ricci et al. 2015).

All possible combinations of s and m regions (s1/m1, s1/m2, s2/m1; and s2/m2) have been found in *H. pylori* clinical isolates; however, the s2/m1 combination is rare (Atherton et al. 1995; Cover and Blanke 2005). The *vacA* s1 and m1 genotypes have been associated with a higher degree of epithelial damage, gastritis severity, gastric atrophy, precancerous intestinal metaplasia, and gastric cancer development (reviewed by Cover 2016; Ricci et al. 2015). A meta-analysis carried out on 44 studies including more than 17,000 patients showed that patients harboring the *vacA* s1 vs. s2, m1 vs. m2, s1/m1 vs. s1/m2, and s1/m1 vs. s2/m2 genotype exhibit a

statistically significant increased risk for development of gastric cancer (Matos et al. 2013).

More recently, two additional polymorphic regions of *vacA* gene have been identified, the so-called intermediate (i) and deletion (d) regions. The i-region is located between the s- and m-region, representing the residues 190–223 of the p33 domain of mature VacA (Rhead et al. 2007). Polymorphism analysis within this area identified clusters A, B, and C. Only clusters B and C influence VacA vacuolating activity, with cluster C showing a greater effect in comparison to cluster B (Rhead et al. 2007). Similar to s- and m-region, two main types of i-region have been recognized and named i1 and i2, respectively (Rhead et al. 2007). Only s1/m2 bacterial strains vary in i-type; s1/m1 and s2/m2 strains being exclusively i1 and i2, respectively. While all s1/i1/m1 strains are vacuolating and all s2/i2/m2 strains are non-vacuolating, the s1/m2 strains that contain the i1-genotype induce vacuolation of RK13 cells in vitro, whereas those that contain the i2-genotype do not (Rhead et al. 2007). Some strains may display discordant pairing of the clusters, in which the amino acid sequence at one cluster is i1-like while one of the other cluster is i2-like; such strains are defined as i3. However, the i3 genotype is rare and phylogenetically closer to the non-vacuolating i2 genotype. The *vacA* i-region seems to be an important determinant of the most severe diseases induced by *H. pylori* and the best independent marker of VacA-associated pathogenicity (Rhead et al. 2007; Ricci et al. 2015).

The *vacA* d-region is located between the i- and m-region, in the area encoding the N-terminal portion of the p55 domain of mature VacA, and is typed as d1 (367–379 bp), if there is no deletion, or d2 (298 bp), if a 69–81 bp deletion is present (Ogiwara et al. 2009). Although the biological role of the d-region remains unclear, the presence of the *vacA* d1 genotype in *H. pylori* strains might be an improved predictor of severe gastric inflammation and gastric mucosal atrophy, and a risk factor for peptic ulcer and gastric cancer development (Ogiwara et al. 2009).

VacA Structure

Purified VacA is a large water-soluble oligomeric complex of 88-kDa monomers (Cover and Blaser 1992). VacA can assemble into different oligomeric structures, including single-layered hexamers and heptamers as well as double-layered structures consisting of 12 or 14 subunits (Chambers et al. 2013; Cover and Blanke 2005; Cover et al. 1997). The structures, of about 30 nm in diameter, resemble “flowers” or “snowflakes” in which a central ring is surrounded by peripheral “petals” or “arms” (Fig. 1b). Each extended arm is about 95 Å long, while the central region shows a diameter of 100 Å. The p55 domains localize within the peripheral arms, while the p33 domains localize within the center of the complexes (Fig. 1b) (Chambers et al. 2013).

Mutant VacA proteins that fail to oligomerize lack vacuolating activity (Ivie et al. 2008; Vinion-Dubiel et al. 1999). Sequences within both the p33 domain (residues 49–57) and the p55 domain (residues 346 and 347) are required for VacA assembly into oligomers (Ivie et al. 2008). Moreover, some inactive mutant VacA monomers (such as the Δ6-27 one) exert a dominant-negative activity, forming mixed oligomers with wild-type monomers (Vinion-Dubiel et al. 1999).

Although the preferred conformation of VacA oligomers in solution appears to be the double-layered one (Chambers et al. 2013), imaging by both deep-etch EM and atomic-force microscopy as well as electrophysiological studies suggest that membrane-associated VacA channels are single-layered hexameric structures with a central pore (Czajkowsky et al. 1999; Iwamoto et al. 1999).

Oligomeric complexes are apparently able to bind to membranes but are not internalized by cells, and lack vacuolating activity unless they are preliminarily dissociated into monomers by brief exposure to either acidic (<5.5) or alkaline (>9.5) pH conditions before toxin addition to cells (Cover and Blanke 2005; Ricci et al. 2015). However, differing from purified VacA, both bacterium-associated toxin and VacA in crude broth culture filtrates are fully active independently of any acidic or alkaline pretreatment (Leunk et al. 1988; Ricci et al. 2000). To be fully active, VacA should thus interact in its monomeric form with the plasma membrane of host cells where it subsequently oligomerizes, giving rise to membrane channels (Cover and Blanke 2005). This model well fits with the finding that a mixture of purified p33 and p55 proteins, which physically interact resulting in formation of 88-kDa VacA monomers, was fully active on cultured cells in the absence of any pH-dependent activation (González-Rivera et al. 2010). This ability to reconstitute a functional protein from two individually expressed component domains is a unique characteristic of VacA, unusual not only among bacterial protein toxins but also among proteins in general.

While still lacking the p33 domain, high-resolution (2.4 Å) crystal structure has been obtained for the p55 domain (Gangwer et al. 2007). The p55 structure is predominantly a right-handed parallel β-helix, a feature characteristic of autotransporter passenger domains but unique among bacterial protein toxins. The p55 β-helix contains regions of plasticity that tolerate alterations without detrimental effects on protein secretion or activity, as well as a C-terminal region that plays a key role in protein folding and secretion (Ivie et al. 2010). All of these deletion mutations were located outside of the VacA region required for cell vacuolation when VacA is expressed in transfected cells (amino acids 1–422; thus encompassing the p33 domain and the first 111 N-terminal residues of p55) (Cover and Blanke 2005; Ricci et al. 2015). Nevertheless, Ivie et al. (2010) observed that the Δ433-461 VacA mutant protein exhibited a reduced vacuolating activity on HeLa cells compared to the wild-type toxin and no vacuolating activity on RK13 or AZ-521 cells. This finding suggests that such a region has an important role in VacA activity mediating interactions with the host cells.

Chambers et al. (2013) propose a model where VacA first oligomerizes into single-layered complexes and then the hexamers or heptamers interact, via structural motifs along the arm domains, to form the double-layered complexes. Comparing non-vacuolating mutant proteins with wild-type VacA, oligomers formed by the Δ6-27 mutant lack an organized p33 core and mixed oligomers containing both wild-type and Δ6-27 mutant also lack an organized core, thus structurally explaining the dominant-negative activity exerted by such a mutant. Surprisingly, oligomers formed by the s2m1 VacA chimera exhibit a p33 central core virtually identical to that of wild-type oligomers, thus suggesting that, unlike the Δ6-27 mutant, its lack of vacuolating activity does not arise from a disorganized p33 region. Chambers et al. (2013) speculate that the N-terminal 12-amino-acid hydrophilic extension characterizing the s2 forms of VacA may interfere with important hydrophobic interactions between p33 and lipids that are required for pore formation in the context of a membrane environment.

The N-terminus of p33 domain of s1-type VacA consists of a unique stretch of 32 uncharged amino acids (Fig. 1a) which represents the only strongly hydrophobic region of VacA, is long enough to span a biological membrane, and might traverse the membrane as an α-helix (Boquet and Ricci 2012; Cover and Blanke 2005). Such a region is crucial for cell vacuolation and membrane channel formation. Thus, it should directly take part in VacA channel formation or, alternatively, to have an indirect key role by favoring channel formation/activity (Boquet and Ricci 2012; Cover and Blanke 2005). This N-terminal hydrophobic region comprises three tandem GXXXG motifs (defined by glycines at positions 14, 18, 22, and 26) (Fig. 1a), which are important in transmembrane α-helix homo-oligomerization (Cover and Blanke 2005; McClain et al. 2003). Single-amino-acid mutagenesis in the N-terminal hydrophobic stretch of VacA holotoxin revealed a key role of glycines at positions 14 and 18, as well as of proline at position 9 for both the vacuolating and the channel-forming activity of VacA. While the lack of activity of VacA containing either G14A or G18A mutation is consistent with the role of GXXXG motifs in protein-protein interactions, P9A mutation may alter the secondary structure of VacA within this region (McClain et al. 2003).

Studying the p33 domain alone, Domańska et al. (2010) found that the N-terminal hydrophobic part of VacA does not significantly affect the electrophysiological characteristics of the channel formed in planar lipid bilayers by p33 alone, which, in turn, are very similar to those reported for the VacA holotoxin. Deletion of 21 residues in the N-terminal hydrophobic stretch of VacA holotoxin (Δ6-27 VacA mutant protein) secreted by genetically-modified *H. pylori* does not block the formation of VacA channels in lipid bilayers, although their formation is delayed, their anion selectivity is decreased, and no vacuolation is found in intoxicated cells (Vinion-Dubiel et al. 1999). The discrepancies between the data on recombinant p33 complex and on VacA holotoxin could be accounted for by direct or indirect interactions between the N-terminal part of p33 and the p55 domain. Moreover, most effects of VacA holotoxin mutated in the p33 N-terminus could be due to an altered membrane interaction or insertion, thus resulting in a functional impairment

of channel activity or regulation, rather than to a defective pore formation (Boquet and Ricci 2012).

VacA Interactions at the Surface of Host Cells

After binding to the cell surface, VacA undergoes assembly into oligomeric structures and forms channels into the plasma membrane (Boquet and Ricci 2012; Cover and Blanke 2005).

In planar lipid bilayers, VacA forms low-conductance, voltage-dependent, anion-selective channels able to conduct Cl^-, HCO_3^-, and small organic molecules (Czajkowsky et al. 1999; Iwamoto et al. 1999; Szabò et al. 1999; Tombola et al. 1999). VacA forms anion-selective channels in the plasma membrane of HeLa cells, resulting in depolarization of the membrane potential (Szabò et al. 1999). VacA channels in the plasma membrane can lead to the diffusion of small ions (such as Cl^- and HCO_3^-) and urea across the cell membrane (Cover and Blanke 2005; Ricci et al. 2015). Mutant VacA defective in the capacity to form membrane channels in planar lipid bilayers also lacks vacuolating activity (Tombola et al. 1999; Vinion-Dubiel et al. 1999). Chloride channel inhibitors block both channel activity and vacuolation induced by VacA, suggesting that VacA channel activity is required for vacuole formation (Iwamoto et al. 1999; Ricci et al. 2015; Szabo et al. 1999). Actin cytoskeleton disruption (which blocks toxin internalization) increases VacA-dependent chloride transport activity at the plasma membrane but inhibits vacuole formation (Gauthier et al. 2004). This finding rules out the possibility that VacA-induced vacuolation may depend on toxin action at the cell surface and suggests that VacA internalization is required for vacuolation.

The Puzzling Story of the VacA Receptor

Whereas some studies suggested a specific binding of VacA to epithelial cells, studies with radiolabeled VacA found no convincing evidence for a saturable, specific binding (reviewed by Boquet and Ricci 2012; Ricci et al. 2015). VacA might thus bind either nonspecifically or to an abundant, low-affinity receptor on host cells. Nevertheless, the list of putative specific VacA receptors is continuously increasing and includes both different lipids and several different proteins, sometimes associated only with a specific action of the toxin in a specific cellular model (Table 1). This is puzzling because for most bacterial toxins studied so far there is only one specific receptor (or a family of closely related molecules).

Among all putative receptors described so far (extensively reviewed in Backert and Tegtmeyer 2010; Boquet and Ricci 2012; Ricci et al. 2015; Yahiro et al. 2016), the receptor protein tyrosine phosphatases (RPTP) α and β have been the ones more deeply investigated. Whereas VacA administration to wild-type mice resulted in severe gastric damage followed by ulcer development, such tissue damage was absent in RPTPβ-knockout mice (Fujikawa et al. 2003). However, both cultured

Table 1 Putative host cell surface receptors for VacA toxin

Host binding partner	Proposed role in VacA-host interaction
1. Epidermal growth factor receptor	Receptor in epithelial cells
2. Fibronectin	Extracellular matrix component whose VacA affects interaction with integrin receptors leading to altered cell signaling
3. Glycosphingolipids	Binding partner facilitating toxin uptake
4. Heparan sulphate	Receptor/co-receptor in epithelial cells
5. Integrin β2 (CD18)	Receptor in leukocytes
6. Lipid bilayer	Binding partner leading to channel formation and toxin uptake
7. Lipid rafts	Primary binding and cell entering site
8. Low-density lipoprotein receptor-related protein-1 (LRP1)	Receptor in epithelial cells mediating toxin-induced autophagy and apoptosis
9. Multimerin 1	Receptor in platelets
10. Phospholipids	Binding partner facilitating toxin uptake
11. Receptor protein tyrosine phosphatases (RPTP) α	Receptor in epithelial cells
12. RPTP β	Receptor in epithelial cells involved in toxin-induced gastric injury in vivo
13. Sphingomyelin	Receptor in epithelial cells required for toxin uptake

gastric epithelial cells from RPTPβ-knockout mice and G401 human kidney cell line not expressing RPTPβ were sensitive to VacA vacuolating activity. These results suggest that RPTPβ is not the only receptor for VacA. Indeed, treatment of RPTP-β-defective G401 cells with RPTPα-silencing oligonucleotides resulted in downregulation of VacA-induced vacuolation. The similarity between the extracellular domains of RPTPα and RPTPβ is limited to a short segment of each protein (less than 30% amino acid identity over a stretch of about 50 amino acids), but an unrelated region of RPTPβ is apparently critical for the interaction with VacA. Five residues (QTTQP) at positions 747–751 of the extracellular domain of RPTPβ play a crucial role in its interaction with VacA, which results not only in cell vacuolation but also in tyrosine phosphorylation of G-protein-coupled receptor-kinase-interactor 1 (Git-1). Git-1 phosphorylation would in turn result in epithelial cell detachment from the extracellular matrix through a pathway apparently independent from that leading to vacuolation (Fujikawa et al. 2003; Nakayama et al. 2006; Ricci et al. 2015).

However, a pivotal role in VacA interaction with the plasma membrane of host cells is played by lipid rafts, unique membrane microdomains highly enriched in saturated lipids such as sphingomyelin and cholesterol (Boquet and Ricci 2012; Cover and Blanke 2005; Ricci et al. 2015).

Since RPTPα and β normally reside outside lipid rafts, it has been suggested that, upon toxin binding, the VacA–receptor complex is recruited to lipid rafts (Nakayama et al. 2006), which could act as concentration platforms favoring toxin oligomerization and channel formation (Ricci et al. 2000). In contrast, other studies suggest

that the VacA receptor permanently resides in lipid rafts (Gauthier et al. 2004) and that sphingomyelin, one of the main constituents of lipid rafts, acts as a VacA receptor (Gupta et al. 2008).

Apparently, VacA exploits different receptors for intoxication or modulation of epithelial or immune cells. The β2 integrin subunit (CD18; not expressed in epithelial cells) of the leucocyte-specific integrin receptor LFA-1 (i.e., lymphocyte function-associated antigen 1; CD11a/CD18) is the functional receptor for VacA on human T lymphocytes (Sewald et al. 2008a, b). Since LFA-1 is present on all human leukocyte subsets, VacA might exploit this integrin receptor also in granulocytes, macrophages, dendritic cells, B cells, or NK cells (Sewald et al. 2008b). However, a residual membrane binding and vacuolating activity in LFA-1-deficient Jurkat T cells suggests that CD18 may not be the only cell surface receptor for VacA on T cells (Sewald et al. 2008b). The specific targeting of human CD18 (but not its murine counterpart) may explain the species specificity of VacA for human T lymphocytes and the reason why murine T cells are resistant to VacA (Sewald et al. 2008b), a fact with major implications taking into consideration the wide use of a mouse model of *H. pylori* infection.

To make the story of VacA receptor even more intricate, a recent paper investigating VacA role in platelet activation found that human platelets expressed both RPTP β and CD18 on their membrane, but no binding between VacA and each of these receptors was observed (Satoh et al. 2013). On the contrary, Satoh et al. (2013) provided evidence that multimerin 1 might play the role of VacA receptor in human platelets and identified residues 321–340 of multimerin 1 as the specific binding site for VacA.

Internalization and Intracellular Trafficking of the Toxin

In epithelial cells, after oligomerization in lipid rafts, VacA is internalized by a clathrin-independent pinocytic mechanism devoted to endocytosis of glycosylphosphatidylinositol (GPI)-anchored proteins and involving the small GTPases Rac1 and Cdc42. The toxin reaches a tubulovesicular compartment located close to the plasma membrane and named GPI-anchored-protein-enriched early endosomal compartment (GEEC) or clathrin-independent carriers (CLICs) (Fig. 2) (Boquet and Ricci 2012; Ricci et al. 2000). The adaptor molecule CD2-associated protein (CD2AP) is required for maturation of VacA patches from GEEC/CLICs, through a subpopulation of early endosomes (EEs) that undergo motility via the formation of actin comet tails at their surface. CD2AP regulates actin polymerization and comet tail formation by bridging the surface of VacA-containing vesicles and dynamic F-actin structures. Mobile EEs can fuse with late endosomes (LEs) where most VacA is delivered and where the toxin exerts its typical vacuolating activity. However, actin-dependent motility of the toxin-containing endosomes would also allow their transport to other organelles such as the mitochondria, where VacA holotoxin or at least its p33 moiety translocates (Fig. 2).

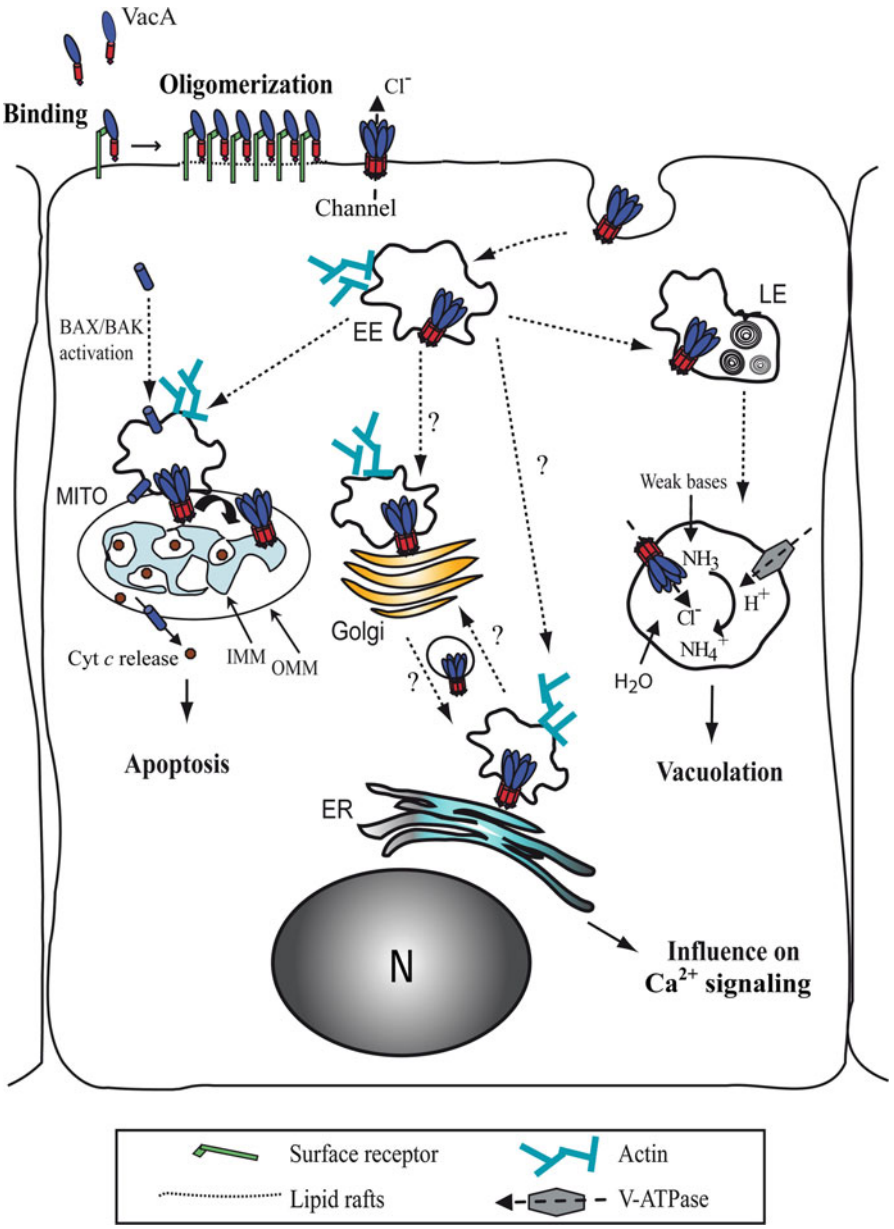

Fig. 2 Working model of VacA internalization and intracellular trafficking in epithelial cells. VacA monomer binds to its cell surface receptor, oligomerizes and is inserted into the plasma membrane at the level of lipid rafts, forming an anion-selective channel. The toxin is then internalized through clathrin-independent endocytosis and reaches a subpopulation of EEs that exhibit a high motility due to the formation of actin comet tails at their surface. VacA-containing endosomes may recruit and activate the pro-apoptotic factors BAX and BAK. Because of their high motility, VacA-containing

VacA might transfer to mitochondria by endosomal–mitochondrial juxtapositional exchange, together with activation of the BAX and BAK pro-apoptotic factors (Calore et al. 2010). Apparently, VacA-containing EEs are able to recruit and activate BAX and BAK. By inserting their hydrophobic C termini into the outer mitochondrial membrane (OMM), BAX and BAK could play a key role in the docking of VacA-containing endosomes with the mitochondria. This could favor their own transfer and that of VacA to the mitochondria, then leading to apoptosis (see below). Mutations of the P9 or G14 amino acids in the N-terminal stretch of p33 domain completely impair BAX/BAK recruitment, endosome–mitochondrion juxtaposition, and BAX/BAK/VacA transfer to mitochondria (Calore et al. 2010). According to Domańska et al. (2010), a key role in mediating VacA transfer to mitochondria is played by the p33 N-terminal stretch of 32 hydrophobic amino acids. This sequence might represent a novel type of mitochondrion-targeting sequence for transfering, through the TOM (for translocase of the outer membrane of mitochondria) complex, p33 alone or the entire toxin to the inner mitochondrial membrane (IMM). However, Jain et al. (2011) suggested that, after reaching the mitochondria through a BAX/BAK-independent pathway, VacA would cause dissipation of the mitochondrial transmembrane electrical potential, resulting in mitochondrial recruitment and activation of dynamin-related protein 1 (Drp1). Drp1, which is a critical regulator of mitochondrial fission through its GTPase activity, causes mitochondrial fragmentation that precedes and is required for BAX/BAK activation and recruitment, which in turn lead to OMM permeabilization and cytochrome *c* release. Inhibition of Drp1-dependent mitochondrial fission within VacA-intoxicated cells prevents toxin-dependent cell death (Jain et al. 2011).

By confocal microscopy in HeLa cells, Kern et al. (2015) recently demonstrated that VacA is also able to reach both the endoplasmic reticulum (ER) and the Golgi apparatus, identifying these cell compartments as novel VacA target structures. It remains, however, to be established how VacA reaches such compartments (through direct transfer from mobile EEs?) and in which order (i.e., first entering the Golgi and then ER, following the so-called retrograde pathway like Shiga toxin, or *vice versa*?).

Fig. 2 (continued) EEs hit the OMM where BAX and BAK might play a key role in the endosomal docking to the mitochondria, thus favouring their own transfer and that of VacA to the mitochondria. Mobile EEs can also fuse with LEs where most VacA is delivered. Here, VacA-induced overactivation of the endosomal proton pump (V-ATPase) through organellar influx of anions (i.e., Cl^-) results in accumulation of weak bases [such as ammonia (NH_3)] by protonation that gives rise to osmotic swelling, finally leading to the typical VacA-dependent cell vacuolation. VacA may be also able to reach other cellular organelles such ER and Golgi apparatus, even though the dynamics and molecular mechanisms of this trafficking pathway are still unclear. Once inside the ER, VacA might affect Ca^{2+}-dependent signaling pathways. See text for further details. Abbreviations: *Cyt c* cytochrome *c*. *EE* early endosome, *ER* endoplasmic reticulum, *IMM* inner mitochondrial membrane, *LE* late endosome, *MITO* mitochondrion, *N* nucleus, *OMM* outer mitochondrial membrane

In immune cells, the mechanism of VacA internalization is very similar to that shown in epithelial cells. While resting primary human T lymphocytes poorly internalize VacA, activated T cells take up the toxin avidly through a Rac1/Cdc42-dependent clathrin-independent pathway (Sewald et al. 2011). In migrating T cells, VacA would exploit integrin recycling vesicles for its cell uptake (Sewald et al. 2008b). A crucial role in VacA uptake seems to be played by kinases of the protein kinase C (PKC) family, in particular PKCζ and PKCη, through phosphorylation of the cytoplasmic tail of the VacA receptor CD18. Phosphorylation of CD18 at T758 is essential and sufficient to induce VacA uptake (Sewald et al. 2011). The following working model for VacA internalization and intracellular trafficking in human T lymphocytes may thus be depicted (Fig. 3): (a) the toxin initially binds to a plasma membrane component such as sphingomyelin or a GPI-anchored protein; (b) then VacA binds CD18, which in activated T cells is released from the cytoskeleton by calpain-mediated cleavage of talin from the CD18 cytoplasmic tail so as to permit its lateral mobility in the membrane and subsequent transfer to the trailing edge (uropod) where lipid rafts are concentrated; (c) oligomerization of the integrin in lipid raft domain leads to PKC-mediated phosphorylation of T758 of the cytoplasmic domain of CD18; (d) the adaptor protein 14-3-3 binds phosphorylated T758 and would initiate recruitment and activation of small GTPases Rac1 and Cdc42; and (e) actin rearrangements may finally result in VacA internalization and transport to late endosomal compartments (leading to vacuolation), to mitochondria (triggering apoptosis), to Golgi apparatus and ER (affecting Ca^{2+} signaling).

Biological Activities of VacA

VacA would play a role in persistent colonization of the human stomach by *H. pylori* through interaction with both epithelial and non-epithelial cells of the gastric mucosa and through different mechanisms, including immunostimulatory and immunosuppressive actions (Cover and Blanke 2005; Oertli et al. 2013; Ricci et al. 2015; Salama et al. 2013).

In a mouse model of infection, Salama et al. (2001) found that, although VacA production was not an absolute requirement for gastric colonization, isogenic *vacA* null mutants were severely defective in the ability to establish initial colonization of the host. Thus, VacA would confer a selective advantage for gastric colonization. This finding well fits with the recent observation (Winter et al. 2014) that bacteria producing the less active s2/i2 form of VacA were able to colonize mice more efficiently than *vacA* null mutants, but also better than strains producing actively vacuolating forms of the toxin. This study provides the first evidence of a positive role for non-vacuolating forms of VacA in *H. pylori* infection. When administered intragastrically in mice, VacA causes gastric damage characterized by ulceration and inflammation, vacuole development in epithelial cells, and marked epithelial infiltration of mast cells and monocytes (Cover and Blanke 2005; Fujikawa et al. 2003;

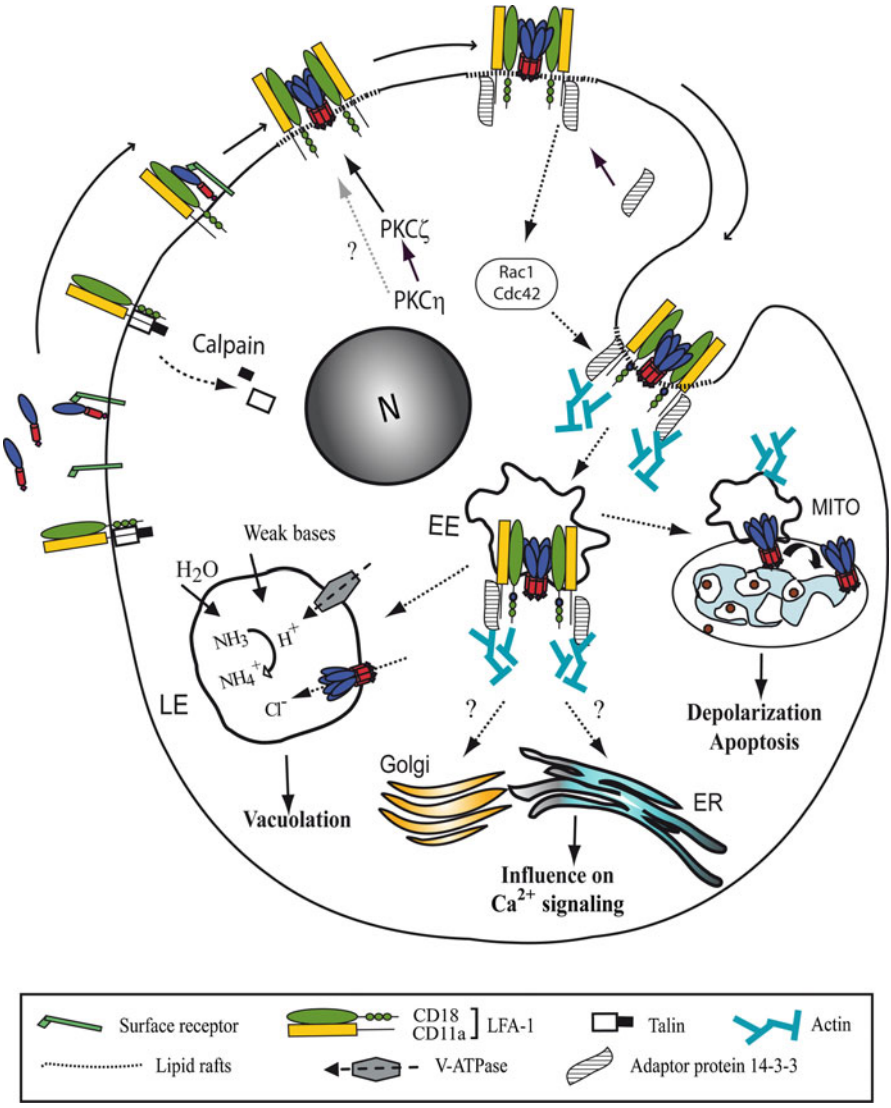

Fig. 3 Working model of VacA internalization and intracellular trafficking in human T lymphocytes. The toxin initially binds to a plasma membrane component such as sphingomyelin or a GPI-anchored protein. Then VacA binds CD18 which, in activated T cells, is released from the cytoskeleton by calpain-mediated cleavage of talin from the CD18 cytoplasmic tail to permit its lateral mobility in the membrane and subsequent transfer to lipid rafts at the uropod of the trailing edge. Oligomerization of the integrin in lipid raft domain leads to PKC-mediated phosphorylation of T758 of the cytoplasmic domain of CD18. The adaptor protein 14-3-3 binds phosphorylated T758 and would initiate recruitment and activation of small GTPases Rac1 and Cdc42. Actin rearrangements may finally result in VacA internalization and transport to late endosomal compartments, mitochondria, Golgi apparatus, and ER. Abbreviations: *EE* early endosome, *ER* endoplasmic reticulum, *IMM* inner mitochondrial membrane, *LE* late endosome, *MITO* mitochondrion, *N* nucleus, *PKC* protein kinase C

Ricci et al. 2015). This suggests a direct role of VacA in the ulcerogenesis of *H. pylori*-colonized patients.

Cytoplasmic Vacuolation

As mentioned above, the characterizing and first identified activity of VacA is its ability to cause formation of large cytoplasmic vacuoles in mammalian cells in culture (Fig. 1c) (Leunk et al. 1988; Cover and Blanke 2005; Boquet and Ricci 2012). Nevertheless, vacuoles virtually identical to those observed in VacA-treated cultured cells are also found in surface gastric epithelial cells in gastric biopsies from *H. pylori*-infected patients (Ricci et al. 2015). In addition, VacA causes vacuolation not only in epithelial cells but also in immune cells (Ricci et al. 2011; Sewald et al. 2008b, 2011). However, the role of VacA-induced cell vacuolation in the overall pathogenetic action of *H. pylori* remains unclear.

It is widely accepted that: (a) VacA-induced vacuoles derive from an acidic late endosomal compartment where the toxin localizes after internalization; (b) such compartment is distinct and different from autophagosomes; and (c) VacA induces the formation of a hybrid compartment with markers of both late endosomes and lysosomes but with a reduced proteolytic activity (reviewed by Cover and Blanke 2005; Ricci et al. 2015).

VacA-induced vacuole development strictly depends on the presence of weak bases such as ammonia produced by *H. pylori* urease, a key enzyme enabling the bacterium to survive in and efficiently colonize the human stomach. VacA by itself is not vacuolating but greatly increases the vacuolating activity of weak bases (Ricci et al. 1997). Weak bases cross cell membranes in an uncharged state and are trapped by protonation within acidic compartments of the eukaryotic cell, thereby inducing osmotic swelling of these compartments resulting in cytoplasmic vacuolation (Ricci et al. 1997, 2015). Internalization and transport to the late endosomal compartment of the anion-selective channels formed by VacA would increase the anionic permeability of this compartment, which in turn would enhance the V-ATPase proton pumping activity leading, in the presence of weak bases like ammonia, to an increased accumulation of osmotically -active ions (like NH_4^+). This finally leads to an increased water influx and swelling (Boquet and Ricci 2012; Cover and Blanke 2005; Ricci et al. 2015).

VacA-induced vacuoles grow in size with time until most of the cytoplasm is occupied by few large vacuoles (Fig. 1c). Vacuolation would thus take place by a progressive addition of membrane, which may result from VacA-induced alteration of membrane traffic along the endocytic-endosomal pathway. Vacuole formation requires the small GTPase Rab7, known to regulate fusion events between vesicles in the late endocytic pathway (Papini et al. 1997). In the absence of weak bases, VacA induces clustering and perinuclear redistribution of late endocytic compartments. Such an action depends on VacA channel-forming activity, is independent of the V-ATPase, and requires Rab7 (Li et al. 2004). In the presence of weak bases, VacA-induced clusters of late endocytic compartments undergo transformation into

typical vacuoles (Li et al. 2004). Clustering of late endocytic compartments may thus be a critical mechanistic step in the process of cell vacuolation induced by VacA.

Terebiznik et al. (2006) suggested that Rab7-dependent homotypic fusion of late endosomal compartments induced by VacA may result in the formation of a unique intracellular niche for internalized *H. pylori*, preventing their lysosomal degradation and thus promoting bacterial survival and infection persistence. Although *H. pylori* is commonly considered an extracellular, noninvasive bacterium, mounting evidence suggests that this bacterium can invade, survive, and multiply in epithelial and immune cells of the human stomach (reviewed in Greenfield and Jones 2013). The ability of *H. pylori* to create an intracellular niche may contribute to infection persistence, evasion from host immune response, and resistance to eradication by membrane-impermeable antibiotics.

A comprehensive proteomic analysis of VacA-containing vacuoles (VCVs) purified from a T-cell line (Jurkat E6-1) revealed a set of 122 VCV-specific proteins not only represented by typical endosomal proteins, but also by defined proteins from other organelles (such as mitochondria and ER) and/or involved in known VacA actions on immune response, cell death, cell signaling, and calcium homeostasis (Kern et al. 2015). This landmark finding strongly supports a specific functional role for VCVs in the VacA intoxication process and suggests that VCVs may act as signaling platforms to trigger specific signaling cascades influenced by the toxin.

Effects on Mitochondria and Cell Death

VacA alters mitochondrial functionality leading to cell death (Boquet and Ricci 2012; Cover and Blanke 2005; Ricci et al. 2015). Gastric epithelial cell death may alter the gastric environment favoring *H. pylori* colonization (e.g., decreasing gastric acidity through death of parietal cells). It may provide more nutrients for the bacteria, and lead to a compensatory faster cell turnover, which predisposes to cancer development. Death of immune cells favors immune response suppression. Therefore, VacA-induced cell death might be an important factor for *H. pylori* infection persistence. VacA causes: (a) decrease in the mitochondrial transmembrane potential (MTP); (b) release of cytochrome *c*; (c) decrease in cellular ATP levels; and (d) apoptosis by triggering the so-called intrinsic (i.e., mitochondrion-dependent) pathway (Boquet and Ricci 2012; Cover and Blanke 2005; Ricci et al. 2015). VacA effects on mitochondria depend on its channel activity but are independent of vacuolation even though increased by ammonia (Chiozzi et al. 2009; Willhite et al. 2003). In comparison to vacuolation, VacA-induced cytochrome *c* release occurs at higher toxin concentrations and at later time points (Willhite et al. 2003). However, the toxin seems to reach and alter mitochondria of cultured epithelial cells in 30–60 min after cell exposure to VacA (Chiozzi et al. 2009; Jain et al. 2011). VacA alters mitochondrial functionality (e.g., decrease in MTP and ATP levels) also in immune cells (Torres et al. 2007). In human B lymphocytes, VacA is reported to cause an increase, rather than a decrease, of MTP (Torres et al. 2007).

After insertion into IMM, a few VacA channels may easily compromise the mitochondrial ion equilibrium of the cell. Considering that a single ion channel producing 1 pA current for 4.4 ms should be sufficient to discharge the membrane of an average mitochondrion, even a single VacA channel might be sufficient to dissipate the MTP of such a mitochondrion (Rassow and Meinecke 2012). However, VacA might trigger mitochondrion-dependent apoptosis also without directly reaching mitochondria but rather activating the proapoptotic protein BAX. Moreover, VacA-induced apoptosis might depend on toxin-induced ER stress, which in turn would lead to induction of the proapoptotic proteins BIM, as well as BAX, activation (reviewed in Foegeding et al. 2016; Ricci et al. 2015).

Based on in vitro findings (such as caspase-independent cell death, cell swelling, release of lactate dehydrogenease and pro-inflammatory proteins, poly(ADP-ribose) polymerase activation), Radin et al. (2011) hypothesized that, in vivo, VacA action on mitochondria might result, at least in part, in programmed necrosis and not apoptosis of gastric epithelial cells. Through release of pro-inflammatory proteins, this type of cell death would increase gastric mucosal inflammation, which contributes to the pathogenesis of peptic ulcer and gastric cancer.

Connexin 43 (Cx43) seems to have an important role in VacA-induced death of epithelial cells (Radin et al. 2014). Cx43 is a host factor that apparently does not serve as a VacA receptor or directly interact with the toxin. It is a multispan transmembrane protein that assembles to form gap junctions at the plasma membrane, but also localizes to intracellular sites such as mitochondria, and is involved in several cell functions (including death and survival) through several mechanisms, some of which are independent of its channel activity. Radin et al. (2014) speculated that VacA-mediated death of gastric epithelial cells through a Cx43-dependent pathway may be important in the pathogenesis of *H. pylori*-associated peptic ulceration. The reestablishment of epithelial integrity in the setting of gastric ulceration and persistent *H. pylori* infection might favor proliferation of cells that have decreased expression of Cx43. These cells may have increased potential for malignant transformation.

Autophagy

An important pathogenic action of *H. pylori* is the modulation of host cell autophagy, and VacA is a key player in such an action.

Terebiznik et al. (2009) found that, in an AGS gastric epithelial cell line infected with *H. pylori*, VacA was the bacterial virulence factor necessary and sufficient to induce autophagy. VacA-induced autophagy was strictly dependent on the channel-forming activity of the toxin, which also partially colocalized with autophagosomes. Based on the observation that autophagy inhibition increased VacA intracellular stability (which in turn resulted in an increased cell vacuolation), Terebiznik et al. (2009) speculated that induction of autophagy is a host mechanism to limit the toxin-induced cell damage by increasing VacA intracellular degradation. Nevertheless, previous studies suggested that, once internalized, VacA persists inside

cultured epithelial cells for hours with little noticeable degradation and without loss of its vacuolating power (Ricci et al. 2015).

People carrying a Crohn's-disease-associated single nucleotide polymorphism in the autophagy gene *ATG16L1* (i.e., rs2241880, which leads to T-to-A substitution at position 300) had increased susceptibility to *H. pylori* infection associated with impaired autophagic response to VacA (Raju et al. 2012). In two separate cohorts, Raju et al. (2012) demonstrated a positive correlation between the presence of the *ATG16L1T300A* risk allele and not just *H. pylori* infection itself, but infection with the more toxigenic *H. pylori* strains. When peripheral blood monocytes isolated from healthy volunteers carrying the 300A risk allele were exposed to VacA, impaired autophagy was observed in comparison to cells isolated from individuals carrying the 300T non-risk allele.

The molecular pathways through which VacA causes autophagy have been investigated by Yahiro et al. (2012) in the AZ-521 cell line (until recently regarded as a gastric epithelial cell line, but now known to be a misidentified HuTu-80 cell line derived from human duodenal carcinoma). Both VacA-induced autophagy and apoptosis were strictly dependent on VacA internalization after binding to the low-density lipoprotein receptor-related protein-1 (LRP1), which in AZ-521 cells apparently plays a key role in toxin binding and internalization leading to vacuole development. Silencing of other VacA receptors, such as RPTPα and β, which are present on the plasma membrane of AZ-521 cells and able to trigger VacA internalization leading to vacuolation, had no effect on VacA-dependent autophagy or apoptosis. LRP1 seems to bind to only the m1 VacA genotype, which, as stressed above, is associated with a higher degree of epithelial damage, gastritis severity, gastric atrophy, precancerous intestinal metaplasia, and increased risk for development of gastric cancer in *H. pylori*-infected patients. Selective inhibition of the autophagy (through siRNA-mediated silencing of *ATG5* gene) inhibited also VacA-induced apoptosis (Tsugawa et al. 2012). Generation of reactive oxygen species (ROS) and AKT activation seem to play a mechanistic role in the autophagy induced by VacA, which would act via decreasing the levels of intracellular glutathione by a yet not fully characterized pathway (Tsugawa et al. 2012).

Nevertheless, VacA is also able to disrupt autophagy. Prolonged exposure (up to 24 h) of human gastric epithelial cells to VacA (to mimic conditions in chronic infection) disrupts autophagy in response to the toxin (Raju et al. 2012). This is because the intoxicated cells lacked the key lysosomal hydrolase cathepsin D in autophagosomes. Loss of autophagy resulted in the accumulation of the sequestosome 1/p62 protein and ROS, both known to promote tumorigenesis. Gastric biopsy samples from patients infected with *H. pylori* strains producing an active (s1/m1) VacA, but not with nontoxigenic strains (i.e., s2/m2 *vacA* genotype), had increased levels of SQSTM1/p62 (Raju et al. 2012).

Jones and coworkers (Greenfield and Jones 2013; Raju et al. 2012) proposed a working model in which the action of VacA on the host autophagic machinery differs according to acute or chronic exposure to the toxin. In acute *H. pylori* infection or with brief (up to 6 h) direct toxin exposure, VacA stimulates autophagy as a cytoprotective reaction to mitigate toxin-induced damage through VacA degradation

and eliminate invading bacteria. In contrast, in chronic *H. pylori* infection or with prolonged direct toxin exposure (both of which may result from a reduced host autophagic response like that associated with the *ATG16L1T300A* risk allele), VacA disrupts autophagy. This may facilitate intracellular bacterial survival and persistent infection but may also create a microenvironment promoting inflammation and carcinogenesis.

Immunosuppressive Activities

Effects on Phagocytosis and Antigen Presentation

H. pylori strategy to achieve a persistent gastric colonization includes the ability to delay its own phagocytosis. In macrophages, delayed phagocytosis results in homotypic phagosome fusion to form large "megasomes" where *H. pylori* may persist because of inhibition of phagosome maturation (Zheng and Jones 2003). VacA contributes to megasome formation by stimulating the recruitment and retention of the tryptophan-aspartate-containing (TACO) protein (also known as coronin 1) to phagosomes, which results in disruption of phagosomal maturation and impaired fusion with lysosomes. Megasomes show a limited lumenal acidification and acquire only limited amounts of late endosomal/lysosomal markers (Zheng and Jones 2003). Moreover, VacA may favor *H. pylori* survival in macrophages by decreasing ROS production in such cells through inhibition of the expression of endothelial nitric oxide synthase, mediated by a suppressive effect of the toxin on the expression of integrin-linked kinase.

VacA interferes with antigen processing in B cells and thereby blocks the subsequent presentation of the resulting peptides to T cells (Molinari et al. 1998). VacA inhibits the Ii-dependent pathway of antigen presentation, which is mediated by newly synthesized major histocompatibility complex (MHC) class II molecules, but does not interfere with the pathway dependent on recycling MHC class II. This suggests that VacA inhibits antigen processing in B cells by interfering with late endocytic membrane traffic.

Dendritic cells (DCs) are antigen-presenting cells playing a key role in regulation of adaptive immune responses. VacA enters and causes vacuolation in human DCs both in vivo and in vitro (Ricci et al. 2011). VacA impairs lipopolysaccharide-triggered DC maturation through the restoration of transcription factor E2F1 signaling, which is normally downregulated during DC maturation. VacA action would result in a sustained immature state of DCs with high endocytosis, and low migratory, capacity (Kim et al. 2011). Moreover, VacA is the virulence factor involved (through a yet unclear channel-independent mechanism) in *H. pylori*-induced reprogramming of murine DCs, both in vivo and in vitro, toward a tolerogenic phenotype which in turn promotes the preferential differentiation of naïve T cells into regulatory T cells (Oertli et al. 2013). This may result not only in persistence of bacterial infection but also in cross-protection against chronic inflammatory and autoimmune diseases.

Effects on Lymphocyte Activation and Proliferation

VacA impairs activation and proliferation of T and B lymphocytes, the major effectors of the adaptive immune response.

In a close dependence on its channel-forming activity, VacA efficiently blocks proliferation of T cells by inducing a G1/S cell cycle arrest by interfering with the T cell receptor/interleukin (IL)-2 signaling pathway at the level of the Ca^{2+}-calmodulin-dependent phosphatase calcineurin (Gebert et al. 2003). This leads to an impaired dephosphorylation of the transcription factor NFAT (nuclear factor of activated T cells) whose nuclear translocation is thus abrogated, resulting in downregulation of the expression of both IL-2 and its membrane receptor (Boncristiano et al. 2003; Gebert et al. 2003; Sundrud et al. 2004).

Recent data by Kern et al. (2015) might have finally mechanistically explained how VacA may inhibit calcineurin activity. After reaching the ER, VacA would interfere with STIM1, a protein that localizes to the ER membrane and senses the Ca^{2+} concentration in the ER lumen. Especially in T cells, STIM1 is important in store-operated calcium entry (SOCE), the Ca^{2+} influx into the cytosol from the extracellular milieu caused by the depletion of intracellular Ca^{2+} stores such as the ER. SOCE-induced high intracellular Ca^{2+} levels are required for calcineurin activity. In this respect, VacA would impair STIM1 movement towards the plasma membrane-localized SOCE channel protein ORAI1 during the process of calcium store depletion; this would result in SOCE inhibition and, in turn, in calcineurin inhibition (Kern et al. 2015).

In addition, VacA impairs T cell activation through a channel-independent mechanism that involves activation of intracellular signaling through the mitogen-activated protein kinases MKK3/6 and p38, as well as the Rac-specific nucleotide exchange factor, Vav (Boncristiano et al. 2003). Nevertheless, in primary human T lymphocytes, VacA inhibits activation-induced cell proliferation also through a mechanism dependent on its channel-forming activity leading to mitochondrial alterations (i.e., depolarization and ATP depletion), but independent of NFAT activation or IL-2 secretion (Torres et al. 2007).

Proinflammatory Activities and Altered Cell Signaling

VacA activates proinflammatory responses in the gastric mucosa and thus contributes to *H. pylori*-induced gastric inflammation (reviewed in Backert and Tegtmeyer 2010; Boquet and Ricci 2012; Ricci et al. 2015; Foegeding et al. 2016). Independently of its channel-forming activity and internalization, in AZ-521 cells VacA activates the mitogen-activated protein kinases (MAPKs) p38 and Erk-1/2 as well as the activating transcription factor 2 (ATF-2) signaling pathway. VacA induces activation of p38 in several other types of cells. In U937 macrophage-like cells, VacA increases IL-8 production by activation of p38 via intracellular Ca^{2+} release, leading to activation of the transcription factors ATF-2, cAMP response element binding (CREB) protein, and nuclear transcription factor kappa B (NF-kB). Worth

noting, VacA induces proinflammatory NF-kB also in Jurkat T cells. VacA thus exerts two opposite activities on T cells, immunosuppression and pro-inflammatory effects. Moreover, VacA stimulates the production of pro-inflammatory enzyme cyclooxygenase 2 (COX-2) in cultured epithelial cells and in human neutrophils and macrophages. COX-2 expression is induced by VacA via the p38 MAPK/ATF-2 cascade and results in increased production of prostaglandin E_2. However, VacA upregulates COX-2 also through activation of the epidermal growth factor receptor (EGFR); this in turn leads to increased expression of vascular endothelial growth factor.

VacA causes degranulation of mast cells and stimulates their Ca^{2+}-dependent production of pro-inflammatory cytokines. Independently of its channel-forming activity, VacA induces a rapid change in cytosolic Ca^{2+} concentrations in mast cells (de Bernard et al. 2005).

VacA may activate the β-catenin signaling pathway, which plays an important role in *H. pylori*-induced carcinogenesis. In the AZ-521 cell line, independently of its channel-forming activity and internalization, VacA activates the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathway, resulting in phosphorylation and inhibition of glycogen synthase kinase-3β (GSK3β), followed by release of β-catenin from the GSK3β/β-catenin complex and subsequent translocation of β-catenin to the nucleus (Nakayama et al. 2009).

Increase in Transepithelial Permeability

In polarized epithelial cell monolayers, through a yet uncharacterized mechanism dependent on its channel-forming activity, VacA induces a decrease in the transepithelial electric resistance which is associated to an increase in the paracellular permeability to ions (such as Fe^{3+} and Ni^{2+}) and molecules with molecular weight <350-400 daltons (Papini et al. 1998). This VacA-dependent increase in paracellular permeability to small molecules and ions may serve in vivo to provide *H. pylori* with nutrients favoring its growth. Probably through its channel-forming activity at plasma membrane level, VacA also increases plasma membrane permeability to small molecules such as urea and bicarbonate (reviewed by Foegeding et al. 2016; Ricci et al. 2015). Nevertheless, the biological relevance of such effects in *H. pylori* infection in vivo is still unclear.

Inhibition of Gastric Acid Secretion

H. pylori-colonized patients may exhibit hypochlorhydria. VacA can inhibit gastric acid secretion by preventing the recruitment of proton pump-containing tubulovesicles to the apical membrane of gastric parietal cells. VacA disrupts apical membrane-cytoskeletal interaction in gastric parietal cells in vitro by acting at the plasma membrane and causing an influx of extracellular Ca^{2+}, followed by activation

of calpain and subsequent proteolysis of ezrin. This in turn causes disruption of the radial arrangement of actin filaments in apical microvilli (Wang et al. 2008).

Functional Interplay with Other Virulence Factors from *H. pylori*

Mounting evidence suggests the existence of a functional relationship between VacA toxin and the bacterial oncoprotein CagA, encoded by the chromosomal pathogenicity island *cag* together with a type IV secretion system that injects CagA into host cells (Backert and Tegtmeyer 2010; Boquet and Ricci 2012; Foegeding et al. 2016; Ricci et al. 2015). Although CagA activates the transcription factor NFAT in cultured gastric epithelial cells, VacA inhibits it. While CagA decreases VacA-induced vacuolation, VacA reduces CagA-induced unique elongation of cultured cells (i.e., the so-called hummingbird phenotype). VacA downregulates the effects of CagA on epithelial cells by interfering with EGFR activation and endocytosis. CagA counteracts VacA apoptotic activity, impairing VacA internalization and intracellular trafficking, as well as stimulating antiapoptotic gene expression. After its entry in gastric epithelial cells, CagA is rapidly degraded by VacA-induced autophagy. However, VacA-induced autophagy of CagA is suppressed in gastric cancer stem-like cells expressing the cell surface marker CD44v9, apparently because these cells are highly resistant to ROS generation (Tsugawa et al. 2012). In these cells, CagA could thus exert a sustained carcinogenic action. In polarized epithelial cell monolayers, VacA and CagA may act in concert to provide specific nutrients such as iron to *H. pylori* attached to the apical cell pole (Tan et al. 2011). Such cooperative action between CagA and VacA could allow *H. pylori* to use the apical surface of the human gastric epithelium as a replicative niche.

The functional interplay between VacA and CagA is an intriguing strategy to achieve the best bacterial fitness with the hostile gastric environment, avoiding excessive cell damage and optimizing bacterial growth. This might explain the almost constant association of active VacA with CagA in highly pathogenic *H. pylori* (i.e., the so-called type I strains), even though the respective genes are distant on the chromosome and their expression levels are not mutually related. This relationship is an additional example of the emerging concept that to improve bacterial colonization and propagation within the host, virulence factors can regulate each other functioning together or antagonizing each other (Ricci et al. 2015; Shames and Finlay 2012).

Conclusion and Future Directions

Compared to any other bacterial protein toxin known, VacA exhibits unique structural and mechanistic properties. Nevertheless, it is still lacking a definitive description of the molecular mechanisms through which VacA interacts with and affects its target cells. Many important questions remain to be answered and a few

discrepancies in the data are emerging from a comparative analysis of the literature. Although the in vitro immunomodulatory activity of VacA has already suggested the use of this toxin as a therapeutic tool in allergen-induced asthma (Engler et al. 2014), it is hotly debated how and to what extent in vitro-derived results apply to in vivo human pathogenesis. Considerable controversies exist about how VacA causes cell death, forms channels in the mitochondria, affects autophagy, and causes channel-independent effects. Thus, much work remains to be done for a complete understanding of the pathobiological role of VacA in the overall strategy of *H. pylori* to colonize the human stomach. The recent finding by Kern et al. (2015) that VacA also reaches the ER and Golgi apparatus, identifying these cell compartments as novel VacA targets, opens new avenues in the study of intracellular trafficking pathway(s) and action mechanisms of VacA. The interaction of VacA with its target cells will thus continue to be a fascinating and rewarding subject for future studies.

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Part VI

Listeria Toxins

Role of *Listeria monocytogenes* Exotoxins in Virulence

16

Juan José Quereda, Pascale Cossart, and Javier Pizarro-Cerdá

Abstract

Listeria monocytogenes is an opportunistic intracellular bacterium responsible for the disease listeriosis. This review will update the knowledge on the four exotoxins secreted by this intracellular pathogen: the cholesterol-dependent cytolysin listeriolysin O (LLO), phosphatidylinositol-specific phospholipase C (PlcA), broad-range phospholipase C (PlcB), and hemolysin listeriolysin S (LLS). Each one of these exotoxins has evolved to perform specific and important functions in the extracellular or intracellular environment during the life cycle of *L. monocytogenes*. LLO, PlcA, and PlcB were discovered decades ago; however, recent studies are revisiting their functions and revealing new, previously unexpected insights. In the same line, LLS was discovered almost a decade ago, but it was recently deciphered that it is not only a toxin for eukaryotic cells but also a bacteriocin targeting bacteria closely related to *L. monocytogenes*. These latest findings, together with the knowledge generated during the history of listeriology, will be discussed in the light of their impact on the infectious process.

Keywords

Listeria monocytogenes • Exotoxins • Listeriolysin O (LLO) • Phosphatidylinositol-specific phospholipase C (PlcA) • Broad-range phospholipase C (PlcB) • Listeriolysin S (LLS)

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Contents

Introduction

Listeria monocytogenes was discovered by E.G.D. Murray in 1926 during the investigation of an epidemic disease of rabbits which induced an extreme monocytosis on the blood, receiving firstly the name *Bacterium monocytogenes* (Murray et al. 1926). J.H.H. Pirie identified the same microorganism in 1927 from the liver of gerbils in South Africa, and the name *Listerella hepatolytica* was adopted in honor to the surgeon J. Lister. Finally, the bacterium was renamed *Listeria monocytogenes*. This Gram-positive facultative intracellular bacterium infects animals and humans and is able to cross the intestinal barrier, the blood–brain barrier, and the fetoplacental barrier. As a consequence, *L. monocytogenes* can cause self-limiting gastroenteritis in healthy individuals as well as meningitis and meningoencephalitis in immunocompromised individuals, and abortions in pregnant women (Cossart 2011). Listeriosis cases are sporadic, although epidemics can occur with a high mortality rate (20–30%) and neurological sequelae in at-risk populations (Cossart 2011). *L. monocytogenes* can be found in nature, including vegetation, water, and soil, and can adopt a planktonic life or form biofilms (Cossart 2011). This bacterium can grow at 4 °C, at extreme pH, and in high salt concentration, ready-to-eat food products being usually involved in listeriosis outbreaks reported worldwide (Cossart 2011).

After ingestion of highly contaminated food, *L. monocytogenes* reaches the intestine where it competes with the intestinal microbiota to survive. From the intestinal lumen, this bacterium is able to cross the intestine invading phagocytic and non-phagocytic cells and then pass from primarily infected cells to neighboring cells, disseminating within tissues without being exposed to antimicrobial molecules and phagocytes of the immune system in the extracellular environment (Stavru et al. 2011).

The intracellular life cycle is critical for *L. monocytogenes* pathogenesis since strains that are incapable of infecting host cells are not able to cause disease (Seveau

2014). *L. monocytogenes* has developed an elaborated arsenal of toxins to successfully colonize the intestine, invade eukaryotic cells, escape from the internalization vacuole, resist autophagic killing, and multiply and spread from cell to cell (Cossart 2011; Stavru et al. 2011). Many *L. monocytogenes* virulence factors are regulated by PrfA and SigB, although small RNAs, antisense RNAs, and riboswitches play an important role during the intracellular lifestyle (Cossart 2011; Quereda et al. 2014). Epidemic *L. monocytogenes* strains possess three pathogenicity islands, LIPI-1, LIPI-3, and LIPI-4. LIPI-1 is regulated by the transcriptional factor PrfA and encodes for three toxins: the cholesterol-dependent cytolysin listeriolysin O (LLO), a phosphatidylinositol-specific phospholipase C (PlcA), and a broad-range phospholipase C (PlcB). LIPI-1 also codes for ActA which plays an important role in actin polymerization, for Mpl which is a metalloprotease associated with PlcB activation, and for PrfA itself (Cossart 2011). The internalin A/internalin B locus encodes for two surface molecules, which respectively bind E-cadherin and the receptor tyrosine kinase Met in the eukaryotic cell to mediate entry (Cossart 2011). LIPI-3 contains listeriolysin S (LLS), a posttranslational modified peptide that exhibits properties of both bacteriocins and hemolytic–cytotoxic factors (Cotter et al. 2008; Quereda et al. 2016). LIPI-4 encodes for a cellobiose PTS system necessary for central nervous system infection (Maury et al. 2016). This book chapter reviews the literature on the exotoxins (LLO, PlcA, PlcB, and LLS) secreted by *L. monocytogenes* and their contribution to the infectious process.

Listeriolysin O

Listeriolysin O is a pore-forming toxin encoded by the *hly* gene (1590 bp) that is transcriptionally regulated by PrfA, the master virulence transcriptional regulator of *L. monocytogenes* (Fig. 1a). This transcriptional factor belongs to the cAMP receptor protein (Crp)/fumarate nitrate reductase regulator (Fnr) family of bacterial transcription factors, and positively controls the transcription of the LIPI-1 and the internalin A/internalin B locus virulence genes of *L. monocytogenes* (Cossart 2011; Seveau 2014). PrfA is under the control of a 5′-UTR thermosensor which allows increased translation at 37 °C when *L. monocytogenes* is inside animal hosts (Cossart 2011; Seveau 2014). Additionally, host cell-derived glutathione contributes towards activating PrfA, which finally leads to higher transcription of virulence genes expression when this bacterium is inside eukaryotic cells (Reniere et al. 2015). *hly* codes for a preprotein of 529 residues with an amino-terminal secretion signal sequence, that after secretion and cleavage releases a mature protein of 504 residues (56kd) (Seveau 2014). LLO is secreted by the general secretory pathway. *hly* is also present in the animal pathogen *Listeria ivanovii*, while it is absent in nonpathogenic *Listeria* species (with the exception of *L. seeligeri*). An epidemiological and comparative genomics study showed that *L. monocytogenes* clonal complexes CC1, CC2, CC4, and CC6 are strongly associated with human clinical cases, and interestingly all of them have a complete *hly* gene, highlighting the importance of LLO for infection (Maury et al. 2016).

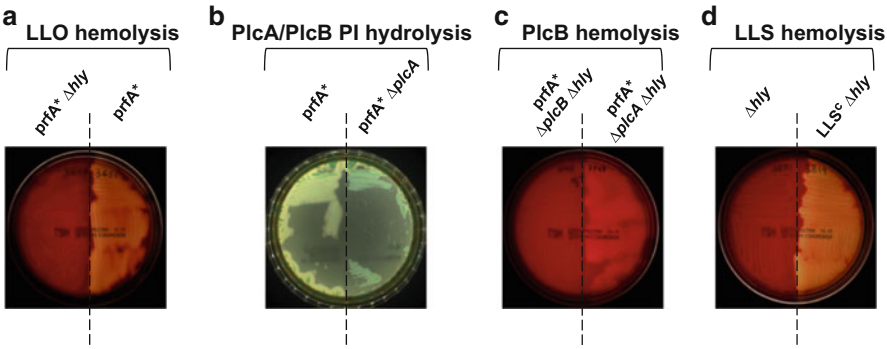

Fig. 1 Lytic activities of *L. monocytogenes* toxins. Hemolytic activities in horse blood agar of LLO (**a**), PlcB (**c**), and LLS (**d**), as well as phosphatidylinositol (*PI*) hydrolysis in ALOA agar of PlcA and PlcB (**b**). Note the powerful hemolysis produced by LLO and LLS (**a** and **d**). Since LLS is not produced in vitro, a synthetic strain that constitutively produces LLS (under the control of the pHELP promoter) was generated in a Δ*hly* background (LLS^c Δ*hly*) (**d**). PlcB is less hemolytic than LLO or LLS (**c**) while PlcA PI hydrolytic activity is higher than that of PlcB (**b**). *L. monocytogenes* strains in plates **a**, **b,** and **d** were streaked and cultured at 37 °C for ≈30 h. *L. monocytogenes* strains in plate **c** were streaked and cultured in anaerobiosis at 37 °C for ≈30 h

LLO belongs to the cholesterol-dependent cytolysin (CDC) family which includes other toxins, such as streptolysin O from *Streptococcus pyogenes*, pneumolysin from *Streptococcus pneumoniae*, suilysin produced by *Streptococcus suis*, intermedilysin from *Streptococcus intermedius*, lectinolysin produced by *Streptococcus mitis*, perfringolysin O from *C. perfringens*, and anthrolysin O synthesized by *B. anthracis* (Schnupf and Portnoy 2007). LLO monomers are secreted as water-soluble monomers that bind to cholesterol-rich regions of eukaryotic membranes, oligomerize, and form large pores of up to 35 nm in diameter consisting of 30–50 monomers (Peraro and van der Goot 2016). Remarkably, pore-dependent membrane damage is reversible. Bacterial membranes lack sterols and are therefore protected from the cytolytic activity of CDCs (Schnupf and Portnoy 2007). Although no CDC has been crystallized in the pore configuration, oligomerization of CDCs seems to follow a sequential accumulation of monomers or multimers. Nevertheless, oligomerization can fail to occur entirely, resulting in the formation of arc-shaped oligomers, faced on the opposite side by a free edge of the lipid membrane that can nevertheless be active pores as confirmed for suilysin (Peraro and van der Goot 2016). A recent study showed by electron microscopy that LLO full-circled pores on the erythrocyte ghost membranes differ in shape, finding occasionally ring arcs, incomplete rings, and slit-shaped structures (Koster et al. 2014). Other studies also showed that LLO can create pores of different size, confirming that diverse-sized pores exist upon infection (Hamon et al. 2012).

LLO activity is regulated by pH due to an acidic triad that triggers a premature unfolding of LLO at neutral pH and pore formation at acidic pH (Hamon et al. 2012). A functional difference between LLO and the rest of CDC toxins is that LLO is

produced by an intracellular pathogen. Thus, LLO activity is increased in acidic environments, such as the phagosomes containing *L. monocytogenes*. Neutral pH causes LLO aggregation and degradation, although it is protected from denaturation and continues to be active if bound to a lipid bilayer. LLO denaturation is slow since several minutes at 37 °C and pH 7.4 in physiological buffer are necessary for its inactivation. As a consequence, LLO secreted by extracellular bacteria can create pores in membranes and affect cellular processes of distant eukaryotic cells in physiological conditions (Seveau 2014). CDCs trigger hemolysis in their reduced state, whereas they are inactive when oxidized. Accordingly, the cellular enzyme gamma-interferon-inducible lysosomal thiol reductase (GILT) present on lysosomes of macrophages contributes to activate LLO in cells (Seveau 2014; Singh et al. 2008) (see below).

Effects of LLO in the Host Organism

Organism Level

Early LLO toxicity studies determined that ≈0.8 μg per mouse was the LD_{50} of LLO administered by intravenous injection in ICR female Swiss mice (Geoffroy et al. 1987). Mice died with convulsions and opisthotonos within 1–2 min after toxin administration. When injected through the intraperitoneal route, the LD_{50} was ≈1.7 μg, and mice died several hours later. LLO is essential for virulence of *L. monocytogenes* in intravenous and oral mouse infection models, as revealed by several logs of reduction of bacterial growth in the spleen and liver of infected animals (Cossart et al. 1989; Lecuit et al. 2007). No mortality was observed after intradermal inoculation of up to 5 μg of LLO (Geoffroy et al. 1987), although it induced a rapid inflammatory response (30 min) mediated by polymorphonuclear cells (Geoffroy et al. 1987). LLO produced during infections induces the production of anti-listeriolysin O antibodies, which have been used for serodiagnosis of human listeriosis (Berche et al. 1990).

Intestine Level

The natural route of *L. monocytogenes* infection is ingestion of contaminated food. Once this bacterium reaches the intestine, it crosses the intestinal barrier through two mechanisms: (1) across ileal Peyer's patches via M cells where InlB plays an important role and (2) upon interaction between *L. monocytogenes* surface protein InlA and E-cadherin, luminally accessible around goblet cells and extruding enterocytes (Nikitas et al. 2011). *L. monocytogenes* is rapidly transcytosed in a vacuole across enterocytes and egresses from them to the lamina propria by exocytosis in a LLO-independent manner (Nikitas et al. 2011). LLO induces chloride secretion and perturbs epithelial barrier function as assessed by transepithelial resistance in HT-29/B6 human colon cells (Richter et al. 2009). Furthermore, LLO is the main determinant of the intestinal transcriptional response to lineage II *L. monocytogenes* infection via the oral route. Histological analyses showed that *L. monocytogenes* Δ*hly* mutants are able to invade ileum enterocytes positioned at

the tips of intestinal villi and accumulate at the lamina propria, although no leukocytic infiltrates are detected in it while present in wild-type infected mice (Lecuit et al. 2007).

Liver, Spleen, and Immune System Levels

After crossing the intestinal barrier, *L. monocytogenes* reaches the liver, spleen, brain, and placenta via the lymph nodes and the blood. *L. monocytogenes* distributes among the liver parenchymal cell and non-parenchymal cell populations in the mouse liver (Cossart and Toledo-Arana 2008). It has been demonstrated that secreted LLO perforates the host cell plasma membrane as a strategy to enhance the internalization of *L. monocytogenes* into human HepG2 hepatocytes. LLO-induced bacterial entry into HepG2 cells occurs upon LLO pore formation in a dynamin-/F-actin-dependent and microtubule-/clathrin-independent way (Seveau 2014; Vadia et al. 2011). Hepatic apoptosis occurs 24 h postinfection as shown by the detection of TUNEL-positive infected hepatocytes with pyknotic nuclei (Carrero et al. 2004).

Splenic white pulp apoptosis is also observed after *L. monocytogenes* infection, and although apoptotic splenic lymphocytes are not infected with *L. monocytogenes*, extracellular bacteria are detected near dying cells (Carrero et al. 2004). Purified LLO produces caspase activation and DNA fragmentation, accompanied by phosphatidylserine exposure and loss of mitochondrial membrane potential 1 hour after treatment of in vitro-cultured T cells and lymph node lymphocytes (Carrero et al. 2004). Apoptosis of lymphocytes decreases host immunity and facilitates *L. monocytogenes* proliferation. Besides apoptosis, LLO influences the inflammatory and immune response of the host through different mechanisms. Firstly, LLO activates Toll-like receptor 4, which subsequently controls the inflammatory response. Secondly, LLO-mediated vacuolar rupture releases *L. monocytogenes* into the host cytosol, activating NOD-like receptors and the inflammasome. Thirdly, LLO perforation of epithelial cell plasma membranes induces NF-kB activation, surface expression of adhesion molecules, and the production of pro-inflammatory cytokines (Seveau 2014). Finally, LLO is the principal source of T cell epitopes during *L. monocytogenes* infection (containing two CD4+ and one CD8+ immunodominant epitopes) (Seveau 2014), but LLO also triggers the expression of negative regulators of TCR signaling which finally affects the proliferation of CD4+ T cells and silences the host adaptive immune responses (Hamon et al. 2012; Gekara et al. 2010).

Intracellular LLO Activities

Vacuolar Escape

In the intracellular environment, LLO function is tightly restricted to the endocytic vacuoles since its activity outside these compartments can damage host organelles and the plasma membrane, leading to cell death and bacterial exposure to the extracellular milieu and immune system effectors. *L. monocytogenes* mutants for which LLO activity is not compartmentalized to vacuoles are cytotoxic and less

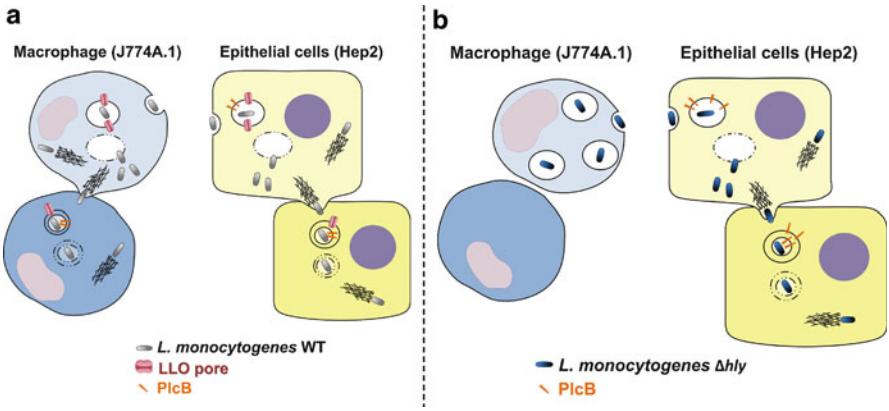

Fig. 2 Graphic representation of the *L. monocytogenes* toxin activities that mediate escape from the intracellular vacuoles (**a**: wild-type *L. monocytogenes*; **b**: Δ*hly L. monocytogenes*). *L. monocytogenes* can be passively internalized by phagocytosis in macrophages or induce its own uptake by receptor-mediated phagocytosis in non-phagocytic epithelial cells. Secreted LLO and PlcB disrupt the phagosomal membrane to allow bacterial access to the cytosol. Once in the cytosol, *L. monocytogenes* replicates and recruits the host actin-polymerization machinery to propel the bacterium in the cytosol and form intercellular protrusions which allow spreading to neighboring cells. In the recipient adjacent cells, *L. monocytogenes* is entrapped in a double-membrane vacuole where PlcA or PlcB cause the dissolution of the inner membrane and LLO disrupts the outer vacuolar membrane (originated from the recipient host cell). Note that LLO is absolutely necessary to escape from vacuoles in macrophages (*right panel*) and that in epithelial cells, PlcB is sufficient for vacuolar escape from the primary phagosome and from secondary phagosomes containing a double membrane generated from cell-to-cell spread. We thank Servier Medical Art (http://www.servier.com/Powerpoint-image-bank) for providing drawings

virulent because they lyse their replicative niche in eukaryotic host cells and cannot avoid extracellular defenses of the host (Schnupf and Portnoy 2007). Ubiquitination and proteasomal degradation, as well as pH sensitivity restricts the activity of LLO in the host cytoplasm (Hamon et al. 2012) Intriguingly, an LLO L461T mutant with hemolytic activity at neutral pH needs acidification of the vacuole for *L. monocytogenes* cytoplasmic escape, suggesting that the acidification requirement for phagosomal rupture does not seem to be caused by the higher hemolytic potential of LLO at an acidic pH (Glomski et al. 2002). Listerial escape from primary and secondary host vacuoles is a multistep process characterized by perforation (mainly dependent on LLO) followed by rupture (Fig. 2). The exact mechanism by which LLO disrupts vacuoles remains unclear since the osmotic pressure inside of the vacuole and in the host cytoplasm is similar, excluding a "lysis"-like mechanism for vacuolar escape (Schnupf and Portnoy 2007; Hamon et al. 2012).

LLO is crucial for *L. monocytogenes* escape from the primary endocytic vacuole in phagocytic and non-phagocytic cells, i.e., human epithelial Caco-2 cells, murine macrophages (BMDM and J774), and fibroblasts (CL.7) (Fig. 2). Furthermore, LLO is sufficient to allow escape of *Bacillus subtilis*, a nonpathogen soil bacterium, from J774 macrophage phagosomes (Schnupf and Portnoy 2007). However, *L. monocytogenes*

LLO deletion mutants are able to escape from internalization vacuoles in non-phagocytic human cell lines such as HeLa, HepG2, Henle 407, HEp-2, HCT116, HEK-293, and also dendritic cells, where PlcB plays a fundamental role in vacuolar rupture (see below) (Fig. 2). Lastly, activated macrophages are less susceptible to *L. monocytogenes*-induced phagosomal degradation. Vacuolar rupture and access to the host cytosol occurs 15–30 min after infection of epithelial cells and macrophages. LLO produced by *L. monocytogenes* slows down the maturation of the vacuole as shown by the delayed recruitment of the late endosomal and lysosomal marker (LAMP)-1 (Henry et al. 2006). LLO also allows *L. monocytogenes* replication in macrophage LAMP-1 (+) vacuoles (termed spacious *Listeria*-containing phagosomes) during persistent infection of severe combined immunodeficient (SCID) mice (Birmingham et al. 2008). Furthermore, extracellular LLO modulates the composition of the host cell endosomal network that will fuse with the *Listeria* phagosome. Particularly, extracellular LLO and PI-PLC regulate the translocation of PKC beta II on J774 early endosomes which finally controls *L. monocytogenes* escape from the phagosome (Seveau 2014).

Although the precise molecular mechanisms that control *L. monocytogenes* vacuolar escape in different species and cell types remain unknown, it is clear that vacuolar rupture is not only dependent on bacterial exotoxins but also on eukaryotic cell factors, i.e., calpain, gamma-interferon-inducible lysosomal thiol reductase (GILT), and cystic fibrosis transmembrane conductance regulator (CFTR) (Seveau 2014; Hamon et al. 2012). Calpain is a cytosolic cysteine protease co-opted to facilitate *L. monocytogenes* escape from the phagosome. As mentioned above, GILT is a thiol reductase enriched in the phagosomes of macrophages which activates LLO by maintaining it in a reduced state. As a consequence, GILT-activated LLO facilitates vacuolar rupture in in vitro and in vivo models of infection (Singh et al. 2008). Another cell factor that controls the activity of LLO is the CFTR. CFTR increases phagosomal chloride concentration, potentiating LLO oligomerization, pore formation, and vacuole escape. CFTR inhibition suppresses *L. monocytogenes* vacuolar rupture in culture and decreases systemic infection in mice (Radtke et al. 2011). Calpain, GILT, and CFTR are highly expressed in macrophages, but their contribution to virulence needs to be assessed in other cell types. Interestingly, human α-defensin HNP-1 blocks LLO-dependent perforation of macrophage membranes and the release of LLO from the bacteria, enabling macrophage control of *L. monocytogenes* phagosomal escape and intracellular growth (Arnett et al. 2011).

Once *L. monocytogenes* has escaped from the internalization vacuole, it replicates and moves intracellularly using a cellular actin-related machinery. The *L. monocytogenes* surface protein ActA mimics the eukaryotic WASP family proteins by recruiting the Arp 2/3 complex and promoting actin polymerization, which finally propels the bacteria inside the cytosol and facilitates cell-to-cell spread (Cossart 2011). Bacteria found in infected neighboring cells are located in a two-membrane vacuole originated from the donor and recipient cells, from which *L. monocytogenes* needs to escape to continue its infectious cycle (Fig. 2). In primary murine macrophages, the bacterial phospholipases (either PlcA or PlcB) cause

rupture of the inner membrane of the spreading vacuole, although they are not sufficient for disruption of the outer membrane (see below). In this scenario, LLO is critical for cell-to-cell spread where it is mainly involved in the disruption of the vacuolar outer membrane originated from the recipient host cell (Alberti-Segui et al. 2007). Furthermore, LLO is unnecessary for cell-to-cell spread between U937 human macrophages and human HEp-2 epithelial cells (a cell line permissive for vacuolar escape of LLO-negative bacteria), where PlcA and PlcB mediate escape from double-membrane spreading vacuoles as described below (Alberti-Segui et al. 2007) (Fig. 2).

Activation of Autophagy

Autophagy is a cytosolic process in eukaryotic cells that degrades and recycles (through a regulated mechanism) intracellular cargos, such as organelles or multiprotein complexes. Moreover, autophagy is an innate defense system for the elimination of microbial invaders. During this process, cellular or foreign material is targeted to double-membrane vacuoles that will fuse with lysosomes. Some intracellular pathogens like *Shigella flexneri* and *Salmonella enterica* induce autophagy, triggering protective innate immune and stress responses. LLO membrane damage activates autophagy both from the extracellular milieu and from the intracellular environment after vacuolar escape (Meyer-Morse et al. 2010; Birmingham et al. 2007) (Fig. 3). *L. monocytogenes* infection of macrophages activates the autophagy system in an LLO-dependent manner at 1 h post infection (p.i.) when bacteria are within vacuoles. At 4 h p.i., only 10% of intracellular bacteria colocalize with the autophagy marker LC3. The role of autophagy has variable effects depending on the cell type: it decreases infection of fibroblasts, but has no effect on infected bone marrow-derived macrophages. Autophagy markers and polyubiquitinated proteins are recruited on the *L. monocytogenes* surface and/or phagosome membranes after disruption, but actin-based motility as well as InlK expression and PlcA secretion counteract the effect of LLO on autophagy and facilitate colonization and replication in the eukaryotic niche (Hamon et al. 2012) (Fig. 3).

Control of the Microbicidal Nicotinamide Adenine Dinucleotide Phosphate (NADPH) Oxidase 2 (NOX2) of Host Cells

The phagocyte NOX2, an isoform of NADPH oxidase, produces reactive oxygen species (ROS) which play a fundamental role in immune responses. *L. monocytogenes* phospholipases C (PlcA and PlcB) activate the NADPH oxidase during infection of phagocytes (Lam et al. 2011). However, LLO suppresses ROS produced by NADPH oxidase by preventing its localization to phagosomes (Fig. 3). This LLO-related activity can be also performed by the cytolysin perfringolysin O, suggesting that other bacteria have evolved similar mechanisms to inhibit the NADPH oxidase and avoid killing (Lam et al. 2011). In vivo studies show that loss of NADPH oxidase activity in mice augments the replication of *L. monocytogenes* compared to wild-type animals (Lam et al. 2011). In vitro research indicates that NOX2 NADPH oxidase limits vacuolar escape of *L. monocytogenes* in macrophages (Hamon et al. 2012; Lam et al. 2011).

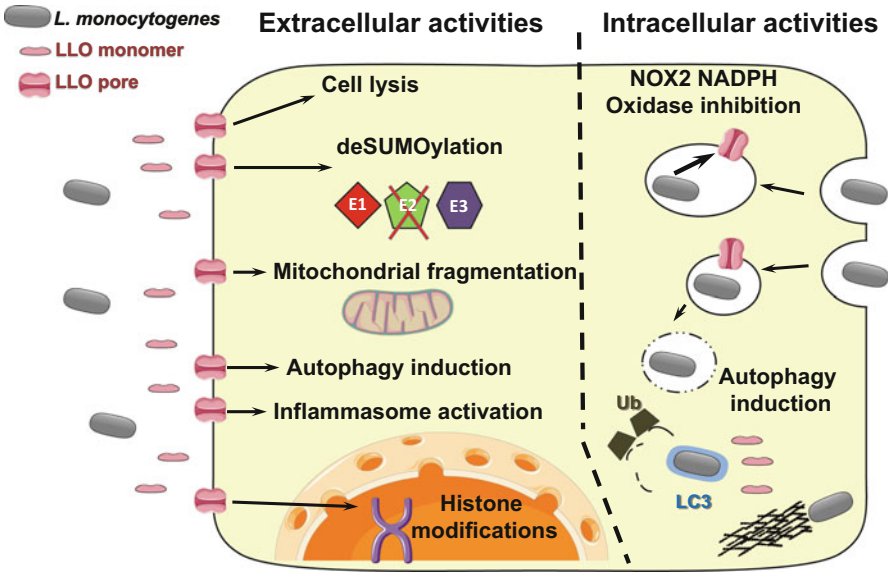

Fig. 3 Role of extracellular and intracellular LLO during infection. Extracellular LLO monomers bind cholesterol and assemble to form the transmembrane pore. Major roles associated with extracellular LLO include global deSUMOylation of host proteins by induced cleavage of the E2 enzyme Ubc9, mitochondrial fragmentation, and histone modifications, among others. Note that all the extracellular effects of LLO depicted require pore formation. Once *L. monocytogenes* is internalized in membrane-bound compartment, the secreted LLO prevents the NOX2 NADPH oxidase localization to phagosomes avoiding bacterial killing by reactive oxygen species during the respiratory burst. As shown in Fig. 2, LLO is critical for phagosomal disruption and *L. monocytogenes* translocation to the host cytoplasm. Additionally, intracytoplasmic LLO induces autophagy, leading to the recruitment of ubiquitin (Ub) to membrane remnants and of the marker LC3 around *L. monocytogenes* autophagosomes. We thank Servier Medical Art (http://www.servier.com/Powerpoint-image-bank) for providing drawings used

Extracellular LLO Activities

SUMOylation

SUMOylation is a reversible posttranslational modification in which proteins of the small ubiquitin-related modifier (SUMO) family are conjugated to proteins to regulate transcription, DNA repair, chromosome segregation, nuclear transport, intracellular transport, stress responses, protein stability, and defense against microbial pathogens among others (Hamon et al. 2012). *L. monocytogenes* infection of human epithelial cells decreases the levels of cellular SUMO-conjugated proteins in a LLO-induced pore-dependent manner (Fig. 3). Interestingly, the *L. monocytogenes* Δ*inlB* mutant (which is impaired in entry into HeLa cells) or noncytotoxic concentrations of LLO decrease the level of SUMO-conjugated proteins, indicating that this decrease is triggered by extracellular LLO. This effect is also shared by the cytolysins perfringolysin O and pneumolysin, and is based on the proteasome-independent degradation of Ubc9, the human E2 SUMO enzyme of the SUMOylation machinery.

Since no other E2 SUMO enzyme exists in the human genome, Ubc9 degradation leads to a general deSUMOylation of host proteins. Moreover, LLO triggers not only a global deSUMOylation event but also degradation of some SUMO-conjugated proteins promoting the infection capacity of *L. monocytogenes*. These in vitro effects of LLO are also observed in vivo since Ubc9 levels in livers of mice infected with *L. monocytogenes* showed a significant reduction at 48 h and 72 h after infection (Ribet et al. 2010).

Mitochondria and Endoplasmic Reticulum

Mitochondria are essential organelles that provide cellular ATP and biosynthetic intermediates. *L. monocytogenes* infection alters mitochondrial dynamics by causing transient mitochondrial network fragmentation through extracellular LLO-mediated calcium influx across the host cell plasma membrane (Fig. 3). The molecular consequence of this LLO effect is a unique dynamin-like protein 1 (Drp1) and optic atrophy protein 1 (Opa1)-independent mitochondrial fission mechanism, where the endoplasmic reticulum marks fragmentation regions. This mitochondrial fragmentation shuts down cellular bioenergetics and could be a strategy used by this bacterium to impair the cell capability to control the onset of infection (Stavru et al. 2013).

LLO produced by intracellular *L. monocytogenes* or secreted by extracellular bacteria injures the endoplasmic reticulum, causing a rapid swelling and activation of the unfolded protein response (UPR). The UPR has a defensive function at the early stages of infection, although continued endoplasmic reticulum (ER) stress favors macrophage death at later time points of infection (Hamon et al. 2012; Gekara et al. 2007).

Histones, DNA Damage Response, and Telomerase Reverse Transcriptase Alterations

Histones contribute to the packaging of DNA in the eukaryotic cell nuclei while retaining the properties of DNA for replication. Nucleosomes are composed of DNA and a histone octamer which consist of two copies each of the histones H2A, H2B, H3, and H4. Histone modifications control DNA accessibility in chromatin. LLO secreted extracellularly mediates a dramatic dephosphorylation of histone H3 and deacetylation of histone H4 during early phases of infection (Fig. 3). Other CDC toxins from extracellular pathogens like perfringolysin and pneumolysin induce similar histone modifications. The reduction of the levels of histone modifications leads to decreased transcriptional activity of some host genes, including key immunity genes (such as *cxcl2*, *dusp4*, or *egr1*) (Hamon et al. 2007). Further research has shown that the efflux of potassium from the eukaryotic cytoplasm is the signal that triggers the dephosphorylation of H3 (Hamon et al. 2012).

The DNA damage response (DDR) is an essential signaling pathway that senses and responds to DNA lesions caused, for example, by UV radiation or bacterial infection, in order to maintain cellular genetic integrity. *L. monocytogenes* induces LLO-mediated degradation of the principal DNA damage sensor, Mre11, blocking downstream signaling and dampening the DNA damage response which finally promotes bacterial replication (Samba-Louaka et al. 2014).

Telomeres are nucleoprotein complexes that protect the ends of chromosomes from degradation. Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of the human telomerase complex. hTERT is involved in telomere maintenance and cell physiology functions, independent of telomere elongation (i.e., mitochondrial functions). Calcium influx through the LLO pores causes a decrease in hTERT levels at early time points of infection of HeLa cells, which surprisingly is detrimental for *L. monocytogenes* replication (Samba-Louaka et al. 2012).

Phospholipases

L. monocytogenes expresses two phospholipases C, a phosphatidylinositol-specific phospholipase C (or PlcA encoded by *plcA*, 954 bp) and a broad-range phospholipase C (PlcB encoded by *plcB*, 870 bp). Both genes are transcriptionally regulated by the transcriptional activator PrfA. These phospholipases hydrolyze phospholipids and damage host membranes.

PlcA is a 33-kd secreted phospholipase that possesses a signal cleavage site between alanine 29 and tyrosine 30. This enzyme is specific for phosphatidylinositol (PI), although it is able to weakly hydrolyze glycosyl PI (GPI)-anchored eukaryotic membrane proteins, with a pH optimum range from 5.5 to 7.0 (Goldfine and Knob 1992; Mengaud et al. 1991) (Fig. 1b). From all known bacterial phosphatidylinositol-specific phospholipases C, PlcA is the only one that lacks a beta-strand, which in the case of *Bacillus cereus* forms contacts with the glycan linker of GPI anchors and presumably enhances its activity on GPI-anchored proteins. Interestingly, expression of *Bacillus cereus* phosphatidylinositol-specific phospholipase in *L. monocytogenes* decreases its virulence, suggesting that loss of the PlcA beta-strand and decrease in GPI cleavage evolved to increase virulence (Wei et al. 2005).

PlcB is a zinc-dependent metalloenzyme secreted as a 264-amino acid inactive proenzyme that is processed in the extracellular medium by proteolytic cleavage to produce the active 29–30-kDa PlcB form. PlcB is secreted as an inactive proenzyme to prevent degradation of the phospholipids contained in the bacterial membrane. The metalloprotease Mpl is encoded in the same operon as PlcB, and it is also a zinc-dependent metalloprotease. Mpl processes the PlcB proenzyme into its active form and controls its cell wall translocation (Vazquez-Boland et al. 2001). Additionally, there is a Mpl-independent activation pathway dependent on acidification of the

vacuolar compartment (Vazquez-Boland et al. 2001). PlcB has a broad-optimum pH range between 5.5 and 8.0. It hydrolyzes phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin. It weakly hydrolyzes phosphatidylinositol and possesses a weak calcium-independent hemolytic activity at 37 °C in guinea pig, horse, and human erythrocytes, but not in sheep red blood cells (Vazquez-Boland et al. 1992, 2001) (Fig. 1c).

plcA and *plcB* are present in pathogenic *Listeria* species (*L. monocytogenes* and *L. ivanovii*) and absent in nonpathogenic species (with the exception of *L. seeligeri*). A large-scale and systematic analysis integrating molecular epidemiological data, as well as comparative genomics, demonstrated that *plcA* and *plcB* are conserved in all the *L. monocytogenes* human clinical isolates (Maury et al. 2016).

Virulence at the Organism Level

Using the intravenous mouse model of infection, it has been shown that PlcA contributes minimally to *L. monocytogenes* virulence as reflected by a threefold increase in mouse LD_{50} for the Δ*PlcA* mutant, whereas PlcB impacts more in virulence since its deletion causes a 20-fold increase in LD50. However, the double mutant Δ*plcA*Δ*plcB* is severely impaired in virulence as demonstrated by a 500-fold increase in LD50. These results demonstrate an overlapping role between the two listerial phospholipases (Smith et al. 1995). PlcB plays an important role in the pathogenesis of meningoencephalitis in an intracerebral infection murine model. Mice infected via intracerebral route with the *L. monocytogenes* Δ*plcB* strain survive longer and have less intracerebral bacterial colony-forming unit (CFU) (≈1 log less), compared to mice infected with the wild-type bacteria. Furthermore, histopathology analyses reveal that the Δ*plcB* strain presents a significantly delayed intracerebral spread (Schluter et al. 1998).

The majority of in vivo experiments performed to evaluate the contribution of *L. monocytogenes* phospholipases to virulence have been performed by the intravenous route in the mouse model. Interestingly, removal of the N-terminal propeptide of PlcB produces a constitutively active PlcB that causes damage to host cell membranes and negatively reduces the virulence in an oral mouse model of infection (200-fold decrease compared with the wild type in a competitive infection assay) (Yeung et al. 2007). It is important to stress that listeriosis is a foodborne disease where the intestinal stage plays a critical role (Quereda et al. 2016), and it would be interesting to evaluate if these phospholipases have an important role during this stage.

Strikingly, purified PlcB is not toxic in mice by intravenous administration. Mice survived after receiving up to 25 μg i.v. (Vazquez-Boland et al. 2001; Geoffroy et al. 1991). No experiments have been performed to evaluate the toxicity of purified PlcA, although due to its low impact in virulence compared to PlcB, it could be speculated that its toxic effect could be negligible.

Activity at the Cellular Level

Vacuolar Escape

PlcB plays a key role in vacuolar escape and cell-to-cell spread, not only between cells of the same tissular origin, but also between macrophages and brain microvascular endothelial cells in vitro, as well as for spread in murine brain tissue as already mentioned (Alberti-Segui et al. 2007; Vazquez-Boland et al. 2001). PlcA slightly contributes to escape from primary phagosomes and secondary double-membrane vacuoles. As for virulence in mice experiments, PlcA and PlcB work synergistically to promote vacuolar dissolution in in vitro experiments (Alberti-Segui et al. 2007). In the absence of LLO, the role of phospholipases is more important, and it has been suggested that a slow vacuolar maturation facilitates phospholipases-mediated escape (Burrack et al. 2009). Importantly, in LLO-deficient strains of *L. monocytogenes*, PlcB is required for rupture of primary vacuoles in human epithelial cell lines (i.e., Henle 407, HEp-2, and HeLa) (Fig. 2). As mentioned above, the proposed model for cell-to-cell spread is that *L. monocytogenes* phospholipases participate in the dissolution of the inner membrane, while LLO is involved in the disruption of the outer membrane (Alberti-Segui et al. 2007) (Fig. 2). This model implies that during listeriosis, spreading of *L. monocytogenes* to distant organs could be dependent on the action of these two phospholipases and that cell-to-cell spread can still occur in cells in which LLO is not strictly necessary for vacuolar escape (Alberti-Segui et al. 2007).

Phospholipid Metabolism

Eukaryotic cell phospholipids are differently generated and distributed at various intracellular locations to regulate a variety of important cellular processes. Some signaling proteins are recruited specifically to membrane sites by direct interaction with phospholipids. Membrane-derived extracellular vesicles also possess phospholipids and regulate important physiological processes. Phospholipid hydrolysis by *L. monocytogenes* phospholipases generates products like diacylglycerol (DAG), ceramide, and inositol phosphates which have important roles in host signaling pathways (i.e., synthesis of cytokines, cell growth, and apoptosis). Both LLO and PI-PLC induce translocation of protein kinase C delta to the periphery of J774 cells, and translocation of PKC beta II to early endosomes (Seveau 2014). In this way, *L. monocytogenes* phospholipases could modulate the host response to facilitate its intracellular survival. LLO and PlcA exogenously added to HUVEC cells synergize to elicit phosphoinositide metabolism and diacylglycerol accumulation. It has been proposed that the pore created by LLO allows access of PlcA to the phosphatidylinositol in the inner monolayer of the plasma membrane. Similarly, this LLO–PlcA synergy was observed in neutrophils where these two *Listeria* toxins provoked phosphoinositide hydrolysis and respiratory burst (Sibelius et al. 1999).

Autophagy

PI3P is generated from PI by the Vps34/Beclin-1 complex and plays an important role in autophagic flux and autophagosome formation. Interestingly, PlcA cleaves PI

into diacylglycerol (DAG) and myo-inositol, which finally affects the PI available for PI3P synthesis. PlcA reduces the cellular levels of PI3P and LC3 autophagic flux causing pre-autophagosomal structure stalling and preventing efficient autophagic targeting of cytosolic *L. monocytogenes* (Birmingham et al. 2007; Tattoli et al. 2013). As mentioned above, *L. monocytogenes* possesses other mechanisms to subvert autophagy defense pathways apart from PlcA, including the bacterial surface proteins InlK and ActA (Cossart 2011).

Listeriolysin S

LLS is an hemolytic and cytotoxic factor encoded by the structural gene *llsA* (150 bp) (Cotter et al. 2008) (Figs. 1d and 4). This toxin was discovered in 2008 and belongs to a family of posttranslationally modified peptides, the streptolysin S-like peptides. In this family there are other toxins produced by Gram-positive pathogens such as S*treptococcus pyogenes* (streptolysin S), *Staphylococcus aureus* str. RF122 (stapholysin S), *and Clostridium botulinum* (clostridiolysin S) (Molloy et al. 2011). In silico analysis of this cluster demonstrated similarities with the clusters responsible for production of streptolysin S and of the bacteriocin microcin B17 (Clayton et al. 2011).

LLS knowledge is very limited due to its recent discovery (2008) and to its absence from the majority of the most frequently used *L. monocytogenes* laboratory

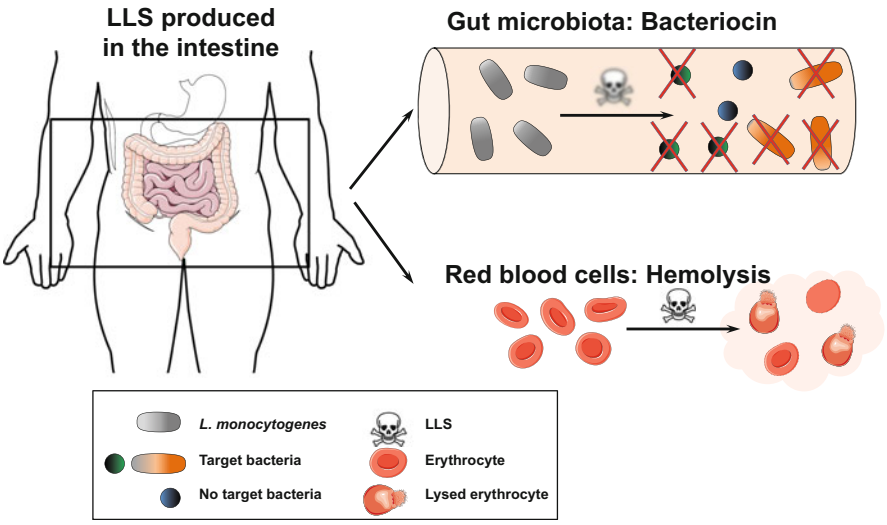

Fig. 4 LLS production is specifically triggered in the intestine of infected hosts. LLS is a bacteriocin that controls protective microorganisms from the gut microbiota and favors *L. monocytogenes* colonization of the intestine. LLS is additionally a potent hemolytic factor (see also Fig. 1). We thank Servier Medical Art (http://www.servier.com/Powerpoint-image-bank) for providing drawings used

strains (EGD-e, EGD, and 10403S) (Cotter et al. 2008). LLS cluster is only present in a subset of lineage I strains responsible for the majority of *L. monocytogenes* epidemics in humans and absent from lineage II and III *L. monocytogenes* strains (Cotter et al. 2008). It was recently shown that *L. monocytogenes* strains that contained LIPI-3 were epidemiologically associated with infections in humans (Maury et al. 2016).

llsA is located within a gene cluster-denominated LIPI-3 that also contains *llsX* (a gene specific of *L. monocytogenes* whose function remains elusive); *llsG* and *llsH* (encoding a putative ATP-binding cassette transport machinery); *llsB*, *llsY*, and *llsD* (these three genes are predicted to form a synthetase complex necessary for the posttranslationally modification of LLS); and *llsP*, a CAAX protease which is a putative immunity protein (Cotter et al. 2008). *llsG*, *llsH*, *llsX*, *llsB*, *llsY*, and *llsD* are essential for LLS hemolytic activity, while *llsP* has no effect on the toxin hemolytic phenotype (Clayton et al. 2011). LLS contains a putative Ala-Gly leader cleavage motif (amino acid 26) as well as a C-terminal core region with an extreme predominance of Cys, Ser and Thr residues which allow posttranslationally modifications resulting in the formation of a distinctive heterocyclic compound. Although the LLS posttranslational modifications are currently unknown, it is probable that like in other toxins of the same family, the Cys, Ser, and Thr residues are converted to thiazole, oxazole, and methyloxazole heterocycles, respectively (Molloy et al. 2011).

One study reported that the *llsA* promoter expression was negligible in *in vitro* laboratory conditions and only induced upon exposure to cumene hydrogen peroxide and hydrogen peroxide (Cotter et al. 2008). Another study also showed that *llsA* expression is negligible in *in vitro* classic laboratory conditions and that the promoter expression only can be observed in infected mice (Quereda et al. 2016). Strikingly, LLS expression was detected in the intestine while absent from the liver and spleen (despite higher CFU *Listeria* numbers can be detected in these two last organs) (Quereda et al. 2016). Hydrogen peroxide-mediated induction of the *llsA* promoter could not be reproduced in this latter study. The discrepancies between these two studies require further investigations.

LLS displays properties of both bacteriocins and hemolytic–cytotoxic factors. Bacteriocins are proteinaceus substances synthesized by bacteria that inhibit the growth of closely related bacteria, favoring the growth of the producing organism in various ecological niches. As a bacteriocin, LLS restricts the growth of other closely related Gram-positive bacteria like *Lactococcus lactis*, *Staphylococcus aureus*, and even *L. monocytogenes* from lineage II (EGD and 10403S) that lack the LLS operon. It has been proposed that in in vitro conditions, LLS hemolytic activity is only active in a bacteria-associated form but inactive in the culture supernatant. Addition of RNA core (ribonuclease-resistant fraction of yeast RNA) in an appropriate buffer containing ammonium acetate as a stabilizer in the culture medium results in hemolytic activity in the supernatant fraction. What remains unknown is if the RNA core acts as an inducer of LLS production or if it releases LLS from the cell wall of the bacteria (Cotter et al. 2008). Finally, liquid cocultures of *L. monocytogenes* constitutively producing LLS and target bacterial species showed

that secreted LLS could inhibit growth of the target species (Quereda et al. 2016). This implies that either LLS-bacteriocin activity is functional in the bacteria-free form against other target bacteria, or that LLS is transmitted upon contact between the LLS producer *L. monocytogenes* strain and the LLS-target strain.

When *L. monocytogenes* reaches the intestine after ingestion of contaminated food, LLS expression is triggered, and as a consequence, the host microbiota is altered (Fig. 4). In particular, LLS expression reduces the number of representatives of the genera *Allobaculum* and *Alloprevotella*. These two genera include species that produce butyric and acetic acid, respectively. These fatty acids negatively impact the ability of *L. monocytogenes* to grow and colonize the intestine. As a consequence, LLS expression in the intestine plays a critical role in *L. monocytogenes* survival within this organ, the first barrier before access to deeper host tissues. Importantly, *L. monocytogenes* lineage I strain F2365 (which contains LLS) colonizes better the intestine than lineage II strains EGD-e and 10403S (which lack LLS) in a mouse oral infection model (Quereda et al. 2016). The higher CFU numbers in the intestine allow the F2365 strain to better colonize deeper organs like the spleen or liver.

One study reported that LLS is also cytotoxic for human cell lines (like C2-Bbe), mouse cell lines (like J774 and CT26), as well as sheep erythrocytes (Cotter et al. 2008). The same study also showed that LLS improves the survival of *L. monocytogenes* in purified polymorphonuclear neutrophils, a crucial component of the immune system for the resolution of *Listeria* infections. Accordingly, LLS plays a role in the intraperitoneal mouse model of infection, where *L. monocytogenes* Δ*llsB* CFU numbers in the spleen and liver are approximately twofold lower compared with the wild-type strain (Cotter et al. 2008).

Conclusion and Future Directions

The exotoxin arsenal of *L. monocytogenes* plays a critical role in its adaptation to mammalian hosts and in particular to the intracellular life. The recent discovery of LLS and its bimodal activity on eukaryotic and prokaryotic membranes opens now new perspectives, not only on *L. monocytogenes* virulence but also on its environmental distribution: how the expression of these different exotoxins is regulated? Do these toxins, and in particular LLS, have a role in *L. monocytogenes* survival in nature? Does LLS cooperate with LLO, PlcA, and PlcB during the *L. monocytogenes* intracellular life cycle? How does LLS perforate red blood cells and what is its bactericidal mechanism in target bacterial species? Addressing all these questions will allow us to better understand the natural history of *L. monocytogenes* and in particular its molecular mechanisms to promote disease.

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Bacterial Pore-Forming Toxin in Macromolecule Delivery: Lessons Learned from Listeriolysin O

17

Manas Mandal

Abstract

Listeriolysin O (LLO) is a pore-forming hemolytic toxin, secreted by the Gram-positive pathogen *Listeria monocytogenes* (LM), responsible for bacterial phagosome escape into the cytosol of an infected host cell and spreading infection. The unique pore-forming ability of LLO has been widely studied to expand our understanding of the host–pathogen interaction, virulence factors, biochemical and signaling events, and modification of host cell membrane leading to pore formation. These elegant studies delineated a clever survival mechanism adopted by this pathogen, that can seemingly be exploited to deliver macromolecules in human antigen-presenting cells (APCs) targeting a specific pathway of antigen processing and presentation to a subset of T lymphocytes. Cytotoxic T lymphocytes (CTLs) protect us against extracellular pathogen infection and cancer by recognizing peptide antigens derived from cytosolic processing and the presentation pathway. It is a daunting task to deliver biologic macromolecules, such as protein or nucleic acid antigen, to prime a CTL response due to the membrane impermeable nature of these macromolecules. In this chapter, different areas of biotechnology applications of LLO as a pH-sensitive, membrane pore-forming hemolysin will be reviewed in the context of novel vaccine delivery strategies to intracellular pathogens, tumor antigens, or DNA.

Keywords

LLO • Pore Formation • Cytosolic Processing • CTL • Vaccine

Dedication: Author wishes to dedicate this chapter in loving memory of his late father, Dr. D. D. Mandal, who remains his life-long inspiration in science.

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Contents

Introduction

Listeria monocytogenes, a Gram-positive facultative intracellular pathogen, is responsible for serious food-borne infections and infections in immunocompromised individuals that include pregnant women (Cossart and Toledo-Arana 2008). *Listeria* infection can cause gastroenteritis in healthy individuals, meningitis in immunocompromised individuals, and abortion in pregnant women. The opportunistic pathogen *Listeria* is capable of replicating in host cells, exploiting its ability to cross the host membrane intestinal, blood–brain, and materno–fetal barriers that causes the infection and disease progression (Cossart and Toledo-Arana 2008; Quereda et al. 2017; Chakraborty 1999). *L. monocytogenes* enters the host normally through the gut following consumption of *Listeria*-contaminated food. The bacteria are subsequently found in regional lymph nodes either as free bacteria or within infected macrophages, following passage through the gut-associated M cells, Peyers patches, or via direct infection of the epithelial lining of the gut. After crossing the intestinal barrier, the bacteria reach liver and spleen in which they replicate and may disseminate to the brain and placenta (Chakraborty 1999). Wild-type *L. monocytogenes* strains possess three pathogenicity islands, *Listeria* pathogenicity island 1 (LIPI-1), LIPI-3, and LIPI-4. LIPI-1 encodes for three toxins: the cholesterol-dependent cytolysin (CDC) listeriolysin O (LLO), a phosphatidylinositol-specific phospholipase C (PlcA), and a broad-range phospholipase C (PlcB). PlcA and PlcB are present in pathogenic *Listeria* species *L. monocytogenes* and *L. ivanovii*, but are usually absent in nonpathogenic species except in *L. seeligeri* (Cossart and Toledo-Arana 2008; Quereda et al. 2017; Vazquez-Boland et al. 2006; Hamon et al. 2006). *Listeria* is capable of crossing the host cell membrane barriers by expressing the cholesterol-dependent cytolysin LLO, triggering various mechanisms that include pore formation in target cell membranes, degradation of membrane lipids, or solubilizing the membranes by detergent-like actions (Vazquez-Boland et al. 2006; Hamon et al. 2006). In a typical listeriosis infection, the bacterium is phagocytosed by an antigen-presenting cell (APC), typically a macrophage. Pore-formation by LLO enables *Listeria* to escape phagolysosomal killing in APC, an innate immune function, via entering the cytosol where it replicates and

then invades the adjoining cell, thus also avoiding host antibody detection-mediated adaptive immune clearance (Dietrich et al. 2001). Bacterial phagolysosomal escape is effected by two secreted bacterial proteins, primarily LLO, the hemolytic toxin of *L. monocytogenes* and PlcA (Cossart and Toledo-Arana 2008; Quereda et al. 2017; Chakraborty 1999; Vazquez-Boland et al. 2006). Interestingly, LLO is optimally active at an acidic phagolysosomal pH of 5.6 (Chakraborty 1999; Hamon et al. 2006; Andrews and Portnoy 1994; Goebel and Kreft 1997). *In vitro* infection and invasion assays demonstrate that *L. monocytogenes* can invade various cell types, including nonphagocytic hepatocytes and fibroblasts with differing efficiencies. Thus, the ability of *L. monocytogenes* to invade both phagocytic and nonphagocytic cells is the hallmark of its successful intracellular lifestyle and infection (Cossart and Toledo-Arana 2008; Vazquez-Boland et al. 2006; Hamon et al. 2006).

Vaccination is the most cost effective and efficient strategy developed for prevention and eradication of infectious diseases. In recent years, vaccine research has advanced beyond traditional preventative immunization to therapeutic vaccination to treat disease conditions such as cancer (Palucka and Banchereau 2012). Immune responses to infection or vaccination can be generated in humoral and/or cellular compartments. Cellular immune response to phagocytosed extracellular antigen predominantly activates T helper (Th) cells, and intracellular pathogen-derived cytosolic antigen primarily activates cytotoxic T lymphocytes (CTLs). Current vaccination strategies mainly target antigens into the phagosomal, major histocompatibility complex class II (MHC II) antigen-processing pathway leading to Th cell activation, with a concomitant predominant humoral immune response. However, certain infections with an intracellular pathogen or tumor are not efficiently cleared by Th cell activation and/or humoral immunity. Rather, it requires a CTL response to clear up intracellular infections and killing of cancer cells. The elicitation of a cytotoxic T-cell response requires introduction of antigens into the cytosol of professional antigen-presenting cells (APCs) such as dendritic cells (DCs), and loading of cytosolic processed peptide antigens onto major histocompatibility complex class I (MHC I) to be recognized by the CTLs (Germain and Margulies 1993; Rock and Goldberg 1999; Kauffman 1998).

As evidenced before in this chapter, and elsewhere in this handbook (Quereda et al. 2017), the intracellular bacterium *Listeria monocytogenes* gains access to the host cell cytosol by secreting LLO. Scientists have successfully employed LLO in novel vaccine delivery approaches to provide access to the cytosol of professional APCs for purified protein antigens, attenuated bacterial vaccine strains, DNA vaccines, and liposome contents (Lee et al. 1996; Dietrich et al. 2001; Mandal and Lee 2002; Mandal et al. 2003; Dietrich et al. 2003). This chapter will review pore-forming properties of LLO in brief, extending the discussion in detail to LLO-mediated cytosolic delivery of macromolecule protein and nucleic acid antigens using various delivery strategies priming antigen-specific immune responses *in vitro*, and *in vivo*, via a CTL response.

Pore-Forming Toxin LLO

Virulent strains of *L. monocytogenes* secrete LLO, an exotoxin that has been identified as a major virulence factor (Cossart et al. 1989; Hamon et al. 2006; Portnoy et al. 2002) and an essential antigen for induction of T-cell mediated immunity (Safely et al. 1991; Darji et al. 1997). Listeriolysin O is the first characterized and purified virulence factor and hemolysin of *Listeria monocytogenes* by Geoffroy et al. (1987), with a molecular weight of approximately 60 kDa. The LLO belongs to a family of cholesterol-dependent cytolysins (CDC) encoded by the *hly* gene that was identified and sequenced by Menguad et al. (1988), and also reviewed by Vazquez-Boland et al. (2006). Analysis of the deduced amino acid sequence for cloned genes of LLO shows similarity with *L. seeligeri* hemolysin, seeligerilysin O (LSO) (Hass et al. 1992). Purified, recombinant LLO expressed in *E. coli* shows a molecular weight of 58 kDa (Lee et al. 1996; Mandal and Lee 2002). Ivanolysin O (ILO), another 58 kDa CDC, has also been purified and characterized (Vazquez-Boland et al. 2006). Recently, the crystal structure of LLO has revealed the importance of monomer interface on oligomerization and pore formation by electron microscopy (Köster et al. 2014). Overall, the structure of LLO resembles other CDCs that consist of four discontinuous and predominantly beta-sheet folded domains (Vazquez-Boland et al. 2006). Biophysical and fluorescent probe studies involving streptolysin O (SLO), pneumolysin O, and perfringolysin O (PFO), members of CDC, demonstrate domain 4 involvement in membrane binding and domains 1–3 involvement in toxin monomer oligomerization and subsequent membrane disruption (Vazques-Boland et al. 2006 and references therein). Therefore, membrane disruption by LLO depends on cholesterol binding and sulfhydryl activated properties of this molecule (Cossart et al. 1989; Portnoy et al. 2002, 1992; Dietrich et al. 2001). Another notable property of LLO is its optimum membrane disrupting activity at pH 5.5–5.6, suitable to perforate the phagolysosomal compartment with almost no activity at the higher cytosolic pH 7.4 (Cossart and Toledo-Arana 2008; Cossart and Cerda-Pizarro 2017; Portnoy et al. 2002, 1992; Beauregard et al. 1997). Toward that end, a single amino acid in domain 4, Leu461, was identified as the responsible factor for low pH optimum for biological activity of LLO (Glomski et al. 2002; Vazquez-Boland et al. 2006; Hamon et al. 2006). Substitution of Leu461 by Thr causes a ten-fold increase in LLO activity at neutral pH (Glomski et al. 2002). Recently, a PEST(P:Pro; E:Glu; S: Ser; T:Thr)-like sequence has been described in LLO which is functionally linked to its short cytosolic half life (Decatur and Portnoy 2000) that provides a significant inbuilt safety mechanism for LLO-mediated cytosolic macromolecule delivery. Therefore, upon infection, water soluble LLO monomers are secreted in a host cell phagolysosomal acidic environment with binding to cholesterol-rich domains of the membrane. Afterward, 30–50 monomers oligomerize to form the pore size of upto 35 nm in diameter (Peraro and van der Goot 2016).

LLO is a Virulence Factor and Also a Protective Antigen

While LLO is a virulence factor that enables *Listeria* to set up intracellular infection, at the same time, LLO is involved in modulating host immune responses

(Quereda et al. 2017; Vazquez-Boland et al. 2006; Dietrich et al. 2001; Portnoy et al. 2002; Peraro and van der Goot 2016 and references therein). LLO-mediated cytosolic release of bacteria, subsequent listerial antigen processing, and MHC class I-restricted presentation to CD8+ CTLs triggering anti-listerial protective immunity in mice have been well documented (Berche et al. 1987; Vazquez-Boland et al. 2006; Hamon et al. 2006; Portnoy et al. 2002; Safely et al. 1991; Harty and Bevan 1992). These studies demonstrated that LLO contains T-cell epitopes that are protective in nature and also correlated LLO production to protective immunity to virulent LLO-producing bacteria. Verma et al. (1995) engineered an attenuated strain of *Salmonella* carrying MHC class I/II restricted T-cell epitopes of LLO (MHC class I-restricted peptide LLO 91–99 or MHC class II-restricted peptide LLO 215–226), generating an epitope-specific protective T-cell response in mice. LLO 91–99 is an immunodominant and protective CD8+ CTL epitope as demonstrated in elegant passive transfer experiments in naïve mice (Palmer et al. 1991; Harty and Bevan 1992). LLO gene expression was only detected in the mice infected with virulent strain that induced protective immunity, while nonvirulent strains or killed bacteria did not generate protective immunity. Protective immunity in nonvirulent strains or killed bacteria could be compensated with LLO in killed bacteria, inducing interferon gamma (IFN-ɣ) and interleukin-12 (IL-12) secretion, which resulted in protective immunity (Vazquez-Boland et al. 2006; Hamon et al. 2006; Cossart et al. 1989; Portnoy et al. 1992, 2002). While LLO demonstrates membrane disrupting activity leading to a strong protective CD8+ CTL response, in cytosol, it also triggers type I interferon gene activation (Vazquez-Boland et al. 2006; Hamon et al. 2006) which is an innate immune response. Finally, a potent and neutralizing anti-LLO humoral immune response was detected after infection with *Listeria* (Vazquez-Boland et al. 2006; Portnoy et al. 2002) or LLO-liposome immunization of mice (Mandal and Lee 2002). Thus, challenging the old paradigm of cell-mediated immune protection of intracellular pathogens (Kauffman 1998) and acknowledging a complementary contribution of cellular and humoral immunity to LLO in *Listeria* infection clearance and protection (Vazquez-Boland et al. 2006).

Taken together, these studies demonstrate that LLO is a major virulence factor of *Listeria* that engineers cytosolic escape of the bacteria triggering LLO-specific innate, and adaptive (cellular and humoral), immunity. Based upon these observations, LLO-mediated macromolecule delivery in vaccine research has been initiated.

LLO in Macromolecule Delivery

Listeria has long been studied as a model pathogen to understand relevance of cell-mediated immunity, specifically a CD8+ CTL response for clearance of intracellular infection and cancer (Harty and Bevan 1992; Kauffman 1998). With ample scientific evidence for an LLO expression-mediated CD8+ CTL response paradigm in mind, researchers attempted to harness the cytosolic escape mechanism of LLO to deliver macromolecule antigen using various platforms such as LLO gene *hly* expressing bacteria, liposome encapsulated LLO for protein antigen and nucleic acid delivery, etc. Mutations within the *hly* gene or heat killed *L. monocytogenes* immunization

failed to deliver the listerial antigen to a cytosolic processing pathway which can otherwise trigger a robust and protective CD8+ CTL activation (reviewed in Dietrich et al. 2001; Dietrich et al. 2003; Portnoy et al. 1992; Barbuddhe and Chakraborty 2008). Therefore, expression of LLO is a necessary component for listerial vaccine-induced protection.

LLO-Engineered Bacteria or Recombinant *Listeria* as a Vector for Vaccine Delivery

Invasive properties of *L. monocytogenes* combined with preferential infection to host monocytes, macrophages, and dendritic cells, accessing both MHC class I and class II pathways of antigen processing and presentation, renders this pathogen an ideal candidate for vaccine carrier. Infection of these cell types with *Listeria* induces their maturation and enhances antigen processing-presentation and secretion of pro-inflammatory cytokines (Dietrich et al. 2001; Dietrich et al. 2003; Portnoy et al. 2002; Gentschev et al. 2001; Barbuddhe and Chakraborty 2008; Hess et al. 2000). These immune-modulatory properties of *Listeria* make it an attractive tool for vaccine delivery with attenuation of virulence factors, by deletion of intracellular motility genes or construction of auxotrophic mutant strains (Dietrich et al. 2001; Mandal et al. 2003; Dietrich et al. 2003; Barbuddhe and Chakraborty 2008 and references therein).

Live, attenuated bacterial vaccines have been licensed for human use for a long time and include typhoid, cholera, and *Mycobacterium bovis* (BCG). Alteration of these live, attenuated bacterial vaccines offers potential development of new vaccines against homologous diseases and also for heterologous pathogens (Dietrich et al. 2003 and references therein). The alpha (α)-hemolysin (*HlyA*) type-I secretion system has been used for incorporation of antigen into the secretion system of *E. coli* for live, attenuated bacterial vaccines. Oral delivery of these live attenuated vaccines can lead to long-term protective immunity via targeting both mucosal and systemic immunity (Dietrich et al. 2003; Gentschev et al. 2001). By using this strategy, heterologous pathogen-specific protein antigens, such as *L. monocytogenes* LLO (*Hly*), p60 and superoxide dismutase (SOD), or BCG antigen Ag85B, were expressed in attenuated *Salmonella* strains that confer complete protection in a murine listeriosis model (Gentschev et al. 2001 and references therein; Dietrich et al. 2003). Whereas, a *Salmonella* strain with a defective transport machinery does not confer protection against listeriosis, mainly due to inefficient CD8+ CTL responses (Gentschev et al. 2001). Thus, *Salmonella* strains secreting a biologically active LLO via the *HlyA* secretion system gained cytosolic access and triggered protective CD4+ and CD8+ T cell responses (Barbuddhe and Chakraborty 2008 and references therein). The observed protection apparently depends on faster availability of listerial antigens secreted by *S. typhirium* strains, enhancing a CD8+ CTL response (Dietrich et al. 2003). Various biotechnological applications of *Listeria*'s sophisticated infection and evasion strategies have been exploited (reviewed in Hess et al. 2000), that includes but not limited to a LLO-expressing tuberculosis vaccine

strain (BCG) tested in clinical trials or attenuated *L. monocytogenes*-mediated functional transfer of a cystic fibrosis transmembrane-conductance regulator (CFTR) gene.

As cellular immunity, specifically, CTL response is a necessary and required component in clearance of virus infection and eradication of tumors (Germain and Margulies 1993), *L. monocytogenes* has been a logical choice for expressing a wide range of viral antigen such as influenza virus, lymphocytic choriomeningitis (LCMV) virus, and human immunodeficiency virus (HIV) (Friedman et al. 2000). In these experiments, antigen encoding genes were either inserted into the listerial chromosome (mostly) or in the episomal plasmids (few instances) that ensured coexpressed vaccine antigen (with LLO) delivery into the host APC cytosol. An antigen-specific CD8+ CTL response protects virus-infected mice (Rock and Goldberg 1999). Importantly, *L. monocytogenes* delivery of the HIV antigen, gag protein, is promising as it generates both systemic and mucosal immunity. Surrogate tumor antigens have also been delivered in mice using recombinant *L. monocytogenes* expressing influenza nucleoprotein (Lm-NP). Mice immunized with the recombinant *Listeria* are protected against nucleoprotein-expressing tumor challenge via an antigen-specific, CT8+ CTL activation. Also, regression of the established tumor is observed upon recombinant *Listeria* vaccination (Pan et al. 1999). Other applications of recombinantly attenuated *Listeria* carrying various tumor antigens has been reviewed by Vazquez-Boland et al. (2006). These studies demonstrate that recombinant *Listeria*, carrying tumor antigens and used as an immunogen via parenteral or oral route, is capable of generating a tumor antigen-specific protective CD8+ CTL response in a prophylactic or therapeutic setting.

Apart from carrying viral or tumor antigen, *Listeria monocytogenes* has also been utilized for delivering a parasite antigen from *Leishmania major* (Soussi et al. 2002). As resistance and protection against *Leishmania* infection depends on a strong CD4+ T helper cell 1 (Th1)-type response, recombinant *L. monocytogenes* expressing leishmanial antigen becomes a natural choice for vaccine delivery as *Listeria* infection induces a strong interleukin 12 (IL-12) secretion. The cytokine profile elicited by recombinant *Listeria* expressing *Leishmania* homologue of receptors for activated C kinase antigen (LACK), in immunized mice, is distinctively of a Th1-type with IFN-ɣ and IL-12 secretion. The Th1-skewed cytokine pattern provides control of lesion progression in the *L. monocytogenes*-LACK immunized mice.

LLO-Liposome-Mediated Protein Antigen Delivery

Liposomes and other nanovesicles are important vaccine delivery vehicles due to their versatility and plasticity; choice of lipids to achieve desired charge, size, entrapment, and location of antigens; and adjuvants. Liposomes are usually internalized by APCs and the lipid bilayer is disrupted inside the endosomal compartment, releasing liposomal contents (Mandal et al. 2003; Provoda and Lee 2000). Efficient cytosolic delivery of membrane impermeable macromolecules requires a

defined endosomolytic mechanism rendered by LLO. Coadministration (Darji et al. 1997), or coencapsulation, of LLO into pH-sensitive liposomes (LLO-liposome) (Lee et al. 1996) efficiently delivers exogenous macromolecule antigens into the cytosolic space of APCs for ubiquitination, proteasomal degradation, and transporter-associated peptide antigen (TAP)-dependent MHC class I presentation for CD8+ CTL recognition. LLO-mediated delivery of exogenous macromolecule antigen triggers an enhanced, protective CD8+ CTL response against murine melanoma antigen or lymphocytic choriomeningitis virus nucleoprotein (LCMV-NP) (Mandal and Lee 2002; Mandal et al. 2003, 2004 and references therein, Provoda and Lee 2000 and references therein). LLO-liposome-mediated enhanced cytosolic delivery of antigen not only confers immune protection by CTL, but also increases CTL frequency, as determined by enzyme-linked immuno spot (ELISpot) (Mandal and Lee 2002) or MHC class I-peptide tetramer binding assays (Mandal et al. 2004). These two ultra-sensitive assays (ELISpot and tetramer binding) unequivocally demonstrate that LLO inside pH-sensitive liposomes not only triggers protective immunity, via antigen-specific CD8+ CTL activation, but also increases frequencies of these CTLs (over threefold), which are usually very low.

The concept of a cancer vaccine involves priming APCs such as DCs with tumor-derived antigens in order to facilitate eradication of tumor cells (Palucka and Banchereau 2012). Stier et al. (2005) studied comparative efficacy of LLO-liposome-mediated exogenous antigen delivery in primary cultured, murine bone-marrow derived macrophages and DCs. These are the two major APCs involved in T cell activation. LLO-mediated cytosolic release of liposomal antigen is more efficient in macrophages compared to DCs, perhaps due to differential activity and varying efficiency of LLO-mediated endosomal escape in different antigen-presenting cell types. As a purified or recombinant protein, LLO induces apoptosis in some primary immune cells, such as bone marrow macrophages or DCs (Hamon et al. 2006; Vasquez-Boland et al. 2006).

LLO-Liposome-Mediated Nucleic Acid Delivery

In recent years, nonviral eukaryotic antigen-expressing vectors called “naked DNA” vaccination has been employed via intramuscular or intradermal routes. Such methods have gained much attention due to ease of development, plasmid vector length reduction, and noninterference by preexisting viral vector-specific immunity in the host. Viral vectors also pose certain issues involving genome integration, the inability to be delivered repeatedly, and possible host rejection. Moreover, nonviral vectors are far less immunogenic than viral vectors (Mandal et al. 2003; Provoda and Lee 2000 and references therein). A major improvement in delivery of plasmid DNA into the cytosol was achieved by attenuated suicide *L. monocytogenes* or by killing the bacteria with antibiotics. In these experiments, delivery of antigen-encoding plasmid DNA was released into host APC cytosol owing to LLO activity and subsequent expression of plasmid DNA antigen for efficient cytosolic processing and MHC class I presentation (Dietrich et al. 2001, 2003; Barbuddhe and

Chakraborty 2008 and references therein). Expression of the MHC class I-restricted model antigen, chicken ovalbumin (OVA) H-2K^b epitope OVA$_{257–264}$, was demonstrated via activation of an OVA$_{257–264}$-specifc T cell hybridoma (Dietrich et al. 2001). Taken together, *in vivo* delivery of DNA vaccines by attenuated suicide *L. monocytogenes* elicits a strong humoral and cellular immune response against plasmid-encoded antigens (Dietrich et al. 2003). Although, the abovementioned studies demonstrate efficient cytosolic delivery of heterologous antigen using a listerial plasmid delivery system, the safety of this type of DNA vaccine comes into question due to potential integration of plasmid DNA into the host cell genome with possible oncogenesis (Dietrich et al. 2003). Hence, DNA vaccine vectors may not be acceptable as a safe vaccine delivery vehicle in the future.

Applications of LLO in delivering oligonucleotides to suppress or enhance biological response have been described. Mathew et al. (2003) successfully delivered murine intercellular adhesion molecule-1 (ICAM-1) antisense oligodeoxynucleotides (ODN) into the cytosol of target cells via LLO-liposomes to mediate escape of ODN from intracellular vesicles achieving improved therapeutic antisense activity in cultured macrophages. Application of LLO encapsulated in pH-sensitive liposomes was demonstrated in delivering the plant-derived toxin gelonin in tumor cells, indicating cytosolic delivery of chemotherapeutic drugs for better outcome (Provoda et al. 2003). Synthetic CpG-ODNs mimicking bacterial unmethylated DNA have been shown to activate APCs and lymphocytes targeting toll-like receptors 9, thus acting as an immunostimulatory adjuvant in vaccines. CpG-ODNs have been coadministered in vaccine delivery systems to enhance antigen-specific immune responses (reviewed by Kreig 2002). Liposomes are usually internalized by endocytosis and disrupted inside the endosomal compartment, thus offering potential for delivery of CpG-ODNs along with cargo antigen for vaccine delivery. Andrews et al. (2012) improved incorporation of CpG-ODN in a LLO-containing liposome that primed a model antigen OVA-specific enhanced CD8+ CTL response, along with a skewed Th1-type immunity in mice. Unique endosomolytic properties of LLO have been used in an anionic liposome-entrapped polycation-condensed DNA delivery system (LPDII) for improved plasmid DNA delivery in P388D1macrophage-like cells (Lorenzi and Lee 2005). By using a DNA prime and protein boost protocol, Sun et al. (2010) reported LLO-LPDII-mediated, OVA-specific higher CD8+ CTL frequency in mice compared to a heat-inactivated LLO-LPDII formulation. Genetically engineered fusion proteins of LLO and protamine enables the endosomolytic function of LLO and DNA condensation of protamine in one fusion protein for enhanced gene delivery (Kim et al. 2015).

Conclusion and Future Directions

Listeria monocytogenes infection is a paradigm for the study of host–pathogen cellular interactions, intracellular bacterial escape and adaptation to mammalian host triggering of cellular immunity. One of the central events of this paradigm is secretion of listeriolysin O that enables *Listeria* to perform many of these functions.

Thus *Listeria* or *Salmonella* expressing LLO, utilized as a DNA vaccine vector, coadministration of purified LLO or LLO-liposome delivered exogenous macromolecules primed cellular immunity, specifically enhanced CTL response demonstrating unique endosomolytic applications of LLO. Future biotechnological applications of *Listeria*-LLO may include development of novel vaccine and drug delivery systems, clinical applications, and tools for deciphering cell biology and cellular immunology molecular mechanisms. As biotechnological applications of *Listeria* increase, one specific area of research would be necessary to determine whether preexisting anti-*Listeria* or anti-LLO immunity is detrimental to *Listeria*/LLO-mediated vaccine/drug delivery efforts. *Listeria* is a food-borne infection, and therefore certain individuals may have had prior infection with neutralizing anti-LLO antibody in circulation (Vazquez-Boland et al. 2006; Portnoy et al. 2002). It has been well documented that LLO contains immunodominant T-cell epitopes (both CD4 and CD8) that trigger strong immune responses (Harty and Bevan 1992; Verma et al. 1995; Palmer et al. 1991), and there is a clear dissociation between cytotoxic properties of LLO and its very high antigenicity (Carrero et al. 2012). Preliminary and unpublished observations (of the author) indicate that preexisting LLO-specific immunity does not abrogate LLO-liposome-mediated vaccine, antigen-specific immune responses in mice. Further research should uncover the full potential of listeriolysin O in macromolecule delivery and other biotechnological applications, such as, its use as a vaccine adjuvant for modulating host immune responses.

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Part VII

General Mechanisms of Toxin Action

Chaperones and ADP-Ribosylating Bacterial Toxins

18

Holger Barth and Katharina Ernst

Abstract

Clostridial binary actin ADP-ribosylating toxins are protein toxins, which consist of an enzymatic active (A-) subunit and a separate binding/transport (B-) subunit. The A- and B-components are secreted by the clostridia as two individual, nontoxic proteins, which assemble on the surface of mammalian target cells to form biologically-active AB_7 toxin complexes. The heptameric barrel-shaped B-components mediate transport of the A-components into the host cell cytosol, where the A-components mono-ADP-ribosylate G-actin, which results in depolymerization of actin filaments and cell rounding. When the cellular uptake of these toxins was investigated in more detail, it became evident that cell-bound B-components bind their A-components and mediate the receptor-mediated endocytosis of the AB_7 toxin complexes. After internalization, the B-components have another crucial function for the transport of the A-components into the cytosol: they form transmembrane pores in the membranes of acidified endosomes and serve as channels for the transport of A-components across endosomal membranes. The A-components unfold during this transport step and require the assistance of host cell chaperones and protein-folding helper enzymes of the family of peptidyl-prolyl *cis*/*trans* isomerases (PPIases) for their translocation and/or refolding. In this review article, the current model of the cellular uptake of binary actin ADP-ribosylating toxins from *Clostridium (C.) botulinum*, *C. perfringens,* and *C. difficile*; their interaction with host cell chaperones/PPIases during intracellular membrane transport of their A-components; and the role of host cell chaperones as drug targets for development of novel pharmacological strategies against diseases associated with these medically relevant toxins are summarized.

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Actin ADP-ribosylating toxin • *C. botulinum* C2 toxin • *C. perfringens* iota toxin • *C. difficile* binary toxin CDT • Chaperone • Peptidyl-prolyl *cis/trans* isomerases

Contents

Introduction

Binary Clostridial Actin ADP-Ribosylating Toxins

The *C. botulinum* C2 toxin, the *C. perfringens* iota toxin, and the *C. difficile* binary toxin CDT are potent enterotoxins and belong to the family of binary clostridial actin ADP-ribosylating toxins. The prototype of this toxin family is the C2 toxin, which affects the intestinal mucosa of mice by causing hemorrhagic lesions and necrosis (Ohishi 1983a, b; Ohishi et al. 1980; Simpson 1982) as well as the intestinal loop of chickens and pheasants, by inducing fluid accumulations (Kurazono et al. 1987). Similar to the C2 toxin, the iota toxin displays enterotoxicity in calves and lambs constituting a severe problem in veterinary medicine regarding live stock (Billington et al. 1998; Songer 1996). In human medicine, CDT might be the most important toxin of this family because there is increasing evidence that CDT contributes to *C. difficile* associated enteric diseases (CDAD), including antibiotic-associated diarrhea as well as severe and potentially life-threatening pseudomembranous colitis (Carroll and Bartlett 2011; Schwan et al. 2009, 2011). These diseases frequently occur in hospitalized patients treated over a time with antibiotics that modulate the normal bacterial gut flora. *C. difficile* expresses and secretes the large, single-chain AB-type toxins A (TcdA, 308 kDa) and B (TcdB, 270 kDa) which glucosylate Rho, Rac, and Cdc42 GTPases in mammalian cells and thereby inhibit signaling via these central molecular switches. The disturbed Rho-signaling leads to a rearrangement of

actin filaments and finally destruction of the actin cytoskeleton. In the gut, this leads to rounding up of enterocytes and loss of barrier function of the intestinal wall, and this contributes to the characteristic symptoms of CDAD (for review, see (Carroll and Bartlett 2011)). In recent years, it has been shown that hypervirulent *C. difficile* strains produce the CDT toxin in addition to toxins A and B, and it turns out that CDT might be an important virulence factor which contributes to more severe forms of CDAD with increasing morbidity and mortality. One important hint that CDT might essentially contribute to the disease is the finding that CDT-treatment of enterocytes induces microtubule-based protrusion on the surface of enterocytes in vitro and in vivo (Schwan et al. 2009, 2011). These protrusions "catch" *C. difficile* bacteria in the gut, which significantly improves colonization of the gut by *C. difficile*. Patients infected with hypervirulent CDT-producing *C. difficile* strains show a more severe course of the disease, as well as an increased recurrence of infection (Carroll and Bartlett 2011; Schwan et al. 2009, 2011).

C2 toxin, iota toxin, and CDT are binary toxins that consist of two separate proteins, an enzymatic active (A-) component and a binding/transport (B-) component. The A- and B-components are secreted by the clostridia as two separate proteins into the environment. After their proteolytic activation, the B-components of C2 toxin and iota toxin form heptameric, barrel-shaped B_7 complexes, which represent the biologically active species. The B-component of CDT is less investigated; however, due to the sequence homology and comparable mode of action and cellular uptake mechanism between iota and CDT toxins, a comparable structure and mode of action should be expected (Barth et al. 2004; Perelle et al. 1997a). The B_7-components of C2 toxin and iota toxin assemble on the surface of mammalian target cells with their respective A-components, forming biologically-active AB_7 toxin complexes (Ohishi 1983a, b; Ohishi and Miyake 1985; Ohishi and Yanagimoto 1992; Ohishi et al. 1980, 1984; Sakurai and Kobayashi 1995; Simpson 1982). The B_7-components mediate transport of the A-components into the host cell cytosol. In the cytosol, the A-components of all these toxins mono-ADP-ribosylate G-actin (Aktories et al. 1986; Popoff et al. 1988; Schering et al. 1988), which inhibits further actin polymerization and results in the depolymerization of actin filaments and cell rounding due to the loss of the actin cytoskeleton. This is the reason for the loss of barrier functions of epithelial or endothelial tissues (see Fig. 1; Aktories and Wegner 1992; Geipel et al. 1989; Wegner and Aktories 1988).

During the last 20 years, the cellular uptake of these toxins was investigated in more detail and novel mechanisms regarding the intracellular transport of bacterial protein toxins and the role of host cell factors during the uptake of the toxins were discovered. Since the A-components must reach G-actin in the host cell cytosol, they require an efficient transport from endosomal vesicles to the cytosol. This transport is achieved by the unique structure of the binary toxins and by exploiting protein trafficking mechanisms of their host cell. The uptake mechanism of C2 toxin, which was investigated in most detail amongst these toxins, is summarized in Fig. 2. The overall uptake of iota toxin is widely comparable with minor differences. The cellular uptake of CDT and the role of its B-component are less investigated, but recent results implicate a widely comparable cellular uptake mechanism as described

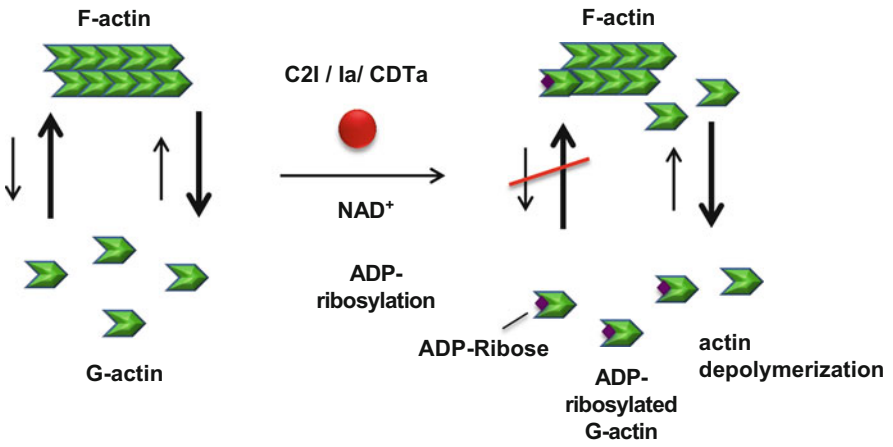

Fig. 1 ADP-ribosylation of G-actin by clostridial C2, iota, and CDT toxins. Detailed explanation is given in the text (Derived from (Aktories and Wegner 1992))

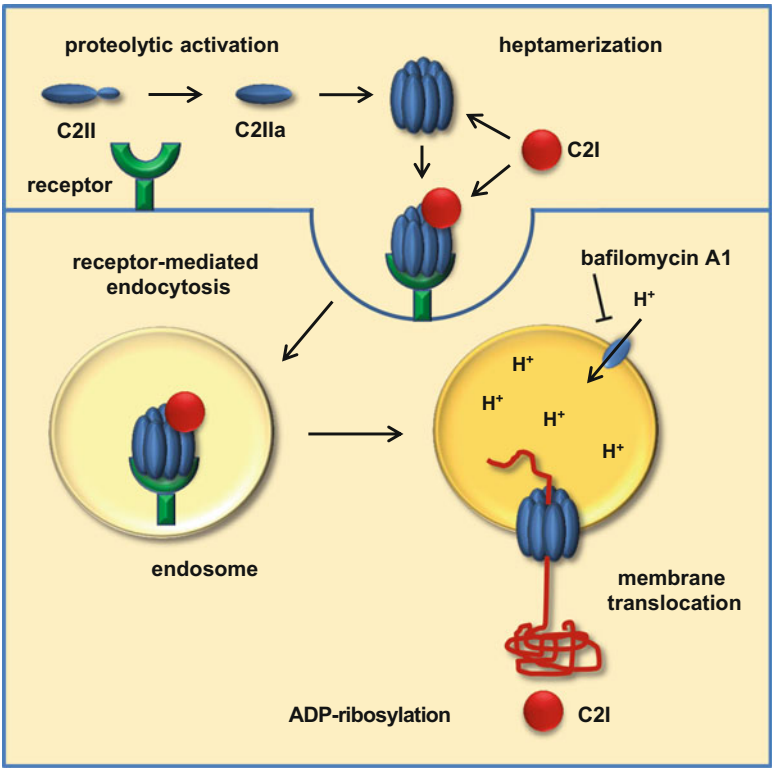

Fig. 2 Cellular uptake mechanism of *C. botulinum* C2 toxin. Detailed description can be found in the text (Derived from Barth and Aktories (2011))

for C2 and iota toxins. Interestingly, the B-component of iota toxin delivers the A-component of CDT into cells and vice versa, while both do not deliver the A-component of C2 toxin. Due to this functional interchangeability of their A- and B-components, their higher degree of sequence homology among each other, and their common protein receptor on the surface of target cells, iota toxin and CDT are closely related and belong to the group of iota-like toxins, together with the *C. spiroforme* binary toxin CST, while C2 toxin stands alone (Barth et al. 2004; Perelle et al. 1997b).

According to the current model, the B_7-components of the binary clostridial toxins are the central molecules during the transport of the A-components into the cytosol. First, the B_7-components mediate assembly of the toxin complexes to their cell receptors. For the C2 toxin, asparagine-linked hybrid and complex carbohydrates are receptors while the B-components of iota toxin and CDT bind to lipolysis-stimulated receptor in gut cells, which is a protein receptor (Eckhardt et al. 2000; Papatheodorou et al. 2011, 2013). Later during cellular uptake, after receptor-mediated endocytosis, the B_7-components of C2 toxin and iota toxin form transmembrane pores in the membranes of acidified endosomes, which mediate transport of the A-components from the endosomal lumen into the cytosol (for review on that topic, see Barth and Aktories 2011; Barth et al. 2004; Stiles et al. 2011). For their translocation through the B_7-pores, the A-components unfold at least partially and host cell chaperones as well as protein-folding helper enzymes of the peptidyl-prolyl *cis/trans* isomerase (PPIase) family facilitate their translocation and/or refolding (Ernst et al. 2015; Haug et al. 2003a, 2004; Kaiser et al. 2009, 2011, 2012).

The binary clostridial actin ADP-ribosylating toxins were the first bacterial toxins for which a role of host cell PPIases during the pH-dependent membrane transport of the A-components was discovered. However, there is increasing evidence that an Hsp90/PPIase-dependent transport of the A-components across intracellular membranes might be a unique and common feature, not only for this toxin family but for bacterial ADP-RTs in general. This would imply that specific pharmacological inhibitors of the involved host cell factors should inhibit the cellular uptake of all bacterial ADP-ribosylating toxins, and thus could be a totally novel therapeutic strategy against diseases associated with these toxins such as CDAD, diphtheria, cholera, or pertussis.

The Structure of Clostridial Binary ADP-Ribosylating Toxins

The binary structures of *C. botulinum* C2 toxin, *C. perfringens* iota toxin, and *C. difficile* CDT toxin are widely comparable because they all are composed of an enzyme component (i.e., an ADP-ribosyltransferase (ADP-RT)) and a separate transport component. The C2 toxin represents the prototype of this toxin family because of several reasons: (i) C2 toxin was the first family member for which the binary structure was described, (ii) the ADP-ribosylation of actin was first demonstrated for C2 toxin (Aktories et al. 1986), and (iii) the role of the B-component

during cellular uptake of the enzyme component (Barth et al. 2000) and the involvement of host cell chaperones was first reported for C2 toxin (Haug et al. 2003a). The C2 toxin consists of the B-component C2II (~80 or 100 kDa, depending on the strain Sterthoff et al. 2010), which binds to cells and mediates the transport of separate A-component C2I (~49 kDa) into cells (Ohishi 1983a, b).

The A-Components

Comparable to the A-components of iota toxin (Ia) and CDT (CDTa), the A-component of C2 toxin (C2I) consists of two domains of similar size and structure, as shown in the crystal structure of C2I by Schleberger et al. (2006). The enzymatically inactive N-terminal domain of C2I (C2IN, ~25 kDa) serves as an adaptor for binding of C2I to the B-component and transport of C2I into cells. Therefore, C2IN was exploited for delivery of pharmacologically active "foreign" proteins into the cytosol of mammalian cells (Barth et al. 1998a, 2002) and recombinant C2IN-based fusion proteins were delivered via the B-component into different types of mammalian cells to specifically modulate cell functions in the context of cell biological or pharmacological studies (for review, see Barth and Stiles 2008). The C-terminal domain of C2I harbors the catalytic center with the ADP-RT activity and the catalytic amino acid residues, which are highly conserved among the family of ADP-RTs (Tsuge et al. 2008). For example, the "catalytic" glutamates within the $_{387}EXE_{389}$ motif are crucial for actin binding (E_{387}) and transferase activity (E_{389}) (Aktories et al. 2011; Barth et al. 1998b; Carroll and Collier 1984; Han et al. 1999; Tsuge et al. 2008), and comparable EXE triads, which are essential for the ADP-RT activity, were identified in Ia (Sakurai et al. 2003) and CDTa (Gülke et al. 2001). Ia and CDTa contain a LKDKE sequence, in their N-terminus, which is important for binding of these ADP-RTs to G-actin (Popoff 2000) and crystallography studies show the interactions between G-actin and Ia (Tsuge et al. 2003, 2008) and CDTa (Sundriyal et al. 2009).

The B-Components and Their Cellular Receptors

The binding/transport component of C2 toxin, C2II, consists of four domains D1–D4, each of them responsible for a specific step in the uptake of C2 toxin into mammalian cells. Here too, the B-component of iota toxin Ib displays significant homology to C2II and both components are proteolytically activated, resulting in a biologically active species (Blöcker et al. 2000; Gibert et al. 2000; Ohishi 1987). However, the receptor-binding domain, which is located in the C-terminus, differs between C2II and Ib (Blöcker et al. 2000). C2IIa is cleaved between residues Lys181 and Ala182 in the N-terminal domain D1 (Barth et al. 2000; Blöcker et al. 2000; Ohishi 1987). During this step, which can be achieved in vitro by trypsin treatment, the N-terminal peptide of C2II of about 20 kDa is removed and the resulting

activated protein (C2IIa) forms barrel-shaped heptamers in solution (Barth and Stiles 2008; Barth et al. 2000). The generated N-terminal domain D1 of C2IIa binds C2I, and under saturating conditions in vitro, up to three molecules of C2I can bind to one heptamer of C2IIa (Kaiser et al. 2006). The D2 domain is crucial for pore formation in membranes and the D3 domain for the heptamerization of C2IIa. The D4 domain mediates the binding of C2IIa heptamers to carbohydrate receptors on the surface of eukaryotic cells (Blöcker et al. 2000). C2IIa binds to asparagine-linked complex and hybrid carbohydrates (Eckhardt et al. 2000), and all tested vertebrate cells respond to C2 toxin (Eckhardt et al. 2000; Ohishi et al. 1984; Sugii and Kozaki 1990). Randomly mutagenized Chinese hamster ovary (CHO) fibroblasts lacking N-acetylglucosaminyltransferase I activity are resistant towards C2 toxin because they cannot produce the receptor for this toxin (Fritz et al. 1995).

However, such cells are still susceptible to iota toxin (Fritz et al. 1995) because Ib binds to a protein receptor, namely, the lipolysis-stimulated lipoprotein receptor (LSR) (Papatheodorou et al. 2011, 2013) and exploit lipid rafts for its binding and cell entry (Hale et al. 2004; Nagahama et al. 2004, 2009). All iota-like toxins including CDT and CST bind to LSR (Papatheodorou et al. 2011, 2013), which is not expressed on all cell types. In polarized human colon carcinoma cells (CaCo-2), this toxin receptor is expressed on the basolateral membrane (Blöcker et al. 2001; Richard et al. 2002). Cell-bound Ib forms heptamers within 1 min at 37 °C (Nagahama et al. 2002; Stiles et al. 2002) but not at 4 °C. Moreover, in Vero cells, Ib complexes are localized in lipid rafts (Blonder et al. 2005). Besides LSR, CD44 plays a role in the uptake of iota toxin, and CDT and CD44-deficient cells show less efficient uptake of the toxins. CD44 knockout mice are also resistant towards iota toxin (Wigelsworth et al. 2012). Thus, CD44 may serve as a co-receptor besides LSR, but the precise role of CD44 in iota toxin and CDT uptake is not clear so far.

The Uptake of Clostridial Binary ADP-Ribosylating Toxins into Mammalian Target Cells

Cell-bound C2IIa serves as a docking platform for the A-component C2I (Barth et al. 2000; Ohishi 1987; Stiles et al. 2002), but C2IIa/C2I complexes are also formed in solution independent of the C2 toxin receptor. Here, the toxin complexes are formed prior to cell binding and one C2IIa heptamer might bind up to three C2I proteins (Kaiser et al. 2006). After receptor binding, C2IIa/C2I complexes are taken up into cells by receptor-mediated endocytosis and there is evidence for clathrin- and Rho-dependent internalization (Nagahama et al. 2009; Pust et al. 2010). After endocytosis, C2I translocates in a partially unfolded conformation across the membranes of acidified endosomes into the host cell cytosol (Barth et al. 2000; Haug et al. 2003b; Nagahama et al. 2014).

The cellular uptake mechanisms of the iota-like toxins are widely comparable to that of C2 toxin, with some differences regarding the endocytosis and translocation. While C2 toxin, as well as the iota-like toxins, exploit Rho-dependent endocytic

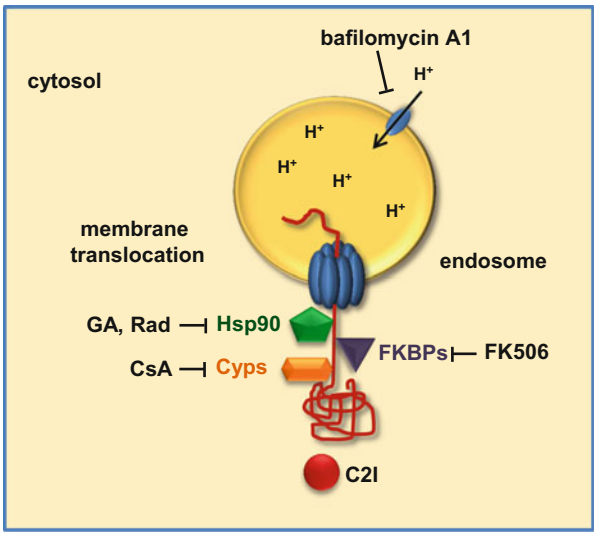

Fig. 3 Membrane translocation of the A-component of clostridial C2 toxin. Details are given in the text

pathways (Gibert et al. 2011; Pust et al. 2010), toxins of the iota family exploit a clathrin-independent endocytic pathway (Gibert et al. 2011). For their translocation across endosomal membranes, the A-components of iota-like toxins require a membrane potential in addition to a pH gradient, while C2I only requires the pH gradient to reach the cytosol (Gibert et al. 2007). However, the pH-dependent membrane translocation of the A-components through the B_7-pores is facilitated by the same set of host cell factors including heat shock protein (Hsp)90, cyclophilins (Cyps), and FK506 binding proteins (FKBPs) (see Fig. 3; Ernst et al. 2015; Haug et al. 2003a; Kaiser et al. 2012).

When the AB_7 toxin complexes reach acidified endosomal vesicles after receptor-mediated endocytosis, the B_7-components insert into and form transmembrane pores in the endosomal membranes. This is due to conformational changes caused by acidic conditions in the lumen of endosomes. The B_7-transmembrane pores much likely serve as translocation channels for the transport of A-components across the endosomal membranes into the host cell cytosol (Blöcker et al. 2003; Bronnhuber et al. 2014; Kaiser et al. 2011; Knapp et al. 2015; Nestorovich et al. 2011), therefore representing central structures and essential prerequisites for the uptake of binary toxins into mammalian cells. The domains of C2IIa and Ib, which are involved in pore formation and membrane spanning, have been characterized and for C2IIa it has been shown that the membrane spanning part of the heptamer is lined up by 14 amphipathic, antiparallel ß-sheets with two provided per monomer (Neumeyer et al. 2008; Schleberger et al. 2006). The A-components unfold during their translocation through the narrow inner diameter of the B_7-transmembrane pores, which might be a reason why host cell chaperones bind to the A-components and facilitate their refolding and/or translocation in living cells.

Role of Hsp90, Cyclophilins, and FK506 Binding Proteins in Uptake of the A-Components of C2 Toxin, Iota Toxin, and CDT into the Cytosol of Mammalian Target Cells

Besides diphtheria toxin (Ratts et al. 2003), the binary actin ADP-ribosylating C2 toxin was the first bacterial toxin for which in 2003 the involvement of the host cell chaperone Hsp90 was discovered (Haug et al. 2003a). Interestingly, both toxins are ADP-RTs and deliver their A-subunit from acidic early endosomes into the cytosol. Moreover, C2 toxin and the related iota and CDT toxins were the first bacterial toxins for which the participation of protein-folding helper enzymes from the PPIase family was reported (Ernst et al. 2015; Kaiser et al. 2009, 2011). The detailed investigation of how Hsp90 and PPIases function during cellular uptake of the clostridial binary toxins became possible because there are specific, cell-permeable pharmacological inhibitors for each of these factors (see Fig. 3). The chaperone activity of Hsp90 is inhibited by geldanamycin (GA) or radicicol (Rad) due to high-affinity binding of these compounds to the Hsp90 ATP-binding site, located within the N-terminal part of Hsp90 (Grenert et al. 1997). Hydrolysis of ATP by the intrinsic ATPase activity of Hsp90 is required for a proper function of Hsp90, as it results in conformational alterations in the client protein and release of a correctly folded client (Pratt and Toft 2003). Pretreatment of cells with one of these substances protects cells from intoxication with C2, iota, and CDT toxins, clearly indicating that Hsp90 plays a crucial role for the mode of action of these toxins (Haug et al. 2003a, 2004; Kaiser et al. 2011). Moreover, in GA- or Rad-treated cells, lesser A-components were detectable in the cytosol, implicating that the transport of the A-components was blocked (Haug et al. 2003b; Kaiser et al. 2011).

Indeed, Hsp90 facilitates the pH-dependent translocation of A-components across cell membranes, as demonstrated by the toxin translocation assay (Haug et al. 2003a; Kaiser et al. 2011). With this assay, translocation of the A-components of C2, iota, and CDT toxins across a membrane can be mimicked at the cytoplasmic membrane by generating an extracellular acidic environment. During normal uptake, the toxins are exposed to these acidic conditions in the endosomal lumen. This assay enables investigation of the membrane translocation step in an isolated manner. Analysis of GA and Rad in this assay shows that both inhibitors impair membrane translocation of A-components into the cytosol (Haug et al. 2003a; Kaiser et al. 2011). Remarkably, other steps of C2, iota, and CDT toxin uptake and mode of action, like receptor binding and enzyme activity, are not impaired by GA or Rad.

In recent years, novel derivatives of GA and Rad have been developed and investigated concerning their effect in anticancer treatment. An involvement of Hsp90 in carcinogenesis was demonstrated earlier, and Rad and GA possess strong antitumor effects. However, high toxicity, poor biological stability, and inadequate solubility of GA and Rad demand the improvement of these inhibitors. The novel GA-derivative 17-AAG efficiently inhibits Hsp90, possesses antitumor properties, and has passed preclinical and clinical trials (Li and Buchner 2013).

In eukaryotic cells, Hsp90 represents an abundant, highly conserved cytosolic protein. It plays a crucial role in various essential processes in the cell such as cycle control, survival, stress response, and hormone signaling. The involvement of Hsp90 in these processes is based on its ability to secure correct protein conformation and activation due to its folding helper activity. Therefore, Hsp90 also participates in folding of proteins, refolding processes, prevention of aggregated unfolded proteins and the transport of proteins from ER to the Golgi apparatus (Li and Buchner 2013). Remarkably, Hsp90 often fulfills its function in a concerted manner together with other protein-folding helper enzymes such as PPIases of the cyclophilin and FK506-binding protein families. The interaction of Hsp90, Cyps, and FKBPs is facilitated via specific interaction motifs: Cyps and FKBPs bind via their TPR-domain to the C-terminal MEEVD motif of Hsp90 (Ratajczak and Carrello 1996). The functional interplay of these co-chaperones is well characterized for the activation cycle of steroid hormone receptors (for review, see (Li et al. 2012)).

Interestingly, Cyps and FKBPs are also required for the membrane translocation of the clostridial binary toxins (Ernst et al. 2015; Kaiser et al. 2009). PPIases function as protein-folding enzymes by catalyzing the *cis/trans* isomerization of prolyl-bonds in proteins, which often represents a rate-limiting step during protein folding (for review, see (Göthel and Marahiel 1999)). They comprise the families of Cyps, FKBPs, and parvulins, with several different isoforms noted for all families (Fischer et al. 1984, 1989; Galat 2003; Lang et al. 1987; Schiene-Fischer 2014).

For Cyps and FKBPs, specific pharmacological inhibitors are available: Cyclosporine A (CsA) inhibits the PPIase activity of Cyps and FK506 prevents activity of FKBPs (Handschumacher et al. 1984; Harding et al. 1989). Pretreatment with either CsA or FK506 protects cultured cells from intoxication with C2, iota, and CDT toxin. This is demonstrated by a significant delay in toxin-induced changes in morphology (i.e., rounding of cells), while the combination of both inhibitors provides a synergistic inhibitory effect. Moreover, in the presence of CsA or FK506, the amount of ADP-ribosylated actin is strongly diminished (Kaiser et al. 2009, 2011, 2012). CsA and FK06 specifically inhibit membrane translocation of C2I into the host cell cytosol and, comparable to Rad, do not impair other steps of toxin action such as enzyme activity or receptor binding (Kaiser et al. 2009, 2012). Moreover, CsA restricts translocation of C2I from isolated early endosomes in vitro (Kaiser et al. 2009). To this end, endosomes loaded with C2I and C2IIa in living cells pretreated with bafilomycin A1 inhibit pore formation by C2IIa and trap C2I in the endosomes. The toxin-loaded endosomes were prepared from cell lysates by a gradient centrifugation. By adding freshly prepared cytosol to these toxin-loaded endosomes, the pore formation of C2IIa in endosomal membranes and the subsequent C2IIa-mediated translocation of C2I across the endosomal membrane into the cytosol are achieved in vitro, implying a role of host cell factors during this step of toxin uptake. The pretreatment of cytosol with CsA or with a specific antibody against CypA inhibits C2I translocation from purified endosomes, demonstrating a pivotal role of Cyps, in particular CypA, for membrane translocation of C2I. This assay established earlier for investigating the translocation of diphtheria toxin by

Lemichez et al. (1997), and employed by Ratts et al. (2003), has now been adapted by the Barth group for C2 toxin (Lemichez et al. 1997; Ratts et al. 2003).

Interestingly, the combination of PPIase inhibitors with Rad, the Hsp90 inhibitor, has a synergistic inhibitory effect on intoxication of cells with C2 toxin, suggesting that PPIases and Hsp90 might act in a multichaperone complex similar to the Hsp90-containing complex described for activating steroid hormone receptors (Kaiser et al. 2009, 2012). The families of Cyps and FKBPs comprise several isoforms; however, CsA and FK506 are nonisoform specific. In vitro analysis employing the dot blot assay and isothermal titration calorimetry reveals direct protein–protein interaction for C2I, Ia, and CDTa with CypA, Cyp40, and FKBP51 (Ernst et al. 2015; Kaiser et al. 2009, 2011, 2012). Until now, 18 different Cyp isoforms are known. Their structure is highly conserved and they can be found in nearly all tissues and compartments, which indicate a crucial biological role (Fischer and Aumüller 2003). Cyp isoforms can be categorized into single- and multi-domain proteins (for review, see Schiene-Fischer 2014). The small isoform, CypA, consists of a single PPIase domain and is the most abundant cytosolic isoform. CypA is involved in many cellular processes such as signal transduction, oxidative stress response, and regulation of cell cycle (Nigro et al. 2013). Moreover, CypA has the highest affinity to CsA amongst the Cyp isoforms (Fruman et al. 1994). Besides the cytosolic Cyps, extracellular Cyps such as CypA and CypB participate in cell–cell communications in inflammatory pathways and can be inhibited by the non-cell-permeable, CsA-derivative MM284 (Hoffmann and Schiene-Fischer 2014; Malesevic et al. 2013). Pretreatment of cells with the non-cell-permeable MM284 prior to intoxication reveals that MM284 does not inhibit uptake of C2 toxin into the cell. Therefore, an involvement of extracellular Cyps can be excluded (Ernst et al. 2015). Cyp40 represents a multidomain isoform and comprises further functional domains additional to the PPIase domain such as the TPR domains, which are required for binding of Cyp40 to Hsp90 (Pratt and Toft 1997). Cyp40 and FKBPs bind to the same MEEVD motif of Hsp90 and compete for the binding site in Hsp90 multichaperone complexes that facilitate activation of steroid hormone receptors in the cell (Pratt and Toft 1997). Moreover, Cyp40 facilitates the transport of glucocorticoid receptors along the cytoskeleton into the nucleus by interacting with the motor protein dynein (Galigniana et al. 2002; Owens-Grillo et al. 1995).

Comparable to the Cyps, FKBPs also comprise single- and multidomain proteins (Schiene-Fischer 2014). The PPIase domain (i.e., FKBP domain) is present in all isoforms and represents the binding site for FK506. The small isoform FKBP12 comprises only the PPIase domain and displays, similar to CypA, the strongest binding to the inhibitor of PPIase activity FK506. FKBP12 has no interaction with A-components of C2, iota, or CDT toxins in vitro (Kaiser et al. 2011). However, the multidomain isoform FKBP51 is an interaction partner of C2I, Ia, and CDTa (Kaiser et al. 2012). Comparable to the Cyps, the multidomain isoforms of FKBPs also contain TPR domains, which enable interaction with Hsp90. Furthermore, Cyp40, FKBP51, and FKBP52 compete with each other for the binding site of Hsp90. Interestingly, FKBP51 and FKBP52 show antagonistic behavior: FKBP52 increases the activity of glucocorticoid and

androgen receptors, whereas FKBP51 decreases affinity of the respective hormone to its receptor (Cheung-Flynn et al. 2005; Denny et al. 2000; Mamane et al. 2000; Riggs et al. 2003). Moreover, FKBP52, when associated with the Hsp90-containing multichaperone complex, interacts with the motor protein dynein to facilitate translocation of the steroid hormone receptor into the nucleus (Galigniana et al. 2001, 2010). Here too, FKBP51 acts in an antagonistic way and delays this translocation (Wochnik et al. 2005). Remarkably, Hsp90 coprecipitated with C2I in these same lysates from toxin-treated cells also shows coprecipitates of Cyp40 and FKBP51 (Ernst et al. 2015; Kaiser et al. 2012). There is also a direct interaction of Hsp90 with C2I, Ia, and CDTa in vitro (Kaiser et al. 2011). In contrast, the B-component of C2 toxin, C2IIa, does not bind to Hsp90 or the PPIases in vitro, suggesting that the interaction of these host cell factors is restricted to the A-component of the binary actin ADP-ribosylating toxins (Ernst et al. 2015). Remarkably, interaction of the A-components of C2, iota, and CDT toxins with Hsp90 and the PPIases is significantly enhanced in vitro when the A-components are denatured (i.e., partially unfolded conformation) (Ernst et al. 2015; Kaiser et al. 2012). This seems plausible since the A-components must be partially unfolded to translocate through the narrow pore formed by the B_7-complexes, and Hsp90 plus PPIases are known to interact with unfolded proteins since they are involved in the refolding processes of proteins in cells (Haug et al. 2003b; Pratt and Toft 1997). As evidenced by Cyp40, a conformation was discovered by crystallography that exhibits a partially unfolded TPR-domain, which could easily recognize other unfolded protein domains (Taylor et al. 2001).

So far, all investigated ADP-ribosylating toxins depend upon Hsp90 and PPIases for their membrane translocation into the host cell cytosol. On the contrary, uptake of other toxins that show high structural homology or similar uptake mechanisms to the clostridial ADP-ribosylating toxins and display a different enzyme activity, such as the *Bacillus anthracis* lethal toxin or the *C. difficile* TcdA and TcdB toxins, are independent of Hsp90 and PPIases (Dmochewitz et al. 2011; Haug et al. 2003a; Kaiser et al. 2009, 2011, 2012; Zornetta et al. 2010).

Further investigations that analyze uptake of recombinant fusion toxins demonstrate that Hsp90 and the PPIases interactions depend on the ADP-RT domain of the toxin. Regarding the C2I protein, the ADP-RT domain is located in the C-terminal part of the protein (Barth et al. 1998b, 2002). Uptake of the N-terminal part, C2IN, shows no enzyme activity and is not impaired when the activity of Hsp90 or the PPIases is inhibited. However, if an ADP-RT domain, such as the Rho-modifying C3 protein from *C. limosum* or the actin ADP-ribosylating domain from the *Salmonella enterica* virulence factor SpvB, is fused to C2IN, these host cell factors are crucial for uptake of these fusion toxins (Kaiser et al. 2012; Pust et al. 2007). However, when other proteins, which are not ADP-RTs, are fused to C2IN, Hsp90 and PPIases are not required for uptake of these fusion proteins into the cytosol of host cells (Kaiser et al. 2012). Taken together, these findings strongly suggest involvement of Hsp90 as well as PPIases of the Cyp and FKBP families for membrane translocation that might be a commonly unique characteristic for ADP-ribosylating toxins.

Chaperones and PPIases as Novel Drug Targets: Pharmacological Inhibitors of Chaperones and PPIases Prevent Uptake of the A-Components of Binary ADP-Ribosylating Toxins from Clostridia and Protect Cells from Intoxication

Finally, novel findings regarding the membrane translocation of clostridial ADP-ribosylating toxins might also have medical implications. If translocation of the A-component into the host cell cytosol can be inhibited by compounds, the intracellular substrate of these toxins cannot be modified. This would prevent the toxin-induced cellular effects and therefore the clinical symptoms caused by these bacterial toxins. However, CsA and FK506 display an immunosuppressive effect and thus are applied as licensed drugs for immunosuppression therapy (i.e., organ transplantation to inhibit rejection) (Borel et al. 1976; Elliott et al. 1984; Krönke et al. 1984; Liu et al. 1991; Schreiber et al. 1991; Swanson et al. 1992). The immunosuppressive effect is based on binding of the CsA/Cyp or analogously the FK506/FKBP complex to the protein phosphatase calcineurin, resulting in inhibition of calcineurin. As a consequence, dephosphorylation of the transcription factor nuclear factor of activated T-cells (NF-AT) by calcineurin cannot occur and NF-AT stays in the cytosol unable to activate the transcription of interleukins leading to a decreased activation of T-lymphocytes (Clipstone and Crabtree 1992; Liu et al. 1991; Schreiber et al. 1991). However, compound VK112, a novel derivative of CsA, was developed lacking the immunosuppressive effect but still potently inhibits the PPIase activity of Cyps (Daum et al. 2009; Fischer et al. 2010; Prell et al. 2013). Importantly, VK112 protects cells from intoxication with C2, iota, and CDT toxins and also prevents membrane translocation of the A-components of these toxins (see Fig. 4; Ernst et al. 2015). VK112 tackles the toxin uptake into cells on a defined molecular level and could therefore be considered a promising starting point

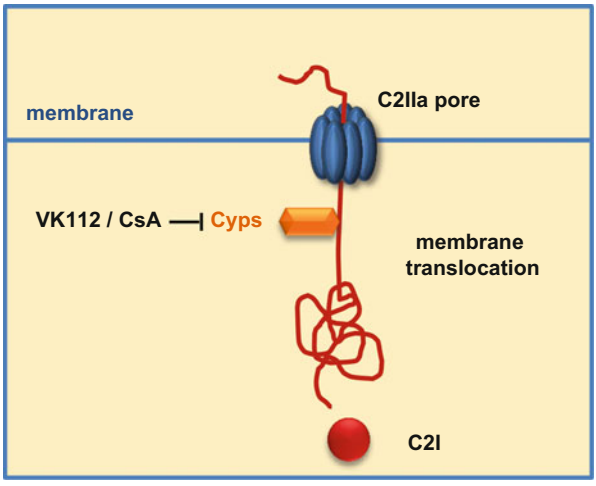

Fig. 4 The membrane translocation of C2I is inhibited by CsA and its nonimmunosuppressive derivative VK112. Further details are given in the text (Derived from (Ernst et al. 2015))

for developing new therapeutic strategies against the secreted binary ADP-ribosylating toxins (e.g., in the context of food poisoning).

However, substances like VK112 could also be applied during infection of humans and animals with toxin-producing bacteria, such as hypervirulent strains of *C. difficile* that occur with increasing frequency in hospitals, posing a severe threat to patients' health and recovery. Here, novel pharmacological inhibitors like VK112 should also protect patients against the symptoms caused by toxin-producing bacteria that already show resistance against many traditional antibiotic treatment regimes (for review, see (Barth 2011)).

Conclusion and Future Directions

Taken together, there is ample evidence from studies with the clostridial binary actin ADP-ribosylating toxins that Hsp90, Cyps, and FKPBs specifically interact with the ADP-RT domains of the A-components and selectively facilitate their intracellular membrane translocation, after internalization of these toxins into mammalian cells.

Recently, it was demonstrated that the actin ADP-ribosylating PTC3 toxin from *Photorhabdus luminescens* (Lang et al. 2010) also requires the activities of Hsp90, Cyps, and FKBPs to translocate into the cytosol of target cells (Lang et al. 2014). Moreover, the isolated ADP-RT domain of this toxin interacts with Hsp90, CypA, Cyp40, and FKBP51 in vitro and coprecipitates with the ADP-RT domain from intoxicated cells (Lang et al. 2014). Furthermore, the enzyme component is delivered into the cytosol by a novel pore-forming translocation component, completely different from the B-components of C2 and iota-like toxins, as recently described (Gatsogiannis et al. 2013; Meusch et al. 2014) and introduced in this chapter. This indicates that the Hsp90/PPIase-facilitated membrane translocation of ADP-ribosylating toxins is not restricted to a particular type of translocation pore, but seems to depend only on the presence of an ADP-RT domain in the toxin. One possible explanation for this specific interaction might be that Hsp90 and the PPIases recognize the highly conserved tertiary structure of mono-ADP-ribosyltransferases with the ß-strand and an α-helix in the catalytic site (Collier 1995; Masignani et al. 2000, 2005).

This hypothesis is also supported by the findings that Hsp90 is an essential cytosolic factor for membrane translocation of the ADP-RT domain of a recombinant diphtheria fusion toxin (Ratts et al. 2003) and of the A-subunit from cholera toxin, another ADP-RT (Taylor et al. 2010). In contrast, bacterial AB-type toxins such as the binary anthrax toxins (for review, see Collier 2009; Young and Collier 2007), which are not ADP-RTs, do not require the aforementioned chaperones/PPIases for delivering enzyme domain into the cytosol (Dmochewitz et al. 2011; Zornetta et al. 2010).

The molecular mechanism underlying the specific interaction between the ADP-ribosylating toxins and host cell chaperones/PPIases requires further

investigation. It is not clear whether the latter are part of multichaperone complexes, comparable to multichaperone complexes which interact with glucocorticoid receptors in mammalian cells and contain FKBP51, FKBP52, Cyp40, and Hsp90 (Ni et al. 2010; Pirkl and Buchner 2001; Pratt and Toft 1997; Stechschulte and Sanchez 2011). These latter components are all identified as interaction partners of the binary actin ADP-ribosylating toxins. Interestingly, an Hsp90-containing protein complex facilitates translocation of a diphtheria fusion toxin across endosomal membranes (Murphy 2011; Ratts et al. 2003), but so far it is not known whether PPIases are part of this complex.

Cross-References

- ▶ *Bacillus anthracis* Toxins: Efficient Biochemical Weapons for the Infectious Battle
- ▶ *Clostridium perfringens* Iota Toxin: A Successfully Shared Template for Common Enteric Pathogens
- ▶ Role of *Clostridium difficile* Toxins in Antibiotic-Associated Diarrhea and Pseudomembranous Colitis

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Repeats-in-Toxin (RTX) Toxins: A Review

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Abstract

Repeats-in-toxin (RTX) exoproteins of Gram-negative bacteria form a steadily growing family of proteins with diverse biological functions. All the diverse RTX toxins share two common features: (i) they are secreted by a single-step export mechanism via the type I secretion system that employs a "channel–tunnel" conduit spanning across the entire Gram-negative bacterial envelope, thus connecting bacterial cytoplasm to extracellular environment; (ii) RTX toxins possess the characteristic glycine- and aspartate-rich repeats that bind numerous calcium ions, typically in the carboxy-terminal portions of the molecule, with the large multifunctional autoprocessing RTX (MARTX) toxins bearing similar repeats also as N-terminal segments. RTX toxins further require physiologically high (>1 mM) Ca^{2+} concentration for proper folding and biological activity on host epithelial or phagocytic cells. Members of the RTX family of toxins act as effective "contact weapons" and have been shown to play a major role in virulence of a broad range of Gram-negative pathogens.

The classical RTX toxins, originally classified as leukotoxins and hemolysins, were shown to penetrate and permeabilize host cell membranes. Research over the past decade, however, revealed that RTX toxins can exert numerous additional activities contributing to cytotoxic action in pathogenesis and virulence of bacterial infections. Some of the RTX toxins affect host cell signaling via uncontrolled conversion of ATP to a key signaling molecule, cAMP (CyaA, $MARTX_{Vc}$), or via interaction with small GTPases ($MARTX_{Vc}$, $MARTX_{Vv}$) and thus effectively subvert host cell physiology. Other RTX toxins induce cytoskeletal rearrangements via covalent cross-linking of actin molecules ($MARTX_{Vc}$, $MARTX_{Vv}$), or inflict an

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apoptotic/lytic program on target phagocytic or epithelial cells (HlyA, CyaA, $MARTX_{Vv}$), thereby promoting disease pathogenesis.

Keywords

Gram-negative bacteria • Hemolysin • Leukotoxin • Membrane permeabilization • Pathogenesis • RTX toxin • Type I secretion system

Contents

Introduction

RTX proteins are secreted by a broad range of Gram-negative bacteria, including *Escherichia coli*, *Bordetella pertussis*, *Proteus vulgaris*, *Morganella morganii*, *Vibrio species, Moraxella bovis, Kingella kingae*, and members of the family *Pasteurellaceae* (*Mannheimia*, *Pasteurella*, *Aggregatibacter*, and *Actinobacillus* species). Most RTX toxins penetrate and permeabilize host cell membranes and share several characteristic features, including (i) posttranslational modification, (ii) C-terminal unprocessed secretion signal, (iii) export out of the cell by type I secretion systems (T1SS), and (iv) a C-terminal calcium-binding domain consisting of acidic glycine-rich nonapeptide repeats with a consensus sequence X-(L/I/F)-X-G-G-X-G-(N/D)-D. Recognition of the last feature allowed Rodney A. Welch to coin the name for the entire protein family (Welch 1991). The number of characteristic RTX repeats varies among different RTX toxins from less than 10 to over 40. RTX toxins can be roughly subdivided into two classes based on their target cell specificity. RTX hemolysins exhibit little target cell specificity, while leukotoxins have pronounced species- or cell-specific effects (Coote 1992; Welch 1991). RTX toxins of this class, comprising the leukotoxins of *A. actinomycetemcomitans* (LtxA) and *P. haemolytica* (LktA), are toxic to restricted groups of cells (Strathdee and Lo 1989; Taichman et al. 1987).

A third group of very large RTX toxins with multiple activities (multifunctional autoprocessing RTX toxins, abbreviated as MARTX) was discovered in 1999 (Lin et al. 1999). Their prototypic representative is the VcRtxA ($MARTX_{Vc}$), a

more than 4,500-residue-long protein from *V. cholerae* (Fullner and Mekalanos 2000).

Characteristic Features of Prototypic RTX Hemolysins and Leukotoxins

Encoding Operons

Although variations do exist, the generic RTX toxin operon consists of four genes that are designated *rtxCABD* (Fig. 1). The RTX toxins require posttranslational fatty-acyl modification of the pro-RtxA protein on internal lysine residues to become a biologically active RtxA toxin. This activation step is accomplished by the *rtxC* gene product, a toxin acyl transferase enzyme using acyl-acyl carrier protein (acyl-ACP) as acyl chain donor (Stanley et al. 1994). Activated RtxA toxins are then transported directly from the cytosol of the producing bacterial cell to its outer membrane surface by the transport proteins encoded by the *rtxBDE* genes. The RtxB proteins are members of the ATP-binding cassette superfamily of transport proteins integrated into the bacterial cytoplasmic (inner) membrane (Higgins 1992). The RtxD proteins belong to the membrane fusion protein family and span from the cytoplasmic membrane across the periplasmic space (Dinh et al. 1994). The *rtxE* gene encoding the T1SS apparatus of outer membrane component, homologous to the *E. coli* TolC protein, is in most cases located outside of the *rtx* locus. Its product, TolC-like protein, may as well be involved in additional processes than RTX protein secretion.

Posttranslational Modification

Posttranslational activation of RTX toxins is mediated by the co-synthesized RtxC proteins (Barry et al. 1991; Goebel and Hedgpeth 1982; Sebo et al. 1991). The mechanism of this novel type of protein acylation was analyzed in substantial detail for the prototype RTX toxin-activating and acyl-ACP-dependent protein acyltransferase HlyC, which acylates the ε-amino groups of internal Lys^{564} and

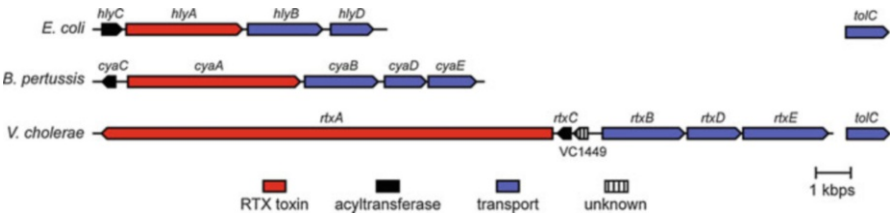

Fig. 1 The schematic representation of the rtx gene clusters of *E. coli*, *B. pertussis*, and *V. cholerae*. The *arrows* represent coding regions and transcriptional directions of the rtx genes

Lys^{690} of HlyA, the *E. coli* α-hemolysin (Greene et al. 2015; Issartel et al. 1991; Ludwig et al. 1996; Stanley et al. 1994). It has been demonstrated that HlyC uses the fatty-acyl group from acyl-ACP to generate an acyl-HlyC intermediate, which transfers its fatty-acyl group to proHlyA (Worsham et al. 2001). Various acyl-ACP-carrying fatty acids, including the most common in *E. coli*, the palmitoyl (C16:0) and palmitoleic (C16:1) residues, were found to be efficiently used in vitro as acyl donors for modification of HlyA (Issartel et al. 1991). In vivo, however, HlyC exhibits a high selectivity for C14:0 myristic acid that was found to constitute about 68% of the acyl chains covalently linked to side chains of Lys^{564} and Lys^{690} residues of native HlyA. Curiously enough, the extremely rare odd carbon-saturated C15:0 and C17:0 fatty-acyl residues constituted the rest of the in vivo acylation of HlyA from two different *E. coli* strains (Lim et al. 2000).

The extent of fatty acylation of the *Bordetella pertussis* CyaA in vivo appears to depend on the producing strain. Initially, the *Bp*-CyaA extracted from a Tohama I-type strain *B. pertussis* 338 was found to be monoacylated by a single palmitoylation at only the Lys^{983} residue (Hackett et al. 1994). The CyaA sequence, however, comprises two characteristic acylation sites conserved in RTX cytolysin sequences, suggesting that CyaA could be acylated on two lysine residues, Lys^{860} and Lys^{983}. The recombinant *Ec*-CyaA toxin co-expressed with CyaC in *E. coli* K12 was, indeed, found to bear a second acylation on the Lys^{860} residue (Hackett et al. 1995). The acylation of Lys^{983} was shown to be necessary and sufficient for the activation and full biological activity of CyaA (Basar et al. 2001). Nevertheless, acylation of either Lys^{860} or Lys^{983} was shown to each individually confer on CyaA a capacity to tightly associate with its receptor CD11b/CD18. Moreover, CyaA acylated only on Lys^{860} still exhibited a reduced but substantial cytotoxic activity toward murine monocytic cells expressing CD11b/CD18 (Masin et al. 2005). The role of Lys^{860} acylation hence remains enigmatic, and one hypothesis would be that it may be required for toxin interactions with some particular cell subsets in vivo.

RTX leukotoxins require fatty acylation for all known cytotoxic activities. The function of the posttranslational modification is, however, incompletely understood. Non-acylated proHlyA or non-acylated proCyaA forms pores in planar lipid bilayers with a reduced specific pore-forming activity, compared to fully acylated proteins. The transmembrane pores generated by the pro-toxins, however, exhibit similar properties as the pores generated by the acylated toxins (Ludwig et al. 1996; Masin et al. 2005). Both non-acylated pro-toxins (proHlyA and proCyaA) also penetrate a naked liposomal membrane (Masin et al. 2004; Soloaga et al. 1996). Recent evidence indicates that acylation is essential for tight binding and productive interaction of the RTX toxins with target cells, which is required for a cytotoxic effect to occur (El-Azami-El-Idrissi et al. 2003; Sun et al. 1999; Thumbikat et al. 2003).

Secretion of RTX Toxins via the Type I Secretion System

The RTX toxins harbor an approximately 60 residue-long C-terminal secretion signal that is not processed during secretion (Gentschev et al. 1990; Sebo and

Ladant 1993; Stanley et al. 1991). This is recognized by the T1SS that mediates translocation of toxins directly from bacterial cytosol into the extracellular milieu. This is accomplished via the T1SS "channel–tunnel" assembly that spans across the entire Gram-negative bacterial cell envelope consisting of a sandwich of inner and outer membrane enclosing the periplasm and peptidoglycan meshwork of cell sacculus (Fig. 2). The T1SS secretion involves three specific proteins:

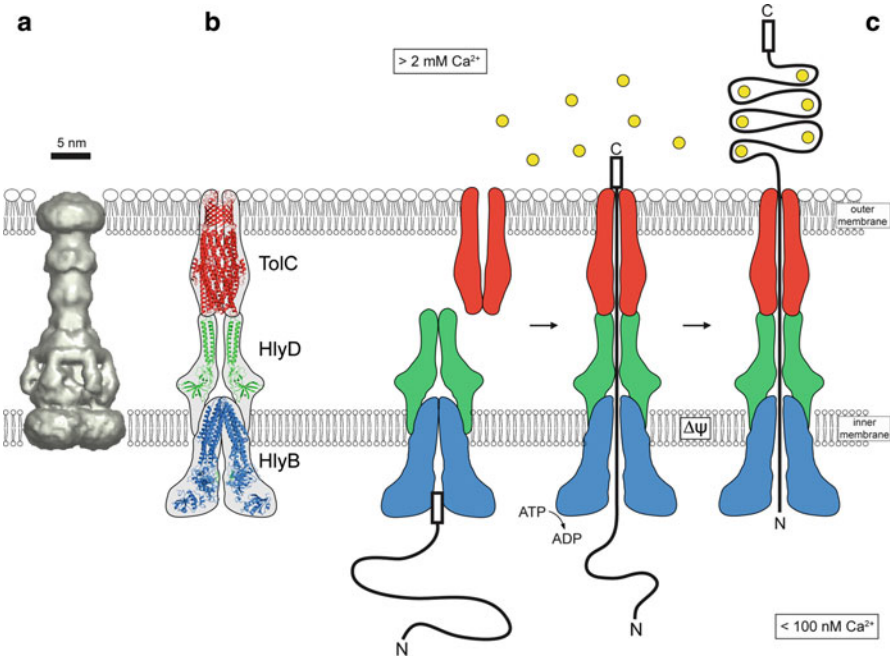

Fig. 2 Structural and functional organization of the type I secretion system (*T1SS*). (**a**) Three-dimensional electron microscopy reconstruction of the AcrA-AcrB-TolC complex (EM Data Bank: EMD-5915), an efflux pump that belongs to a family of a resistance–nodulation–division (*RND*) multidrug efflux pump, of which tripartite organization is closely related to that of T1SS. (**b**) Putative structure of individual protein components of prototypical T1SS apparatus from *E. coli*. An outer membrane TolC component is shared by both RND efflux pumps and T1SS, while a periplasmic membrane fusion protein (HlyD) and an inner membrane component (HlyB) are exclusive for the T1SS. The model of the T1SS is based on X-ray structure of *E. coli* TolC (PDB: 1EK9, *red*); HlyB (*blue*) and HlyD (*green*) are represented by X-ray structure of the human mitochondrial ABCB10 ATP-binding cassette (ABC) transporter (PDB: 4AYT) along with NMR structure of the C-39-like domain of HlyB (PDB: 3ZUA) and the *E. coli* AcrA (PDB: 2F1M) of the RND efflux pump, respectively. (**c**) Schematic depiction of the T1SS assembly operation. Upon recognition of a C-terminal secretion signal of a T1SS substrate, the inner membrane HlyB-HlyD complex contacts the outer membrane trimeric TolC and together form a sealed channel–tunnel assembly spanning across the entire Gram-negative bacterial cell envelope. Low concentration of Ca^{2+} ions in the bacterial cytoplasm (<100 nM) prevents folding of the C-terminal RTX domain, and ATP hydrolysis by HlyB and transmembrane potential ($\Delta\psi$) enable the transfer of the unfolded substrate through the translocon. Loading of RTX repeats of the secreted protein by Ca^{2+} ions in the extracellular environment (>2 mM) then promotes folding and acquisition of biological activity of the translocated substrate

(i) a polytopic inner membrane protein with a cytoplasmic ATPase domain operating as an ATP-binding cassette (ABC) exporter, (ii) a membrane fusion protein (MFP), and (iii) an outer membrane protein (OMP). The MFP spans out from the inner membrane into the periplasm and contacts both the inner membrane ABC exporter and the OMP. The paradigm of the T1SS is based on the analysis of the mechanism of secretion of the HlyA toxin of *E. coli*. The ABC exporter (HlyB) and the MFP protein (HlyD) are both encoded within the *hly* operon, while a multifunctional OMP component (TolC) is encoded elsewhere on the *E. coli* chromosome (Fig. 1; Wagner et al. 1983; Wandersman and Delepelaire 1990). In several other species, such as *B. pertussis* or *M. bovis*, the gene for a TolC homologue is comprised in the *rtx* operon (Angelos et al. 2003; Glaser et al. 1988a). Determination of the TolC structure by X-ray crystallography demonstrated that the protein forms a trimeric export channel in the outer membrane (Delepelaire 2004; Koronakis et al. 2004). It was suggested that the inner membrane proteins HlyB and HlyD form a complex that subsequently recognizes the C-terminal secretion signal peptide of HlyA. Upon binding of HlyA, the HlyD trimer interacts with the trimeric TolC protein of the outer membrane, inducing its conformational change and export of HlyA (Andersen et al. 2001). Contact with HlyD was suggested to affect directly, or indirectly, the folding of HlyA following or during its transit through the translocator (Pimenta et al. 2005). The secretion complex appears to be transient, with the inner membrane complex of HlyB, HlyD, and TolC disengaging and reverting to the resting state, once the toxin has been transported (Thanabalu et al. 1998). The energy necessary for the secretion process depends not only on ATP hydrolysis mediated by HlyB but also on the proton motive force on the inner membrane (Koronakis et al. 1991, 1993, 1995). Recently, it was shown that binding of extracellular Ca^{2+} ions to the emerging RTX protein segments drives folding of the C-terminal RTX domains into β-barrel structures at the outer T1SS mouth. This yields formation of intramolecular Brownian ratchets that prevent backward movement of the RTX chains in the T1SS channel and allow their directional diffusion out of the bacterial cell (Bumba et al. 2016). The Hly T1SS was shown to promote to some extent also heterologous secretion of some other RTX toxins expressed in *E. coli*, such as the CyaA of *B. pertussis* (Sebo and Ladant 1993) or PaxA of *P. aerogenes* (Kuhnert et al. 2000).

An atypical T1SS has been described for the large MARTX proteins in *Vibrio*. This apparatus consists of four proteins, an analogue of HlyB (RtxB), an analogue of HlyD (RtxD), a TolC-like protein, and an additional ATP-binding protein RtxE, where both ABC exporter proteins, RtxB and RtxE, appear to be necessary for MARTX protein secretion (Boardman and Satchell 2004).

Interaction of RTX Toxins with Target Membranes

In the case of RTX toxins, their interaction with target cell membrane can be subdivided into two steps: (i) a reversible cell surface adsorption, sensitive to electrostatic forces, and (ii) an irreversible membrane insertion (Bakas et al. 1996;

Ostolaza et al. 1997). Once the toxin is inserted in the membrane, it appears to undergo an irreversible conformational change (Moayeri and Welch 1997), after which it cannot be recovered from the membrane without the use of detergents (Bhakdi et al. 1986). In case of RTX toxins, the detailed mechanism of membrane insertion and pore formation remains unclear. Several studies showed that the hydrophobic regions of *E. coli* HlyA and *B. pertussis* CyaA domains are responsible for insertion of the toxins into target membranes (Hyland et al. 2001; Schindel et al. 2001). These domains are further critical for the ability of the toxins to form transmembrane pores (Benz et al. 2014). Biophysical studies demonstrated that RTX toxins form cation-selective pores that have a defined size and a short lifetime of only a few seconds (Benz et al. 1989, 1994). Dose–response dependencies of cell lysis by RTX toxins exhibit a high cooperativity number, indicating that oligomerization of RTX toxin molecules is involved in RTX pore formation (Bhakdi et al. 1989; Cavalieri and Snyder 1982; Gray et al. 1998). This is supported by mutagenesis studies on CyaA (Vojtova-Vodolanova et al. 2009). Indeed, complementation of individually inactive truncated variants of *E. coli* HlyA or *B. pertussis* CyaA in vitro produces hemolytic activity, suggesting that two or more toxin molecules may aggregate before pore formation occurs (Iwaki et al. 1995; Ludwig et al. 1993). Indeed, the exposure of membranes to RTX toxins at high concentration or for longer periods of time may yield lesions through a detergent-like mechanism (Moayeri and Welch 1994; Ostolaza et al. 1993). Shortly after insertion into target membrane, RTX toxins stimulate cationic fluxes of Ca^{2+} and K^{+} across cell membrane and the release of host metabolites such as ATP (Linhartova et al. 2010; Skals et al. 2014; Wald et al. 2014). Depending on the size of the pores formed by the RTX toxin, leakage of ATP may activate P2X receptors, which, in turn, may exacerbate cell permeabilization through ion channel opening and accelerate the lytic effects of RTX toxin action (Masin et al. 2013; Munksgaard et al. 2012; Skals et al. 2009).

It has been repeatedly shown that β_2 integrins can serve as RTX toxin receptors on leukocytes. These integrins share a common β_2 subunit, CD18, which is combined with either one of the unique α chains, αL (CD11a), αM (CD11b), αX (CD11c), or αD (CD11d). The CyaA toxin from *B. pertussis* has been shown to use the complement receptor 3 (CR3, also called the $\alpha_M\beta_2$ integrin CD11b/CD18 or Mac-1) as a specific host cell receptor on myeloid phagocytes (Guermonprez et al. 2001). Binding of other RTX toxins to other members of the β_2 integrin family has been reported (Ambagala et al. 1999; Lally et al. 1997; Li et al. 1999). The *E. coli* HlyA, which shares homology with the CyaA hemolysin moiety, exhibits specificity for leukocytes but only at low toxin concentrations (Welch 1991). This relative specificity was shown to be mediated by the interaction of HlyA with CD11a/CD18, the LFA-1 integrin of leukocytes (Lally et al. 1997). *Actinobacillus actinomycetemcomitans* LtxA and *Pasteurella haemolytica* LktA are RTX leukotoxins specific for human and bovine leukocytes and interact with LFA-1 as well (Ambagala et al. 1999; Lally et al. 1997; Li et al. 1999).

RTX toxins require Ca^{2+} ions for activity, as first shown for *E. coli* α-hemolysin (Short and Kurtz 1971) and *B. pertussis* CyaA (Hewlett et al. 1991). Several studies revealed that the Ca^{2+} ions bind within the nonapeptide repeats with the consensus

sequence X-(L/I/F)-X-G-G-X-G-(N/D)-D in the C-terminal moieties of these toxins (Ludwig et al. 1988; Rhodes et al. 2001; Rose et al. 1995).

The RTX domain of CyaA from *B. pertussis* is organized into five successive blocks of about eight nonapeptide RTX motifs each, with the blocks separated by linkers of variable length (Bauche et al. 2006; Glaser et al. 1988b; Rhodes et al. 2001). The repeat domain of CyaA contains a small number (three to five) of high-affinity and about 35 low-affinity calcium-binding sites (Rose et al. 1995). Biological activity of CyaA, such as target cell binding and translocation into cells, strictly depends on presence of calcium ions in the millimolar concentration range. The RTX domain harbors the low-affinity Ca^{2+}-binding sites, the loading of which is required for conformational changes of the toxin molecule and adenylate cyclase (AC) domain translocation into target cells (Hanski and Farfel 1985; Hewlett et al. 1991; Rose et al. 1995). The binding of calcium ions to the high-affinity sites is required for structuring of the RTX domain and binding of CyaA to cellular membrane. Recent unpublished data show that the calcium-triggered folding of the C-terminal RTX repeat blocks of the CyaA toxin on the outer bacterial surface during secretion drives T1SS secretion, and release of the toxin. This yields formation of intramolecular Brownian ratchets that direct vectorial translocation of the secreted RTX polypeptides through the T1SS conduits (Bumba et al. 2016).

RTX Toxins

CyaA Toxin: A Fusion of an Adenylate Cyclase Enzyme with an RTX Hemolysin

The bifunctional adenylate cyclase toxin-hemolysin (CyaA) of *B. pertussis* is a prototype RTX toxin, as it harbors features of both the hemolysin and leukotoxin families of RTX toxins (Linhartova et al. 2010; Satchell 2011). CyaA is a key virulence factor of the whooping cough agent and it is a multifunctional protein of 1,706 residues in length (Fig. 3). It consists of an N-terminal enzymatic adenylate cyclase (AC) domain of about 400 residues that is fused to a pore-forming RTX family hemolysin/cytolysin moiety of about 1,300 residues. The latter itself consists of a hydrophobic pore-forming domain (residues 500–700), a fatty-acyl-modified domain (residues 800–1,000), a vast calcium-binding domain characteristic of RTX proteins (residues 1,000–1,600), and a C-terminal secretion signal (Linhartova et al. 2010). The hemolysin part of CyaA is involved in cell binding and enables delivery of the enzymatic AC domain into the cytosol of host cells to convert ATP to cAMP upon activation through binding of eukaryotic calmodulin to the AC domain (Bellalou et al. 1990). In parallel, CyaA is able to oligomerize into pores that permeabilize cell membrane and allow efflux of potassium ions from cells (Gray et al. 1998; Vojtova-Vodolanova et al. 2009). Both toxin activities then depend on the posttranslational activation of proCyaA to CyaA (Hackett et al. 1994; Masin et al. 2005) and on calcium ion loading of the ~40 binding sites in the RTX domain (Rose et al. 1995). CyaA was shown to exert a complex array of cytotoxic and

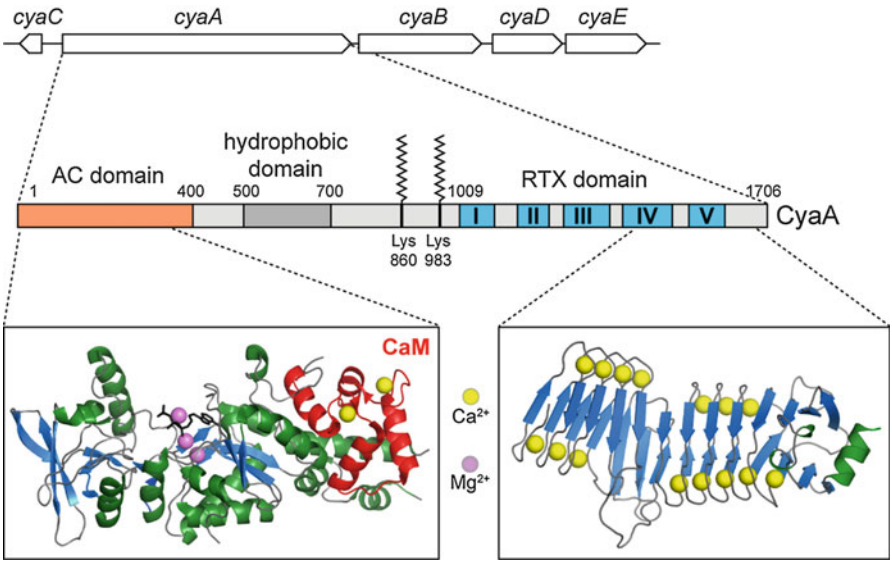

Fig. 3 Schematic representation of the *cyaA* locus and of the CyaA molecule. The *cyaA* gene is located within the *cyaCABDE* locus. The *cyaBDE* genes encode for components of the T1SS apparatus. The *cyaC* gene is transcribed in the opposite direction and encodes the 22-kDa CyaC protein responsible for the posttranslational modification of proCyaA by fatty-acid residues (amide-linked palmitoylation of ε-amino groups of lysine residues 860 and 983). CyaA harbors an N-terminal and cell-invasive adenylate cyclase enzymatic domain, the atomic structure of which was determined by X-ray crystallography (Guo et al. 2005) (*left bottom* panel). The RTX hemolysin contains a hydrophobic pore-forming domain (residues 500–700, an activation domain with two lysine residues (Lys860 and Lys983) modified by acylation), five blocks of RTX nonapeptide repeats with consensus sequence G-G-X-G-X-D, and an unprocessed C-terminal secretion signal. The atomic structure of RTX blocks IV and V has been recently solved by X-ray crystallography (Bumba et al. 2016) (*bottom right* panel)

immunosubversive activities on host phagocytes, to which the toxin specifically binds with high affinity through the integrin CR3 (Guermonprez et al. 2001). Prior to membrane insertion, CyaA toxin initially interacts with N-linked oligosaccharides (Hasan et al. 2015; Morova et al. 2008) and recognizes CR3 through its CD11b subunit (Guermonprez et al. 2001; Osicka et al. 2015; Wald et al. 2016). Over the past decade, evidence has been accumulated that at least two alternative and distinct conformers of CyaA coexist and operate within the target cell membrane. One conformer accounts for translocation of the AC-domain polypeptide across cellular membrane, and the other yields formation of a cation-selective membrane pores (Basler et al. 2007; Osickova et al. 1999, 2010). AC-domain translocation across lipid bilayer of cells does not require endocytosis of the toxin (Gordon et al. 1988) and proceeds directly across the cytoplasmic membrane, with a very short half-time of about 30 s (Rogel and Hanski 1992). The AC translocation process appears to be driven by membrane potential (Otero et al. 1995) and does not involve membrane permeabilization by the CyaA pore (Osickova et al. 2010). It was recently shown

that a membrane translocation intermediate of the AC polypeptide itself participates in formation of a novel type of conduct enabling calcium ion translocation across the membrane of monocytic cells (Bumba et al. 2010; Fiser et al. 2007, 2012). Its formation plays, indeed, a key role in the mechanism of toxin action on CR3-expressing cells, as CyaA-mediated influx of external Ca^{2+} into the submembrane compartment of cells induces activation of the protease calpain and cleavage of the talin tether that anchors CR3 to actin cytoskeleton. As a result, the toxin-receptor complex is mobilized for relocation into the cholesterol-rich lipid microdomains, where the translocation of the AC domain into cell cytosol is completed (Bumba et al. 2010). Supraphysiological levels of cAMP produced by the AC enzyme inside the cytosol of target cells then interfere with signaling of opsonins through complement and immunoglobulin receptors of neutrophils, macrophages, or dendritic cells. As a result, production of bactericidal reactive oxygen and nitrogen species and neutrophil extracellular trap formation by cells are rapidly inhibited (Cerny et al. 2015; Confer and Eaton 1982; Eby et al. 2014). In macrophage cells, the produced cAMP causes transient inhibition of RhoA GTPase activity and elicits massive actin cytoskeleton rearrangements and unproductive membrane ruffling (Kamanova et al. 2008). As a result, phagocytosis and killing of complement-opsonized bacteria by neutrophils and macrophages are blocked (Confer and Eaton 1982; Kamanova et al. 2008; Weingart et al. 2000).

Given the protective immunogenicity of CyaA and the major immunosuppressive role played by the AC toxin in *Bordetella* infections, it appears logical that CyaA toxoid would be considered as a first choice antigen for inclusion in the next generation of acellular pertussis (aP) vaccines. The most advanced application of nonenzymatic CyaA toxoids (genetically detoxified, abbreviated as CyaA-AC^{-}) is their use as a T-cell antigen delivery tool in anticancer immunotherapeutic T-cell vaccines for induction of potent antigen-specific, cytotoxic $CD8^{+}$ T lymphocyte immune responses (Linhartova et al. 2010). Most importantly, the CyaA-AC^{-} toxoid carrying inserted antigens was demonstrated to elicit both protective and therapeutic immune responses against HPV-16-induced tumors and melanoma in mice (Mackova et al. 2006). Phase I/II clinical trials are currently underway to examine CyaA-AC^{-}-based vaccines for immunotherapy of cervical tumors (www.genticel.com) and metastatic melanoma (EU FP6 Theravac Consortium). Recently, the AC domain of the toxoid could be replaced by a synthetic polyepitope construct that could be used for ex vivo expansion of cytomegalovirus-specific human cytotoxic T lymphocytes, thus opening the design of polyvalent therapeutic T-cell vaccines (Adkins et al. 2012; Holubova et al. 2012).

The RTX Domain of CyaA

The most prominent structural feature of CyaA is the presence of calcium-binding repeat domain encompassing the last 700 amino acid residues of the toxin molecule. It consists of five distinct repeat blocks (I–V) of a nonapeptide RTX motif separated by linkers of variable lengths (from 23 to 49 residues) (Osicka et al. 2000). By analogy with several 3-D structures of proteins containing RTX motifs, such as metalloproteases and lipases from Gram-negative bacteria like *P. aeruginosa*

(Baumann et al. 1993) and *Serratia sp.* (Baumann 1994; Hamada et al. 1996), consecutive RTX nonapeptides are arranged in a parallel β-roll structure characterized by two facing sheets of parallel β-strands linked by calcium-loaded turns. A turn is made by the first six amino acids *GGXGXD* from the RTX motif, while the last three residues *XUX* of the RTX motifs are involved in the formation of β-strands and stabilize the hydrophobic core of the β-roll structure. The stacking of consecutive turns and β-strands builds up a right-handed helix of parallel β-strands. One turn of this helix consists of two consecutive RTX motifs. Calcium is usually coordinated between two spatially adjacent turns by the conserved aspartic acids and backbone carbonyl groups (Linhartova et al. 2010).

In the presence of calcium, the whole RTX domain exhibits a stable and compact β-sheet conformation that is further required for efficient binding to target cells (Bauche et al. 2006; Chenal et al. 2009). Deletions of two nonapeptide repeats at the beginning of the repeat block III (residues 1245–1273) or insertions of hexa- or nonapeptides at specific positions (1166, 1281, 1416, 1548) significantly affect the toxin activity (Osicka et al. 2000). Moreover, the residues between 1,166 and 1,281 within the repeat blocks II and III are the major integrin-binding domain of CyaA (El-Azami-El-Idrissi et al. 2003). Of the five repeat blocks, the last repeat block (block V; amino acids 1,523 to 1,638) and its structural integrity appear to be essential for CyaA activity. Deletion of 15 amino acids encompassing the last nonapeptide repeat of block V (residues 1,636–1,650) results in a complete loss of toxin activity (Bejerano et al. 1999). This is due to inability of this mutated RTX domain to fold and adopt a proper conformation required for binding to CR3 on the target cell surface. In line with that, the residues 1,632 to 1,650 were found to be essential for forming of a folding nucleus required for calcium-dependent folding of the isolated $CyaA_{1530–1680}$ segment (Chenal et al. 2010). Calcium loading into RTX repeats and formation of the β-roll structure is then a highly cooperative process within a single RTX block. It appears, moreover, that a high degree of folding cooperativity exists between individual RTX blocks, where folding of block V affects successive folding of the other RTX blocks. Such a chaperone-like activity of block V in folding of the entire RTX domain of CyaA is consistent with predictions made for other members of the RTX protein family (Zhang et al. 2012).

RTX Leukotoxins

The typical RTX leukotoxins (~1,000 residues long) are genetically related and share common, highly conserved motifs (Narayanan et al. 2002). These comprise the N-terminal region, the central region, and the RTX nonapeptide repeats, followed by the C-terminal secretion signal. The N-terminal regions (first ~400 residues) harbor amphipathic α-helices that are involved in the interaction of the polypeptide with the host cell membrane, and formation of the transmembrane pores. The central regions of RTX leukotoxins (residues 400 to 700) consist of large hydrophilic domains with one (LktA) or two (LtxA) internal lysine residues that are covalently modified with fatty-acyl chains. The acylation is required for full biological activities

of the toxins, and the fatty-acyl residues are proposed to play a crucial role in the insertion of toxins into the cytoplasmic membrane of the host cells (Stanley et al. 1998). The RTX domains (residues 700 to 950) are involved in calcium binding and play a vital role in folding of the RTX protein molecules to a proper tertiary conformation that is required for cytotoxicity toward leukocytes. The target host cell receptors bind the RTX repeat domains and this interaction is responsible for the host cell specificity of the RTX leukotoxins.

LktA is a 953 residue polypeptide produced by all serotypes of *M. haemolytica* (formerly *P. haemolytica*). This pathogen is the major cause of fibrinous and necrotizing lobar pneumonia and pleuropneumonia of cattle (known also as shipping fever) and has a marked economic significance in the cattle industry (Rice et al. 2007; Singh et al. 2011). LktA is synthesized in bacterial cytoplasm as a protoxin that is acylated by the *lktC* gene product at the conserved Lys-554 lysine residue, and a second functionally redundant acylation site may also be present. LktA is cytotoxic only for leukocytes derived from a very narrow spectrum of ruminant species, such as cattle, sheep, and goats (Highlander 2001). This species specificity stems from the selective interaction of LktA with the CD18 subunit of the heterodimeric β_2 integrin receptors. The LktA-binding site is formed by amino acids 5–17 of bovine CD18 signal peptide that, in contrast to other nonruminant CD18 molecules, is not cleaved by the signal peptidase in the endoplasmic reticulum during export of the CD18 protein and remains intact on the CD18 subunits on the cell surface (Shanthalingam and Srikumaran 2009). However, the CD11a subunit of the heterodimeric CD11a/CD18 (LFA-1 or $\alpha_L\beta_2$) receptor appears to be involved in the interaction of LktA with host cell surfaces of ruminant leukocytes (Dileepan et al. 2005).

LktA follows a species-specific dose-dependent activation–inhibition paradox. At low concentration, LktA can activate bovine neutrophils and alveolar macrophages to undergo respiratory burst and degranulation. Upon binding to LFA-1, LktA induces tyrosine (Y735) phosphorylation of the CD18 cytoplasmic tail by PI3K and Src kinases. This leads to influx of extracellular Ca^{2+} ions into cell cytoplasm via voltage-gated channels. The elevation of intracellular Ca^{2+} concentrations is essential for triggering NF-κB translocation into the nucleus and production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-8. Phosphorylation of the Y735 residue also stimulates the activity of phospholipase A2 (PLA_2) and C (PLC), which results in production of arachidonic acid derivatives, such as leukotrienes, prostaglandins, and hydroxyeicosatetraenoic acids (HETEs) (Zecchinon et al. 2005). These proinflammatory lipid mediators serve as potent chemoattractants and contribute to the overaccumulation of neutrophils in the lung and subsequent destruction of alveolar epithelium. High concentrations of LktA then induce formation of transmembrane pores that lead to the efflux of K^+ ions, colloidal osmotic swelling, and oncotic cell necrosis. The subsequent cell lysis of activated macrophages and neutrophils causes leakage of inflammatory mediators into the surrounding pulmonary parenchyma, contributing to fibrinous and necrotizing lobar pneumonia (Jeyaseelan et al. 2002). The effects of LktA are further enhanced by

lipopolysaccharide, which is associated with the release of proinflammatory cytokines from leukocytes, activation of complement and coagulation cascades, and cell cytolysis (Singh et al. 2011).

LtxA is a 1,055 residue-long leukotoxin produced by *A. actinomycetemcomitans.* The bacterium colonizes the oral cavity of a large portion of humans and is a major etiologic agent in some aggressive forms of periodontitis (Aberg et al. 2015; Raja et al. 2014). The contribution of *A. actinomycetemcomitans* to disease progression is poorly understood, but its presence in the oral biofilm, together with lifestyle and genetic predisposition, is among the factors that determine disease outcome. Aggressive forms of periodontitis are then associated with the highly leukotoxic JP2 strains of *A. actinomycetemcomitans* that are characterized by a 530 bp deletion in the promoter of the *ltxCABD* operon that is required for the regulation of LtxA secretion (Haubek 2010). Two internal lysine residues (Lys-562 and Lys-687) have been identified as targets for the covalent fatty-acyl modification of LtxA (Balashova et al. 2009). The LtxA protein exhibits a unique specificity against leukocytes from humans and some other primates (Lally et al. 1997). This restricted host cell specificity is mediated through the N-terminal 128 amino acids of human CD11a that are missing in the murine LFA-1 counterpart (Kieba et al. 2007). The cysteine-rich tandem repeats of human CD18 subunit have also been reported to contribute to LtxA binding to the integrin (Dileepan et al. 2007).

Interaction of LtxA with human neutrophils and macrophages leads to cell lysis. LtxA binds to LFA-1 and induces elevation of cytosolic Ca^{2+} concentrations, thus provoking cortical cytoskeleton rearrangements and mobilization of the toxin-receptor complex into cholesterol and sphingolipid-rich membrane microdomains (lipid rafts) (Fong et al. 2006). In neutrophils, these events are followed by an extracellular release of proteolytic enzymes (matrix metalloproteinase 8) from both the primary and secondary granules, likely contributing to resulting tissue damage (Claesson et al. 2002). In macrophages, oligomerization of LtxA-LFA-1 complex within lipid rafts contributes to formation of transmembrane pores that mediate release of cytosolic ATP (Aberg et al. 2015). This binds to the purinergic receptors, such as $P2X_7$ that subsequently trigger efflux of K^+ ions. Efflux of K^+ is associated with formation and activation of the inflammasome complex, which promotes activation of caspase-1 and excessive secretion of interleukin-1β (IL-1β) and interleukin-18 (IL-18). Secreted IL-1β then acts as the major activator of bone resorption and tissue destruction in the host organism. The formation of transmembrane pores also leads to increased osmotic pressure, water influx, cell swelling, and eventual colloid osmotic lysis of the target cells (Johansson 2011).

The very narrow specificity of LtxA for LFA-1 could help in the development of LtxA as a therapeutic agent for leukemia treatment. LFA-1 receptor is upregulated in many lymphomas, and thus malignant cells are more sensitive to LtxA-mediated cytotoxicity than normal cells (Kachlany et al. 2009). In addition, LtxA is highly effective in a SCID mouse xenograft model of human leukemia and shows targeted activity when administered intravenously to a nonhuman primate (DiFranco et al. 2015).

RTX Hemolysins

RTX hemolysins are secreted by Gram-negative pathogenic and commensal bacteria that colonize the respiratory, gastrointestinal, or urinary tracts as well as ocular structures of humans and animals. Production of these toxins accounts for the hemolytic halos of bacterial colonies grown on blood agar plates. In vivo, the RTX hemolysins induce production of inflammatory mediators or display cytotoxic and cytolytic effects on host cells of a broad range of species, thus inducing inflammation, apoptosis, and/or necrosis (Czuprynski and Welch 1995; Frey and Kuhnert 2002; Welch 2001).

HlyA (α-hemolysin) is the best-characterized RTX hemolysin prototype protein. It is secreted by uropathogenic isolates of *E. coli* (UPEC) that account for the majority of urinary tract infections in humans (Wiles et al. 2008). HlyA is a 1,024-residue-long polypeptide consisting of the N-terminal hydrophobic domain containing predicted amphipathic α-helices (residues 177 to 411) (Hyland et al. 2001), a central region including two lysine residues (Lys^{564} and Lys^{690}) that become posttranslationally acylated prior to secretion (Stanley et al. 1994), and the C-terminal calcium-binding domain containing 11 tandemly repeated sequences consisting of nine amino acid residues (residues 739 to 849) (Ludwig et al. 1988). It has been proposed that both the amphipathic α-helical domain and the RTX domain of HlyA are directly involved in membrane interaction (Hyland et al. 2001; Sanchez-Magraner et al. 2007). While the RTX domain is responsible for the early stages of HlyA docking to the target membrane (Sanchez-Magraner et al. 2007), the hydrophobic domain mediates irreversible insertion of HlyA into the membrane, and its structural elements are essential for transmembrane pore formation (Hyland et al. 2001; Valeva et al. 2008).

HlyA is rather promiscuous, exhibiting a well-detectable cytotoxic activity on a wide spectrum of cells from various species, including monocytes, granulocytes, erythrocytes, endothelial cells, or renal epithelial cells from mice, ruminants, and primates (Bhakdi et al. 1989; Gadeberg and Orskov 1984; Keane et al. 1987; Suttorp et al. 1990). It was demonstrated that HlyA recognizes the LFA-1 integrin CD11a/CD18 on the surface of promyelocytic leukemia HL60 cells (Lally et al. 1997). Another study, however, showed that binding of HlyA to granulocytes occurs in a non-saturable manner and that toxin does not interact with a specific protein receptor (Valeva et al. 2005). On erythrocytes that lack LFA-1, HlyA binds the surface sialoglycoprotein glycophorin through a short sequence (residues 914–936) located within the C-terminal RTX domain (Cortajarena et al. 2001, 2003). Besides, HlyA can also bind artificial lipid bilayers or liposomes lacking any protein receptor and can still form functional transmembrane pores (Benz et al. 1989). This indicates that HlyA may bind target cell membranes both in a specific proteinaceous receptor-dependent and receptor-independent manner.

It is assumed that the steps leading to pore formation involve association of HlyA monomers with the membrane through electrostatic interactions, including both the N- and C-terminal domains, membrane anchoring via acyl groups linked to the lysine residues, and insertion of amphipathic α-helical segments of the N-terminal

domain into the membrane. Toxin monomers would then transiently oligomerize into pores by an association–dissociation equilibrium mechanism (Forestier and Welch 1991; Herlax et al. 2009; Hyland et al. 2001; Sanchez-Magraner et al. 2007; Valeva et al. 2008). Shortly after interaction with a target cell, HlyA triggers influx of extracellular calcium ions and efflux of intracellular potassium ions, which both can deregulate host cell signaling cascades (Kloft et al. 2009; Koschinski et al. 2006). HlyA also stimulates the release of ATP from target cells. This may activate P2X receptors and subsequently accelerate the cytolytic effects of HlyA (Bhakdi et al. 1989; Skals et al. 2009). HlyA can induce proinflammatory responses, including the release of IL-1β, secretion of multiple lipid mediators such as prostaglandins and leukotrienes, and the generation of superoxide species (Bhakdi and Martin 1991; Bhakdi et al. 1990; Grimminger et al. 1990; Seeger et al. 1989; Sibelius et al. 2003). Recently, it was demonstrated that in epithelial cells and macrophages, the HlyA activates host serine proteases that specifically cleave components of the proinflammatory NF-κB signaling cascade, the cytoskeletal scaffolding protein paxillin, and other host regulatory proteins (Dhakal and Mulvey 2012).

EhxA (EHEC toxin) exhibits 61% identity with HlyA of UPEC and is produced by the enterohemorrhagic isolates of *E. coli* (EHEC) that provoke diarrhea, hemorrhagic colitis, or hemolytic-uremic syndrome (Karch et al. 2005; Schmidt et al. 1994). EhxA is expressed from an EHEC-*hlyA* gene localized within an operon consisting of four genes (EHEC-*hlyCABD*) located on the 90-kbp pO157 plasmid (Schmidt et al. 1995, 1996). Like HlyA, also EhxA has an N-terminal hydrophobic domain mediating insertion of the toxin into the target cell membrane and forming a transmembrane pore. EhxA has two potential acylation sites on Lys^{550} and Lys^{675}. The RTX portion harbors a tandem array of 13 nonapeptide repeats binding calcium ions and a C-terminal secretion signal recognized by the T1SS apparatus (Schmidt et al. 1995; Stanley et al. 1998). In contrast to HlyA, however, EhxA displays a narrow target cell specificity for human intestinal epithelial and microvascular endothelial cells (Aldick et al. 2007). EhxA exhibits very little activity against human lymphoma cell lines and binds sheep as well as human erythrocytes much less efficiently than HlyA (Bauer and Welch 1996a, b).

EhxA was found in two biologically active forms, one including a free, soluble EhxA protein and the other associated with outer membrane vesicles (OMVs) released by EHEC during growth (Aldick et al. 2009). The OMV-associated EhxA results from a rapid binding of free EhxA to OMVs upon its extracellular secretion and is substantially more stable under physiological conditions than the free EhxA (Aldick et al. 2009; Bielaszewska et al. 2013). Both free and OMV-associated EhxA bind human erythrocytes in vitro and cause hemolysis, which strictly depends on acylation of the toxin and on the presence of calcium ions (Aldick et al. 2009). A role of EhxA in the pathogenesis of EHEC-mediated diseases was investigated using human epithelial colorectal adenocarcinoma cells (Caco-2) and human brain microvascular endothelial cells (HBMEC) as surrogate models for intestinal epithelial and microvascular endothelial cells (Bielaszewska et al. 2013). In contrast to free EhxA, which remains on the cell membrane, the OMV-associated EhxA is internalized by

cells via dynamin-dependent endocytosis of OMVs and trafficked with OMVs into endo-lysosomal compartments. Upon endosome acidification and subsequent pH drop, EhxA separates from OMVs; escapes from the lysosomes, most probably via its pore-forming activity; and targets mitochondria. The presence of EhxA in mitochondria reduces the mitochondrial membrane potential and releases cytochrome c to the cytosol. Subsequent activation of caspases 9 and 3 leads to apoptotic cell death (Bielaszewska et al. 2013). The proinflammatory potential of EhxA may contribute to the pathogenesis of EHEC-mediated diseases through release of the proinflammatory cytokine interleukin-1β from human monocyte/macrophage cells (Taneike et al. 2002).

Hemolysins MmxA of *M. morganii* and PvxA of *P. vulgaris* are homologues of HlyA (Koronakis et al. 1987). Both bacteria are a common cause of opportunistic infections in humans (McDermott and Mylotte 1984; Toth and Emody 2000), and both form cation-selective pores in lipid-bilayer membranes, exhibiting pore characteristics similar to those of *E. coli* HlyA (Benz et al. 1994).

The MbxA hemolysin secreted by *M. bovis* is implicated in the pathogenesis of infectious bovine keratoconjunctivitis, the most important ocular disease affecting cattle worldwide (Brown et al. 1998). MbxA promotes the development of corneal ulcers by lysis of corneal epithelial cells (Beard and Moore 1994; Kagonyera et al. 1989; Rogers et al. 1987).

A large group of homologous cytolytic RTX hemolysins is secreted by bacteria of the genus *Pasteurellaceae*. These include ApxIA, ApxIIA, and ApxIIIA of *Actinobacillus* spp. (Frey and Kuhnert 2002; Chang et al. 1989; Rycroft et al. 1991), PaxA of *P. aerogenes* (Frey and Kuhnert 2002), and AqxA of *A. equuli* (Berthoud et al. 2002). Genetic analysis suggested that RTX determinants might have evolved in *Pasteurellaceae* and spread to other Gram-negative bacteria by horizontal gene transfer.

The most studied RTX toxins of the genus *Pasteurellaceae* are the three different ApxA exotoxins (ApxIA, ApxIIA, and ApxIIIA). These are secreted by the bacterium *A. pleuropneumoniae*, the etiological agent of porcine pleuropneumonia, which is a severe, highly contagious, and economically significant infectious disease (Chiers et al. 2010). ApxIA shows strong hemolytic activity, while ApxIIA exhibits weaker hemolytic activity (Frey and Nicolet 1990; Kamp et al. 1991). Both are cytotoxic and active on various cells of different types and species (Rosendal et al. 1988). The ApxIIIA protein is strongly cytotoxic and targets mainly porcine alveolar macrophages and neutrophils (Rycroft et al. 1991). ApxIIIA is nonhemolytic on erythrocytes but shows a significant co-hemolytic reaction dependent on the sphingomyelinase from β-hemolytic *Staphylococcus aureus* (CAMP reaction) (Frey et al. 1994; Kuhnert et al. 2000). It was demonstrated that ApxIIA is essential in the pathogenesis of porcine pleuropneumonia, and the combination of ApxIA and ApxIIA secreted by certain serotypes of *A. pleuropneumoniae* accounts for the severe course of the disease with a fatal outcome (Reimer et al. 1995). A fourth secreted RTX protein of *A. pleuropneumoniae*, ApxIVA, is produced in vivo but not under in vitro conditions. Its biological activity remains unknown (Cho and Chae 2001; Schaller et al. 1999).

PaxA is secreted by *P. aerogenes*, which is known as a commensal bacterium or opportunistic pathogen causing abortion in humans, swine, and other mammals. PaxA is structurally similar to ApxIIIA and also shows functional analogy to ApxIIIA, since it is devoid of a direct hemolytic activity and shows co-hemolytic activity with the sphingomyelinase of *S. aureus* (Frey et al. 1994; Kuhnert et al. 2000). PaxA is also produced by *P. mairi* but its cytotoxic activity has not yet been analyzed in detail (Frey and Kuhnert 2002). While all *P. aerogenes* and *P. mairi* strains isolated from aborted fetuses and neonatal septicemia of pigs produce PaxA, strains devoid of the *paxA* gene have been isolated as opportunistic pathogens or commensals (Kuhnert et al. 2000).

The AqxA hemolysin is produced by *A. equuli*, the etiologic agent of a frequently lethal neonatal septicemia in foals as well as other more chronic diseases like pleuritis, pneumonia, peritonitis, or arthritis (Berthoud et al. 2002). It is supposed that AqxA could be a crucial virulence factor (Berthoud et al. 2002).

Multifunctional Autoprocessing Large RTX Toxins

A quite different RTX toxin subfamily consists of as yet 18 described multifunctional autoprocessing RTX toxin (MARTX) proteins. These extremely large toxins differ from all other previously described RTX proteins by the molecular structure and *rtx* gene cluster organization. MARTX have thus far been identified in 14 different Gram-negative bacterial species isolated as mammalian, aquatic, and insect pathogens of the genera *Vibrio*, *Photorhabdus, Proteus, Aeromonas, Xenorhabdus*, and *Yersinia* (Satchell 2011).

The MARTX toxins are often encoded by two divergent operon loci – *rtxHCA* and *rtx*B*DE* (Fig. 1). The toxin gene is found as the third gene downstream of the *rtxC* homologue (a putative acyl transferase) and a conserved hypothetical gene (*rtxH)* of unknown function, which is found only in the *martx* gene clusters. If acylation of the MARTX toxin is required for toxin function, it has not been demonstrated yet. Moreover, deletion of the *rtxC* gene has no effect on virulence of *V. vulnificus* (Cheong et al. 2010; Lee et al. 2007; Liu et al. 2007). This suggests that acylation must not be essential for toxicity of MARTX proteins.

The divergent operon *rtxBDE* encodes homologues of the ATP-binding transporter protein RtxB, the membrane fusion protein RtxD, and a second ATPase, RtxE (Boardman and Satchell 2004). This atypical four-component T1SS seems to be a conserved feature across the entire MARTX family (Boardman and Satchell 2004).

Production of MARTX T1SS components is regulated by growth phase. The repressor regulating *rtxBDE* expression is encoded outside of the *rtx* locus and is not directly linked to quorum sensing, while *V. cholerae* may apparently couple the regulation of the *rtx* locus to stress (Boardman et al. 2007).

All the *rtxA*-like genes encode proteins, ranging from 3,500 to 5,300 residues. Relatively well-conserved RTX-repeat regions are at both the C- and N-termini. Contrary to other toxins, the C-terminal repeats, revealing membership of MARTX proteins to the RTX family, exhibit an 18-residue-long consensus sequence X(V/I)

XXGXXNX(V/I)XXGDGXDX and share a common G-7X-GXXN central motif, instead of the typical nonapeptide repeats. Their function in Ca^{2+}-binding and toxin secretion seems similar as for RTX toxins (Kim et al. 2015; Lin et al. 1999; Satchell 2007). Moreover, MARTX proteins possess additional N-terminal repeats, which fall into two classes. The first has a 20-residue consensus sequence GXXG(N/D)(L/I)(T/S)FXGAG(A/G)XNX(L/I)X(RH) and the second a 19-residue consensus T(K/H)VGDGX(S/T)VAVMXGXAN (I/V)X.

The proposed function of both the N- and C-terminal RTX repeats is the formation of a pore, or pore-like, structure at the host cell membrane that promotes translocation of effector domains into the target cell cytosol (Kim et al. 2015).

The central effector regions of MARTX are composed of one to five differing activity domains that upon entry into the eukaryotic cell may exert different cytotoxic activities (Boardman and Satchell 2004; Satchell 2007). Although ten various effector domains are known until now, only six of them have been characterized for function, the remaining four are often similar to other toxins (Antic et al. 2015; Satchell 2011).

A cysteine protease domain (CPD) essential for autoprocessing and releasing of effector domains into host cytoplasm is a conserved feature of MARTX proteins. The cysteine protease is activated by binding of inositol phosphate molecules on the cytosolic side of the cell membrane, preferentially inositol hexakisphosphate ($InsP_6$). It can be reactivated by cooperative binding of another substrate and $InsP_6$ molecule (Prochazkova and Satchell 2008; Prochazkova et al. 2009). Because $InsP_6$ is exclusive to eukaryotes and present at cytosolic concentrations exceeding 10 μM, the evolution of a proteolytic biosensor responding to $InsP_6$ appears to be an ingenious strategy for assuring that functional activation of a secreted toxin occurs only once it has reached host cell cytosol (Lupardus et al. 2008).

The mechanism of MARTX molecule translocation through the cell membrane prior to CPD processing remains unknown. Low thermostability of the MARTX effector domains might play an important role in this process. A fully unfolded protein appears to be a better substrate for translocation through a narrow pore in the eukaryotic membrane (Kudryashova et al. 2014).

The actin cross-linking domain (ACD) mediates covalent cross-linking of actin molecules, actin net depolymerization, formation of actin multimers, and blocking of the actin cytoskeleton. The ACD of $MARTX_{Vc}$, located between residues 1,963 and 2,375, catalyzes a unique intermolecular iso-peptide bond between the γ-carboxyl group of glutamic acid residue 270 and the ϵ-amino group of lysine residue 50 of actin (Kudryashov et al. 2008).

The Rho GTPase-inactivation domain (RID) has been linked together with the ACD domain to cell rounding and inactivation of the Rho family GTPases causing cytoskeleton depolymerization (Sheahan and Satchell 2007). The mechanism of Rho inactivation by the RID has not been revealed. RID protein is a thiol protease (Pei and Grishin 2009), but there is no evidence that Rho itself is processed. Given that cells activate Rho after removal of bacteria in the absence of protein synthesis, the inactivation of Rho by RID is probably mediated by an indirect mechanism and not by processing (Sheahan and Satchell 2007).

The most virulent strains of *V. vulnificus* produce a 5,206-amino acid MARTX toxin with effector domain in the fifth position termed $DUF5_{Vv}$. Bacterial strains producing DUF5 domain were found to be 10- to 50-fold more virulent in mice. Recently, it was shown that $DUF5_{Vv}$ has a site-specific endopeptidase activity. It cleaves the Switch 1 region of Ras and Rap1, inactivates ERK1/2, and subsequently inhibits cell proliferation (Antic et al. 2015).

By homology to other toxins, an adenylate cyclase domain was identified, which converts ATP to cAMP. Another abundant domain of MARTX protein has strong homology to the αβ-hydrolase family of enzymes. Nine of the MARTX toxins share with *P. luminescens*, a "makes caterpillars floppy" (MCF) domain. Within MCF_{Vv} and MCF_{Ah} (from the *A. hydrophila* $MARTX_{Ah}$ toxin), a cysteine protease catalytic site essential for autoprocessing activation of the toxin was identified, which is essential for toxin activation and processing of cellular target protein that results in cell rounding (Dolores et al. 2015; Dowling et al. 2004, 2007). Another "*Pasteurella multocida* toxin" (PMT) domain has similarity to the toxin of *P. multocida* (Kamitani et al. 2010).

The MARTX prototype gene *rtxA* was found in both clinical and environmental isolates of *V. cholerae*, but not in the O1 classical biotypes (Chow et al. 2001; Lin et al. 1999). The 4545-amino acid RTX toxin was initially characterized for its ability to round epithelial *cells* in vitro (Lin et al. 1999). Even though this more common variant of the two known *V. cholerae* MARTX toxins carries both ACD and RID cell rounding domains, histological studies on intestinal tissues of cholera patients revealed minimal tissue damage (Mathan et al. 1995). The most crucial target for $MARTX_{Vc}$ in vivo hence seems to be knocking down of the innate immune system (Ma et al. 2009; Satchell 2011).

Novel variants of $MARTX_{Vv}$, designated biotype 3, have now been added to the four previously known variants of $MARTX_{Vv}$ of *V. vulnificus*. All of these RTX toxins have been proposed to be essential virulence factors inducing severe destruction of intestinal tissue. Their actions lead to rapid bacterial dissemination in vivo and rapid cell lysis, actin depolymerization, Rho GTPase-inactivation, caspase 3/7-dependent apoptosis, cAMP production, and induction of reactive oxygen species in vitro (Fan et al. 2001; Liu et al. 2007; Satchell 2011; Ziolo et al. 2014).

Conclusion and Future Directions

The members of the unique RTX family of toxins have been shown to play a major role in virulence of Gram-negative pathogens. Recent discovery and advances in characterization of the very large MARTX toxin family represent a breakthrough revelation on the degree of sophistication and complexity of bacterial protein toxin action, introducing the concept of a "multi-warhead" protein toxin manipulating and conditioning host cell physiological functions for the benefit of the pathogen.

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Bacterial Genotoxins as the Interphase Between DNA Damage and Immune Response

20

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Abstract

Bacterial genotoxins are a class of molecules that have the ability to enter the nucleus of a host cell and cause DNA damage by introducing single- and double-strand DNA breaks, leading to various effects, including activation of DNA damage response, senescence, apoptosis, and genetic aberrations. There is emerging evidence for an intricate connection between the DNA damage response and immunity, and it is becoming increasingly clear that bacterial genotoxins can act as potent immunomodulatory factors, which bacteria use in order to tailor the host immune response. This chapter will review some of the basic structural and functional characteristics of bacterial genotoxins and the internalization pathway used to reach the host DNA within the nuclear compartment. Special focus will be given to the connection between the genotoxin-induced DNA damage response and modulation of the host immune responses. Since it is not entirely clear what is the evolutionary advantage for bacteria that express these effectors, the possibility that they might play a role in influencing host immune response in order to promote stealth invasion and establishment of persistent infections will be explored.

Keywords

Bacterial genotoxins • DNA damage response • Inflammation • Immunosuppression

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Contents

Introduction

The outcome of an infection is dictated by the nature of host-pathogen interactions and firstly depends on efficacy of the host immune system to clear or control the invading pathogen. However, bacteria have evolved an arsenal of sophisticated mechanisms to overpower the host immune response and establish a successful infection (do Vale et al. 2016). A pathogenic bacterium is able to infect the host, to persist and proliferate in the body, to avoid the immune system, and to induce pathology by damaging the host either at the cellular level, in the tissue, or in the whole organism (Sansonetti 2011). Bacterial toxins, which can act as virulence factors, contribute to the bacterial pathogenicity (do Vale et al. 2016; Lemichez and Barbieri 2013) and, for certain bacteria, are the main cause of diseases, such as diphtheria, cholera, anthrax, botulinum, and tetanus (Dobrindt et al. 2015).

This review will focus on an unusual mechanism for modulating the host immune response induced by a family of bacterial toxins that are collectively known as genotoxins, since their mode of action is to directly promote DNA damage (Frisan 2016). Bacterial genotoxins belong to a larger group of cyclomodulins, as they have the ability to negatively or positively interfere with progression of the cell cycle (Gargi et al. 2012). The most studied genotoxin is a protein trimer and belongs to the cytolethal distending toxin (CDT) family, produced mainly by extracellular Gram negative bacteria such as *Escherichia coli*, *Aggregatibacter actinomycetemcomitans*, *Haemophilus ducreyi*, *Campylobacter sp.*, and *Helicobacter sp.* (Guerra et al. 2011a). The intracellular pathogen, *Salmonella enterica* serovar Typhi, produces a protein genotoxin named typhoid toxin (Haghjoo and Galan 2004), while colibactin is a peptide-polyketide genotoxin, produced by strains belonging to the phylogenetic group B2 of *Escherichia coli* (Guerra et al. 2011a).

This chapter will firstly describe some general aspects about the link between DNA damage and immune responses, and then will focus on how genotoxins can act as immunomodulators of the host response.

Relationship Between Host DNA Damage Response and Immunity

The DNA Damage Response: A Powerful Host Defense

Cells are constantly exposed to various endogenous and exogenous agents that jeopardize the integrity of genetic information. To cope with DNA damage, cells have evolved a complex response network called DNA damage response (DDR) (Ciccia and Elledge 2010). Since bacterial genotoxins primarily induce single- and double-stranded DNA breaks, special focus will be given to these types of DNA damage.

Single-strand breaks (SSBs) can be generated in a variety of ways, including ionizing radiation (IR), reactive oxygen species (ROS), as well as an intermediate in the processing of various lesions by base excision repair (BER). SSBs are initially sensed by the poly(ADP-ribose) polymerase (PARP) family members, PARP1 and PARP2, via their zinc-finger motifs. Once at the site of DNA damage, they rapidly (within seconds) assemble poly(ADP-ribose) chains (PAR chains) on histones H1 and H2B, which are thought to promote chromatin reorganization and recruitment of repair factors. Some of the proteins that promote repair of SSBs include DNA polymerase β; PNK; the nucleases APE1, APTX, and APLF; and the ligation factors XRCC1 and DNA LIG3 (Caldecott 2014).

DNA double-strand breaks (DSBs) are one of the most dangerous lesions, as they can lead to chromosomal translocations, loss of genetic material, genomic instability, and potentially to cancer development. Some of the effects of DDR induced by DSBs include upregulation or activation of DNA repair genes, damage tolerance, cell cycle checkpoint activation, apoptosis, and senescence. In case of DSBs, the two major pathways that repair these lesions are nonhomologous end joining (NHEJ) and homologous recombination (HR); however, some other nonconventional mechanisms have been identified, such as single-strand annealing (SSA), microhomology-mediated end joining (MMEJ), and alternative-NHEJ (alt-NHEJ) (Mladenov et al. 2016).

NHEJ does not rely on homology, thus it can operate at any phase of the cell cycle, and it is generally considered to be error-prone, resulting in small deletions or insertions following repair of the break. In contrast, HR requires extensive homology from a donor sequence, limiting it to the S and G2 phases of the cell cycle, when sister chromatid is available as the source of genetic information required for the successful repair.

Regardless of the source of DSBs (e.g., programmed DSBs during V(D)J recombination, class switch recombination and meiosis, IR, replication fork collapse, bacterial genotoxins), the breaks are initially sensed by several different sensory proteins which then transduce the signal through mediators to the effector proteins.

Rapidly after creation of DSBs, the lesions are bound by either Ku70/Ku80 or by PARP1/2 proteins. Broken DNA ends can also be recognized by the MRN complex (consisting of Mre 11, Rad50, and Nbs1 proteins in mammalian cells),

which tethers DNA ends and promotes recruitment of downstream signaling and repair factors. Three main apical protein kinases act immediately downstream from DNA break recognition: ataxia-telangiectasia mutated (ATM), ATM and Rad3 related kinase (ATR), and DNA-dependent protein kinase (DNA-PK). They belong to the phosphatidylinositol 3-kinase-related family and, upon activation, phosphorylate a wide range of targets on serine or threonine residues (Awasthi et al. 2015).

The main signaling pathway that is activated by the MRN complex during the initial stages of repair by HR is the ATM-Chk2 pathway. After generation of DSB, MRN complex recognizes the break and both recruits and induces activation of ATM. In its inactive state, ATM is a dimer that can undergo autophosphorylation on serine (Ser) 1981 following DNA damage. One of the most important targets of ATM is the histone variant H2AX, whose phosphorylated version is named γH2AX. Since γH2AX can extend up to a few megabases around the lesion, forming discernible nuclear foci easily detectable by fluorescent microscopy, it is commonly used as a marker for DNA damage. γH2AX serves as a platform for recruitment of many more downstream factors in a positive-feedback loop manner, including MDC1, RNF8, RNF168, 53BP1, and BRCA1. In contrast to these factors that remain localized close to the DNA break, several others have the ability to diffuse through the nucleus and influence targets that are situated away from the break, including the Chk1 and Chk2 kinases that help regulate cell cycle arrest (Bekker-Jensen and Mailand 2010). During HR, DSBs can be resected, and the long stretches of single-strand DNA (ssDNA) exposed are bound and protected by the RPA protein. The resection was recently shown to occur in two steps, called short-range and long-range resection. Short-range resection is mediated by the Mre11 protein, which possesses both endo- and exo-nuclease activity, and by the CtIP protein. Subsequently, long stretches of ssDNA will be exposed in a long-resection step dependent on EXO1, BLM, and WRN helicases/nucleases (Cejka 2015). The presence of ssDNA coated with RPA promotes recruitment and activation of ATR, which can subsequently phosphorylate its downstream targets. One of the principal mediators activated by ATR is the Chk1 kinase, a diffusible protein that has the ability to prevent DNA replication and cell cycle progression in case of persisting DNA damage.

Alternatively, DSBs can be repaired by NHEJ, where DNA-PK plays an important role in stabilizing DNA ends and inhibiting their resection. Loading of the DNA-PK is followed by the recruitment of the XRCC4/DNA Lig4, which seals the end with the help of the stimulatory factor XLF. In case it is not possible to directly ligate the DNA ends, the nucleases ARTEMIS and APLF, as well as PNK kinase/phosphatase will further process the end prior to ligation. Because this process of limited editing does not rely on a template with original genetic information, as is the case in HR, it often results in micromutations. In the series of elegant studies using either “clean” or “dirty” DNA ends in yeast cells (i.e., ones that do not or do require processing before ligation), it was clearly shown that mutations arise only in the case of later kinds of damage (Ciccia and Elledge 2010).

Interphase Between DNA Damage and Host Immune Response

Both DDR and an immune response are needed for the preservation of homeostasis. It is becoming increasingly clear that these two systems interact and influence each other. In particular, exposure to various DNA-damaging agents can influence both innate and adaptive immune responses.

DNA damage is perceived as a danger signal by the organism, and it is this perception of danger that ultimately triggers activation of the immune response. It is important to note that, in contrast to the classical "self-nonself" model (where foreign substances are the prerequisite to prime the immune response), this "danger model" assumes that "cellular discomfort" is the key trigger of immune system activation, where "danger" is defined as anything that can cause tissue stress or destruction (Matzinger 2001). An example of an interesting link between DNA damage and regulation of immune response is the recent observations showing that exposure to UVB irradiation can lead to both immunostimulation or immunosuppression effects, mediated through Toll-like receptors (TLRs) TLR3 and TLR4 (Ahmad et al. 2014; Bernard et al. 2012). Ahmad et al. have shown that a self, noncoding RNA released from keratinocytes after exposure to UVB stimulates production of inflammatory cytokines IL6 and TNF-α in nonirradiated keratinocytes and peripheral blood mononuclear cells in a mechanism dependent on TLR3. Bernard et al. showed that TLR4 plays a major role in UVB-induced immunosuppression through regulating IL12 and IL23 production, which ultimately reduces expression of DNA repair factors and prolongs persistence of DNA damage in the cells, by interfering with the nucleotide excision repair (NER) mechanism.

There are several other examples of DDR components influencing immune responses. One of the apical kinases for DSB detection and repair is the ATM kinase. Research by Croxford et al. has shown that DDR induced by sustained *myc* expression leads to upregulation of DNAM-1, an adhesion molecule constitutively expressed by the majority of immune cells, in an ATM-dependent manner. DNAM-1 upregulation then in turn activates $CD4^+$ and $CD8^+$ T lymphocytes and NK cells, leading to tumor regression in mouse lymphoma models (Croxford et al. 2013). Furthermore, ATM activates the NKG2D ligand after DNA damage or replication stalling in human dermal foreskin fibroblasts, and this in turn recruits NK cells to the injury sites. This mechanism might be extremely important in alerting the immune system to the presence of potentially dangerous (premalignant or tumor) cells in case of constitutive DNA damage (Gasser et al. 2005).

NFkB is a family of transcription factors that acts as a key regulator of immune system development, immune response, inflammation, and cancer. Interestingly, ATM has been shown to influence the activation of NFkB in several different contexts; this activation happens mainly through interaction of NEMO ("NFkB essential modulator") with ATM; however, the molecular mechanisms of this interaction are still not fully characterized. This so-called inside-out signaling, where the nuclear ATM influences cytoplasmic NFkB, is particularly important during lymphocyte differentiation and potentially during development of human lymphatic malignancies (reviewed in McCool and Miyamoto 2012).

The MRN complex, main sensor of DSBs, also has the ability to activate NFkB. Upon induction of DSBs, Rad50, one of the components of MRN, binds to the innate immune system adaptor CARD9, forming a dsDNA-Rad50-CARD9 signaling complex that can activate NFkB and cause production of pro-IL1 in primary cells and mice models (Roth et al. 2014). It must be stressed at this point that there is a growing body of evidence for the role of NFkB in the induction of so-called senescence–associated secretory phenotype (SASP). Senescence is usually considered a mechanism that is tumor suppressive due to induction of an irreversible cell cycle arrest. However, senescent cells secrete proinflammatory factors (like IL1) that can have protumorigenic effect (Salminen et al. 2012).

One of the most important proteins that participate in regulating immune responses following DNA damage is tumor suppressor p53, one of the main downstream effectors of the DDR. p53 was shown to potently activate immune response by causing upregulation of many macrophage cell surface molecules involved in antigen presentation, including ICAM-1, CD58, CD59, and LFA-3 (Gorgoulis et al. 2003). A similar effect was observed for fibroblasts, which gain the ability to activate naïve $CD8^+$ lymphocytes through ICAM-1 upregulation caused by DNA damage. By using an in vivo approach, Xue and collaborators have shown that the induction of p53 in mouse models of liver carcinoma leads to induction of cellular senescence, accompanied by the upregulation of inflammatory cytokines (Xue et al. 2007).

Bacterial Genotoxins Induce DNA Damage and Can Modulate Host Immune Response

So far three groups of bacterial genotoxins have been identified: cytolethal distending toxin family, typhoid toxin, and colibactin. This part of the review will describe the structure, the activity, and the internalization into cells of the three known genotoxins. Since the structure and internalization pathways of bacterial genotoxins have been extensively characterized elsewhere (DiRienzo 2014; Frisan 2016; Grasso and Frisan 2015), we will briefly review these issues, before discussing their role as immunomodulators.

Genotoxins: A Novel Family of Microbial Effectors

Structure and Activity

CDTs are comprised of so-called A and B components, where "A" stands for active and "B" for binding. They have an AB_2 configuration, meaning they are made of one active (CdtB) and two binding (CdtA and CdtC) subunits (Fig. 1). All of the studied CDTs are transcribed from three linked genes and code for the proteins whose respective molecular weights are approximately 25 kDa (CdtA), 30 kDa (CdtB), and 20 kDa (CdtC). Sequence homology studies have shown that CdtB is the most

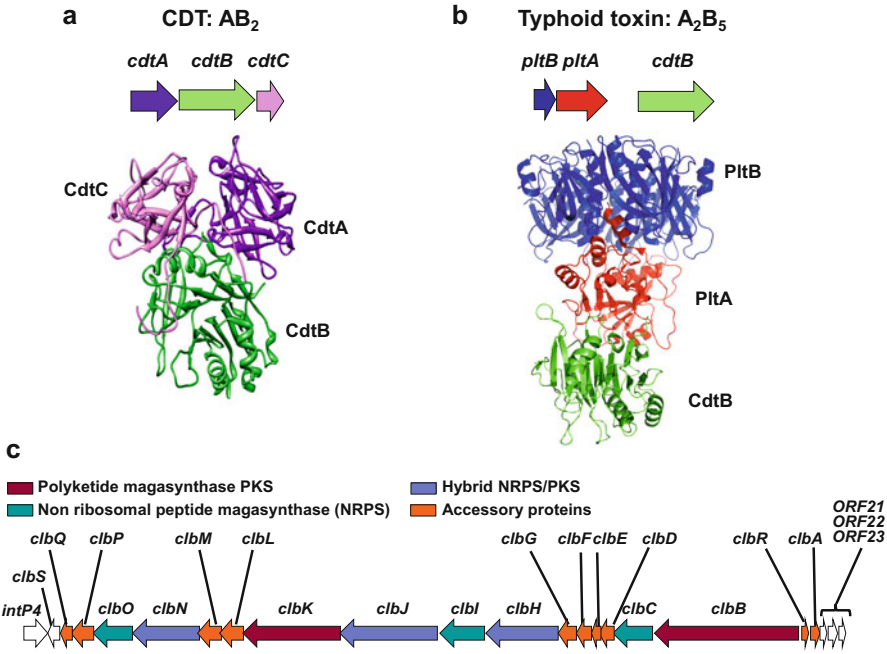

Fig. 1 Structure of the three known genotoxins: CDTs (cytolethal distending toxins), typhoid toxin, and colibactin. (**a**) Schematic representation of the CDT genes from *H. ducreyi* and the crystal structure of the holotoxin (Adapted from Nesic et al. 2004), PDB access number: 1SR4. The CdtB is the active subunit, possessing DNase activity, while the CdtA and CdtC accessory subunits constitute the binding component of this AB_2 toxin. (**b**) Schematic representation of the typhoid toxin genes from *Salmonella enterica* serovar Typhi and crystal structure of the holotoxin (Adapted from Song et al. 2013), PDB access number: 4K6L. CdtB is connected by PltA to a pentameric ring made by five PltB monomers (the "B" moiety). This A_2B_5 toxin contains two active subunits: CdtB, homologous to mammalian DNase I, a characteristic shared with CDTs, and the ADP-ribosyl transferase PltA. (**c**) Schematic representation of the *pks* genomic island that encodes the enzymes and accessory proteins required for synthesis of an active colibactin in the *E. coli* strain Nissle 1917 (Adapted from Homburg et al. 2007) with permission from the authors)

conserved subunit, with 45% sequence similarity between most distant members, while CdtA and CdtC are more divergent.

As the number of CDT-producing species described increases, there is a need for consistent and precise annotation of these toxins. The currently proposed system of nomenclature uses a capitalized first letter of the bacterial genus followed by the first three letters (in lower case) of the bacterial species (e.g., EcolCDT for CDT produced in *E. coli*). In case there is more than one CDT produced in particular bacterial species, it is marked by roman numerals (e.g., EcolCDT-I, EcolCDT-II, EcolCDT-III). This system will be used throughout this review.

CdtB shares structural and functional homology with the human DNase I enzyme, and thus has the capacity to induce either single- or double-strand DNA breaks, although with approximately 100 times less efficiency than DNase I.

Biochemical studies have shown that the intoxicating effect of CDTs on yeast and mammalian cells can be completely abolished by introduction of mutations in either catalytic or Mg^{2+} binding domains of CdtB (Elwell and Dreyfus 2000). In vitro studies have shown that the exact ratio of single-stranded versus double-stranded DNA breaks induced by CdtB in host cells depends on toxin concentration, where lower amounts of CDTs mostly induce SSBs, and higher amounts induce DSBs, possibly due to the juxtaposition of two close SSBs in the two opposite strands of the DNA molecule; however, whether CDT holotoxin can directly induce DSBs is not entirely clear (Bezine et al. 2014). Besides having DNase activity, CdtB from certain species was also shown to be a phosphatase. CdtB from *A. actinomycetemcomitans* possesses PI-3,4,5-triphosphate phosphatase activity in human leukemia T cell lines, which expresses high amounts of PI-3,4,5-triphosphate due to mutations in the PTEN gene. Mutation analysis has shown that CDT toxicity correlates with the CdtB phosphatase activity in lymphocyte cell lines. However, the DNase activity of AactCDT is sufficient to induce cell death in proliferating monocytes, and studies performed in yeast (which do not have the substrate for phosphatidylinositol-3,4,5-triphosphate phosphatase) have shown that G2 arrest and cell death depend exclusively on the CdtB nuclease activity (Shenker et al. 2016). Future research will have to clarify how various cell lines respond to the phosphatase activity of CdtB.

CdtA and CdtC proteins share structural similarity and are both similar to the ricin toxin B-domain. They are required for the binding and internalization of the CDT holotoxin, probably by binding to the sugars on the cell surface, although the exact mechanism(s) remains to be elucidated (Frisan 2016).

Typhoid toxin is produced by the intracellular pathogen *Salmonella typhi* and can either intoxicate the infected cell or be released into the extracellular environment and intoxicate noninfected, bystander cells. The typhoid toxin is composed of three subunits: CdtB, PltA, and PltB, arranged in an A_2B_5 configuration (Fig. 1). The active subunits are: CdtB, similar in sequence and function to the CdtB subunit belonging to CDTs, and PltA, which is a functional ADP-ribosyl transferase; however, its cellular targets are still unknown. Finally, PltB acts as a binding subunit and forms a pentameric ring that participates in internalization of typhoid toxin (Grasso and Frisan 2015; Song et al. 2013).

Colibactin is the only member of the bacterial genotoxins family that is not a protein, but a peptide-polyketide compound produced as a secondary metabolite by certain members of human gut microbiota that include the B2 phylogenetic group of *E. coli*. The gene cluster that produces colibactin (Fig. 1) was discovered in 2006 in a study that found that HeLa cells undergo formation of DSBs, cell cycle arrest and distension of both cell body and nucleus if exposed to certain strains of *E. coli*, reminiscent of classical responses to bacterial genotoxins (Nougayrede et al. 2006). The gene cluster responsible for colibactin synthesis was shown to be the *pks* "pathogenicity island" (*pks* island); mutations in any of its 23 genes render colibactin inactive (Balskus 2015).

Internalization of Genotoxins into the Cells

In order to induce DNA damage in host cells, genotoxins have to be transported from the cell exterior into the nucleus by crossing several membrane compartments, including the cell membrane, endosomal and Golgi compartments, endoplasmic reticulum (ER), and finally the nuclear membrane. Here, this journey will be briefly described, as this topic was discussed in more details in a recent review (Frisan 2016). CDTs are produced and secreted mainly by extracellular bacteria and subsequently bind to target membranes. Lipid rafts that contain cholesterol and sphingolipid rich domains most probably mediate the initial binding of CDTs to target membrane (Boesze-Battaglia et al. 2006). As to the exact nature of the CDT receptor(s), this is still unknown. One assumption is that it should be commonly expressed, as CDTs have the ability to intoxicate a wide range of cell lines, but given the low degree of sequence similarity of the CdtA and CdtC subunits, it is possible that each CDT binds to a specific receptor.

There is only limited data on the nature of the receptor for the typhoid toxin. One study has suggested that this effector preferentially binds to glycoproteins instead of glycolipids, based on the crystal structure of the PltB pentameric ring (Song et al. 2013). Consistent with this, epithelial cell lines cannot be effectively intoxicated when mutations are introduced into the sugar-binding pocket of PltB, and removal of surface glycans from the cell surface or use of cell lines that lack complex and hybrid N-glycans on glycoproteins results in decreased binding of typhoid toxin to the cell membrane.

The mechanisms of intercellular trafficking of genotoxins is incomplete and only partially elucidated. The best-studied toxins in term of internalization are from the CDT family. In HeLa cells, HducCDT is internalized by dynamin-dependent and clathrin-independent endocytosis (Cortes-Bratti et al. 2000). HducCDT is further translocated from early to late endosome, as the expression of a dominant negative mutant of Rab7 (marker of late endosomes) prevents induction of DNA damage in intoxicated cells. This might, however, not be the universal mechanism, as the intracellular trafficking of EcolCDT-III does not depend on late endosomes (Gargi et al. 2013).

After the endosomal compartment, CDTs are translocated to the trans-Golgi network in a manner similar to several other bacterial toxins, including shiga and cholera toxins. From trans-Golgi, CDTs travel to the ER, and biochemical assays and microscopy analysis show the CdtB subunit of EcolCDT-III and HducCDT entering the ER (Gargi et al. 2013; Guerra et al. 2005). What is not completely clear is the intracellular trafficking of the binding subunits CdtA and CdtC. It was shown that the CdtA subunit of AactCDT is not internalized following binding to the cell membrane, and CdtC enters the cytoplasm but not the nucleus (Damek-Poprawa et al. 2012). This could suggest that the proper internalization and nuclear localization of CdtB at least partially depends on CdtC.

The last step in CDT internalization, translocation to the nucleus, is the least characterized to date. By using two genetic screens, AactCDT, CjejCDT, EcolCDT-

III, and HducCDT were shown to utilize components of ERAD (endoplasmic reticulum-associated degradation pathway) in order to translocate from ER to the cytoplasm, from where they presumably enter the nucleus (Eshraghi et al. 2014). On the other hand, a different study that used a combination of confocal microscopy analysis and biochemical assays did not reveal ERAD-dependent HducCDT translocation in the cytosol (Gargi et al. 2013). CdtB of EcolCDT-III were shown to contain two nuclear localization signals (NLS), and deletion of each signal results in a different localization pattern of CdtB: perinuclear distribution, corresponding to trapping of CdtB in the ER and trans-Golgi network, as well as diffuse cytoplasmic staining (McSweeney and Dreyfus 2004). Further studies will be needed to fully characterize the translocation steps of CDT into the nucleus.

Genotoxins Target the Nucleus in Order to Induce DNA Damage

DNA Damage and DNA Damage Response Induced by Genotoxins

Cellular responses to CDTs are similar to those of ionizing radiation and include phosphorylation of H2AX histone, recruitment of MRN complex to sites of DNA damage, as well as activation of ATM, p53, and p21. Depending on the exact dose of CDT, the result can be either transient block of the cell cycle progression, senescence or apoptosis. Several lines of research also point to a differential response of different cell lines: CDTs mainly induce apoptosis in lymphoid cells, whereas they induce senescence in cells of epithelial, endothelial, and mesenchymal origin (Grasso and Frisan 2015).

Although it is clear that colibactin can induce DSBs in host cells, it is not obvious whether this happens directly or through some indirect mechanism. It is possible that colibactin acts by directly alkylating DNA, inducing the formation of cyclopropane and bis-thiazole and thiazolinyl-thiazole heterocycles, and further processing of these lesions by base excision and nucleotide excision (BER and NER, respectively) repair pathways lead to production of DSBs (Balskus 2015). Infection of HeLa and nontransformed rat intestinal crypt IEC-6 cell lines by an *E. coli* strain harboring a bacterial artificial chromosome (BAC) with the *pks* island resulted in induction of DSBs, with all the classical markers of DDR. The latter include γH2AX formation, Chk2 activation, cell cycle arrest, and senescence induction (Nougayrede et al. 2006).

Cell Cycle Arrest

Normal cell cycle progression in eukaryotic cells is tightly regulated and mainly depends on interaction between cyclin-dependent kinases (CDKs) and their respective cyclins. For example, CDK4/cyclin D and CDK2/cyclin E regulate transition through G1 phase and entry into S phase, whereas CDK1/cyclin B regulates entry into M phase (G2/M phase transition). Transitions into the different phases of the cell cycle can be blocked by various proteins that participate in cell cycle regulation, many of which are activated by DNA damage to insure proper DNA repair before replication or cell division (Jackson and Bartek 2009).

Cells intoxicated with bacterial genotoxins can activate either G1/S or G2/M checkpoint. Activation of G1/S checkpoint depends on the activation of p53 and p21 proteins. There have been several conflicting studies regarding activation of this checkpoint by CDTs, and the difference can probably be explained by p53 status: those cells that lack p53 will progress through cell cycle even with DNA damage induced by CDTs, whereas p53-proficient cells stop their cycling at the G1/S border (Cortes-Bratti et al. 2001; Hassane et al. 2003; Liyanage et al. 2010). This relatively simple picture is complicated by the fact that p21 is activated independently of p53 under some conditions, and one study showed that intoxication of plasmacytic cells by AactCDT can lead to p21 activation irrespective of p53 status (Sato et al. 2002). Upon CDT treatment, activation of ATM and subsequently Chk2 leads to inactivation of CDC25C phosphatase and its sequestration to the cytoplasm, rendering the CDK1/Cyclin B complex inactive. It was also shown that overexpression of CDC25C can override CDT-induced G2/M checkpoint induction (Escalas et al. 2000).

Besides cell cycle arrest, CDT intoxication can also lead to cell death or senescence (Blazkova et al. 2010; Cortes-Bratti et al. 2001; Guidi et al. 2013). These pathways are mainly activated through ATM-Chk2 and ATR-Chk1 branches of DDR. Cell death occurs primarily via a mitochondrial-mediated apoptotic pathway that involves proteins of the Bcl2 family, such as BAX, cytochrome C release, and activation of caspases; however, several cell lines like Jurkat and MOLT-4 T cells utilize an extrinsic apoptotic pathway mediated through caspase 8 cleavage (Hickey et al. 2005; Ohara et al. 2004; Shenker et al. 2001). Alternatively to apoptosis, cell death can occur through necrosis, possibly during an abortive mitosis when the G2/M checkpoint is bypassed (Bezine et al. 2014). Interestingly, in the majority of cell lines tested, cell death occurs 2–4 days post intoxication. However, in the case of hematopoietic cells like monocytes and T cells, apoptosis is observed less than 24 h after intoxication and usually without cell cycle arrest (Gelfanova et al. 1999; Li et al. 2002; Ohguchi et al. 1998). One explanation for this difference might be that the CdtB phosphatase activity plays a key role in intoxication of hematopoietic cells, and further research will be needed to establish the exact nature of this interesting phenomenon. Alternatively, lymphoid cells may not activate survival pathways which can regulate the choice between apoptosis and senescence (Bezine et al. 2014).

Survival Signals

One of the potential consequences of DNA-damaging agents is cell survival, despite the fact that the host genome accumulates genetic lesions. If the cells manage to avoid apoptosis and senescence, they can continue to proliferate and acquire characteristics of malignant cells, like chromosomal instability or anchorage independent growth. In vivo, this could potentially lead to tumor initiation/progression (Guerra et al. 2011b).

CDT intoxication in epithelial and mesenchymal cells is characterized by cell distension and formation of actin stress fibers. Cell distention requires functional PI3-kinase and downstream effector mTOR. Actin cytoskeleton reorganization and

stress fiber formation is mediated by RhoA activation, which also promotes survival. Both processes are dependent on NET1, a RhoA-specific guanine nucleotide exchange factor. Following intoxication by CDTs, NET1 is dephosphorylated at the inhibitory site (Grasso and Frisan 2015). The NET1 signaling pathway diverges into two separate downstream cascades; the first one is dependent on the RhoA kinases ROCKI and ROCKII, and the other involves mitogen-activated protein kinase (MAPK) p38, together with its downstream target MAPK-activated protein kinase 2 (Guerra et al. 2008). This activation promotes cell survival. RhoA and MAPK p38 activation, as well as cell survival, depend upon FEN1 protein. The latter is an endonuclease that normally participates in the removal of Okazaki fragments during DNA replication and in certain pathways of DNA damage repair, that includes microhomology-mediated end joining (Guerra et al. 2011c).

Genotoxins Can Act as Host Immunomodulators

Genotoxins, which can directly promote DNA damage, can also modulate the host immune response, and the outcome of this immunomodulatory activity can be either proinflammatory or immunosuppressive. In vitro studies have shown that several members of the CDTs family enhance the host inflammatory response, which is in line with the perception of a danger signal. It is likely that induction of DNA damage activates an inside-out signaling that results in the release of damage-associated molecular patterns (DAMPs), which in turn promotes secretion of proinflammatory mediators as previously shown for UV irradiation (Ahmad et al. 2014; Bernard et al. 2012).

It has been shown that CjejCDT stimulates IL8 production, a major chemokine, which is responsible for neutrophil recruitment into inflammed areas composed of intestinal epithelial cells. Moreover, AactCDT induced the synthesis of IL1β, IL6, and IL8, three highly proinflammatory cytokines, in human peripheral blood mononuclear cells (Belibasakis and Bostanci 2014). Treatment of human macrophages with AactCDT resulted in IL1, tumor necrosis factor (TNF)α, IL6, and IL18 production via activation of the NLRP3 inflammasome (Shenker et al. 2015). Infection of human intestinal and hepatic epithelial cells using two CDT-producing *Helicobacter* species, *H. pullorum* and *H. hepaticus*, induced an upregulation of genes associated with NFkB signaling and a proinflammatory profile. This is done mainly through the expression of T-helper (Th) 17 genes, which are involved in antimicrobial peptide synthesis (Pere-Vedrenne et al. 2016).

The proinflammatory activity of genotoxins may be potentiated by the acquisition of a senescence phenotype. Indeed, it has been shown that human cells exposed to HducCDT and survive the acute intoxication possess hallmarks of cellular senescence including induction of proinflammatory cytokine production, such as IL6, IL8, and IL24 (Blazkova et al. 2010). Moreover, infection with *pks*-positive bacteria

induces an irreversible cell cycle arrest associated with the hallmarks of senescence and production of ROS, as well as secretion of proinflammatory cytokines like IL6, IL8, monocyte chemotactic protein (MCP)-1, and the matrix metalloproteinase (MMP)-3 (Secher et al. 2013).

The proinflammatory activity of CDTs characterized in the in vitro models has also been demonstrated in in vivo models of acute and chronic infections. In 3X mice, homozygous deficient for p50 and heterozygous for p65 ($p50^{-/-}$ $p65^{+/-}$), infection with a *C. jejuni* strain expressing a functional CDT significantly enhanced gastritis and hyperplasia at 4 months postinfection (p.i.) compared to mice colonized with an isogenic *cdtB* mutant strain, indicating that CDT promotes proinflammatory responses (Fox et al. 2004). Similarly, infection with the toxigenic *H. hepaticus* or *H. cinaedi* promoted severe inflammation of the cecum and colon (Pratt et al. 2006; Shen et al. 2009), thus enhancing the capacity to induce lesions in the C57BL/6 $IL10^{-/-}$ murine model for inflammatory bowel disease (IBD) (Young et al. 2004). In these models, infection with the strain expressing a functional CDT enhanced Th1 and Th2 responses, induced higher levels of mucosal IgA and proinflammatory mediators, and lowered levels of the anti-inflammatory cytokine IL10 (Ge et al. 2005; Pratt et al. 2006; Shen et al. 2009).

Thus, evidence from several in vitro and in vivo studies shows that CDT-producing bacteria can enhance the host inflammatory response, and it is now well recognized that chronic inflammation is strongly linked to cancer development. This outcome may apply also to long-term infection with CDT-producing bacteria. Indeed, Ge et al. (2007) have shown that the presence of an active toxin was necessary for the development of hepatic dysplasic nodules in A/JCr mice 10 months after infection with a toxigenic strain of *H. hepaticus*. This effect was associated with enhanced hepatic transcription of proinflammatory (TNFα, IFNγ, Cox-2, IL6 and TGFα) and antiapoptotic (Bcl-2 and Bcl-X_L) genes, upregulation of hepatic mRNA levels of p65 and p50, components of the nonclassical oncogene NFkB, followed by enhanced hepatocyte proliferation (Ge et al. 2007). Similarly, the carcinogenic potential was also demonstrated for colibactin in an animal model of IBD and colitis-associated colorectal cancer. Germ-free $IL10^{-/-}$ mice treated with the colon-specific carcinogen azoxymethane (AOM) had a higher incidence of invasive adenocarcinoma when monocolonized with *pks*-positive *E. coli* versus monocolonization with an isogenic, *pks*-deficient strain or a control *pks*-negative commensal bacterium like *Enterococcus faecalis* (Arthur et al. 2012). Interestingly, transcriptomic analysis of germ-free mice models of inflammation ($IL10^{-/-}$), colitis-associated colorectal cancer (AOM-treated $IL10^{-/-}$ mice) or control (AOM-treated $IL10^{-/-}$-$Rag2^{-/-}$ mice) monocolonized with the *pks*-positive *E. coli* NC101 strain, shows that 66 *E. coli* genes were significantly deregulated in the colitis-associated colorectal cancer model, and among them five belonged to the *pks* island (cblG, cblH, cblL, cblM, and cblN), suggesting that the intestinal microenvironment during cancer development may maintain or enhance *pks* transcription (Arthur et al. 2014).

The significance of the role of genotoxins in inflammation and cancer described above is supported by the detection of a higher frequency of genotoxin-producing bacteria (both CDT and colibactin) in *E. coli* isolates from colorectal cancer and IBD patients versus control subjects (Arthur et al. 2012; Buc et al. 2013; Prorok-Hamon et al. 2014).

Genotoxins as Immunosuppressors

In vitro results suggest that genotoxins could also induce a localized immunodepletion during the early phase of bacterial infection. Indeed, CDTs (i) block proliferation and induce apoptosis of T and B lymphocytes, (ii) inhibit IFNγ secretion of T lymphocytes, (iii) induce apoptosis of monocytes and immature monocyte-derived dendritic cells (DC), and (iv) impair the stimulatory activity of DCs, which are key activators of the adaptive immune response (Cortes-Bratti et al. 2001; Shenker et al. 2001; Li et al. 2002; Bezine, Vignard et al. 2014). AactCDT was initially described as an immunosuppressive factor (Shenker et al. 1999), thus the characteristic mode of action of these toxins may play a key role in establishing a successful, long-term infection. This is supported by several lines of evidence demonstrating that the presence of CDT in *C. jejuni* and *H. hepaticus* contributes to long-term persistence. Gastric colonization of mucin-deficient 129/SvJ mice by *C. jejuni* was enhanced when the bacteria produced a functional CDT (McAuley et al. 2007), while in the C57BL/129 mouse, *C. jejuni* carrying a wild-type CDT colonized the gastrointestinal tract in 50% of the mice 4 months postinfection (Fox et al. 2004). Similarly, a toxigenic *H. hepaticus* strain colonized C57BL/6 IL10$^{-/-}$ mice up to 8 months p.i., whereas presence of the functional toxin did not influence initial colonization (6 weeks p.i.). Long-term colonization (6 months p.i.), but not short-term infection, was also observed in Sv129 mice infected with a strain of *S. typhimurium* expressing a functional typhoid toxin. In the latter case, presence of the functional toxin was also associated with a significant decrease in intestinal inflammation characterized by a reduced recruitment of leukocytes, specifically neutrophils, T lymphocytes, and macrophages in the colonic mucosa of infected mice. Additionally, there is a significant downregulation of transcription factors, TLR signaling effector molecules, interferon responses, and Th1-mediated responses 10 days p.i. (Del Bel Belluz et al. 2016). In line with the suppressive effect of the toxin, it was shown that intravenous injection of purified typhoid toxin in C57Bl/6 mice induces a significant reduction in circulating immune cells, resulting in the almost complete depletion of circulating neutrophils (Song et al. 2013).

S. typhimurium mimics in rodents the symptoms of typhoid fever caused by *S. typhi* infection, which is strictly a human pathogen. It is noteworthy that the initial stages of infection by *S. typhi* in humans are usually asymptomatic due to a suppressed inflammatory response (Monack 2013), allowing the bacteria to establish a systemic and persistent infection. Thus, it is likely that the role of the typhoid toxin

is to promote a stealth infection, which is systemic and persistent, while limiting the host tissue damage.

The anti-inflammatory activity of DNA-damaging bacterial toxins has also been reported for colibactin encoded within the *pks* genomic island of the probiotic *E. coli* strain Nissle 1917. A nongenotoxic mutant carrying a deletion of the *cblA* gene was impaired in its ability to prevent colitis in two models: the dextran sodium sulfate (DSS)-induced acute colitis in rats and a T cell-dependent model of chronic colitis induced by the adoptive transfer of naive $CD4^{+}$ $CD45RB^{high}$ T cells in immunocompromised SCID mice (Olier et al. 2012). The immunosuppressive effect of colibactin is further supported by *pks*-positive *E. coli* strains that induce cell death by apoptosis in Jurkat T lymphocytes and primary lymphocytes from splenocytes (Marcq et al. 2014).

Conclusion and Future Directions

In the past several decades, there has been a rapid expansion of the knowledge on bacterial genotoxins, including their structure and effect on in vitro and in vivo experimental systems. Despite these advances, many interesting and clinically relevant questions remain unanswered. Bacterial genotoxins target the host cell nucleus, induce DNA damage, and trigger the DNA damage response, including the recruitment of the MRN complex and activation of ATM and p53. It is becoming clear that DNA damage and immune responses interact and influence each other. For example, recruitment of the MRN complex activates NFkB, leading to the secretion of proinflammatory cytokines. Moreover, several studies have shown that activation of ATM enhances the immune response by activating T lymphocytes, NK cells, or NFkB signaling. Finally, p53 can also stimulate the immune response leading to T lymphocyte activation (Fig. 2). All of this evidence is in line with in vivo and in vitro results showing that infection with genotoxins modulate the host immune response. Since the connection between DNA damage, inflammation, and cancer is well established, it is entirely possible that the bacterial genotoxins could contribute to tumor development in patients through multiple mechanisms. This important question needs to be addressed in further studies. Conversely, it has also been shown that genotoxins can act as immunosuppressors mainly by inducing apoptosis of immune cells or inhibiting secretion of proinflammatory effectors. The consequence is that the producing bacteria are able to overpower the host immune response, promote stealth invasion, and establish a successfully persistent infection.

Elucidation of the molecular mechanisms by which bacterial genotoxins can modulate the host response, leading to opposite effects (inflammation versus immunosuppression) like a Janus Bifrons, still remains an interesting and exciting challenge in the field.

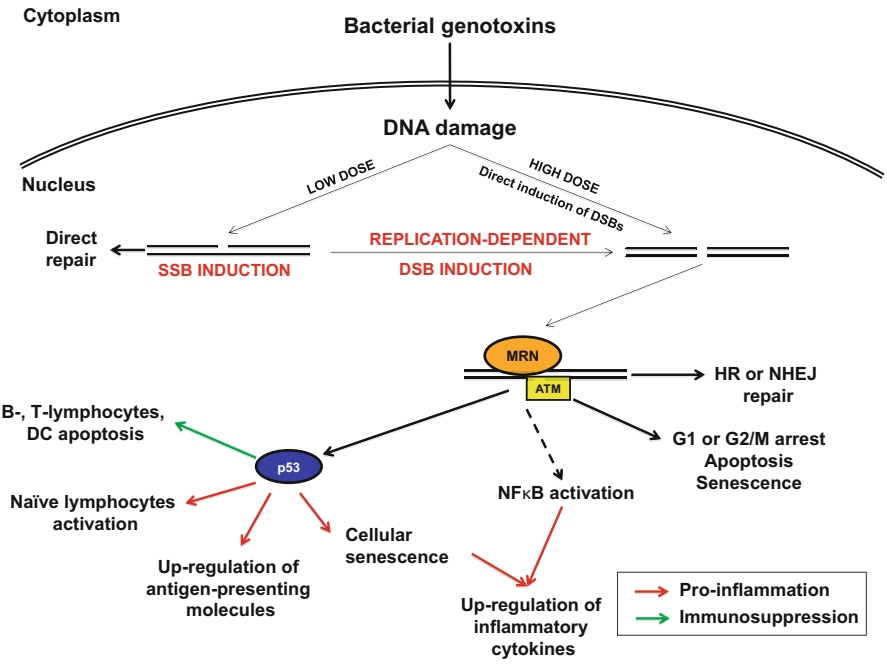

Fig. 2 Schematic representation of the possible roles of bacterial genotoxins as immunomodulators. After induction of DSBs (either directly or through replication-dependent mechanism), various components of the DNA damage response (DDR) are activated, including the MRN complex, the ATM kinase, and the tumor suppressor p53. Downstream signaling pathways of the DDR will contribute to either a proinflammatory response, with production of proinflammatory cytokines and activation of innate and adaptive immunoresponse cells (*red arrows*), or an immunosuppressive response, possibly through direct killing of immune cells (*green arrow*). See main text for details

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Translocation of Toxins by Gram-Negative Pathogens Using the Type III Secretion System

21

Arjan J. Vermeulen, Yuzhou Tang, and Alejandro P. Heuck

Abstract

The type 3 secretion (T3S) system is a syringe-like proteinaceous apparatus used by several Gram-negative bacteria to inject toxic/effector proteins into eukaryotic cells. Three proteins are essential for protein translocation into the host cell. One of these proteins forms a tip complex at the end of a needle that extends from the bacterial surface and plays a role in regulating secretion in response to host cell contact. The other two proteins, or translocators, insert into the target membrane and form a pore through which proteins are injected. Compared to other components of the T3S machinery, the needle tip and translocators show low levels of sequence identity among different T3S families, which suggests that these essential components adapted to the specific needs of the bacteria that use these T3S systems.

Keywords

Type 3 secretion • Translocators • Needle tip protein • Pore forming

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Contents

Introduction

The Type 3 Secretion (T3S) system is used by many Gram-negative pathogenic bacteria to translocate toxins/effector proteins into host cells (Hueck 1998). T3S plays a key role in diarrheal diseases caused by enteric pathogens like *Salmonella enterica* and *Shigella flexneri*. T3S is also essential in diseases like the plague, caused by *Yersinia pestis*, and infections caused by the opportunistic pathogen *Pseudomonas aeruginosa*. A precise understanding of the T3S system is therefore essential to discover new therapeutic targets to combat these pathogens.

The T3S system itself is a complex multiprotein machinery that allows for an efficient transport of proteins across the bacterial inner membrane, the periplasmic space, and the outer membrane to the extracellular milieu or into a target cell (Galán et al. 2014). Secreted proteins are recognized by an N-terminal sequence that is not cleaved during the secretion process. It is not entirely clear how this secretion signal is recognized by the T3S system, as a consensus sequence seems to be missing. T3S is used by bacteria in two different settings: in the assembly of flagella and as a mediator of pathogenesis or symbiosis. T3S that involves injection of proteins into a target eukaryotic cell is called virulence T3S or nonflagellar T3S system. In this chapter, the term T3S system will be used in reference to the nonflagellar T3S system.

T3S can serve several purposes depending on the organism and the situation. In *S. flexneri* and *Salmonella* sp., T3S allows the bacteria to enter the host cell. *Yersinia* sp. on the other hand use their T3S system to ward off immune responses. In all of these situations, the T3S system allows the bacterium to deliver toxins or protein effectors directly into the host cell's cytosol.

The T3S systems can be classified into families based on the phylogenetic analysis of their proteins. The focus of this chapter is the animal-pathogen associated families, including the Ysc, Inv-Mxi-Spa, and Ssa-Esc families (Cornelis 2006). A fourth animal-pathogen associated family is the *Chlamydiales* family. This is the only family found outside the proteobacteria phylum and was initially thought to be an early ancestor of the T3S systems (Mueller et al. 2014). A more enigmatic fifth

family is the *Myxococcales* family. This family was thought to be an evolutionary remnant and no complete systems were thought to exist (Pallen et al. 2005; Abby and Rocha 2012). However, more complete systems have been found recently in this family, and it is now thought that it represents an early form of T3S system (Abby and Rocha 2012). The phylogeny of T3S systems is markedly different from the bacterial phylogeny based on 16S RNA, indicating an evolutionary origin after bacterial speciation took place, followed by distribution among different species through horizontal gene transfer (Cornelis 2006). Lastly, three more families are found in plant-associated bacteria (the so-called Hrc1, Hrc2, and *Rhizobiales* families).

Some bacterial species contain T3S systems from different families (Cornelis 2006). For example, *S. enterica* has two T3S systems. The first T3S system is a member of the Inv-Mxi-Spa family and plays a role in host cell invasion, while the second one belongs to the Ssa-Esc family and is activated after the bacteria enter the host cell. Besides phylogenetic differences, the different families also feature distinctive needle structures. Members of the Ysc and Inv-Mxi-Spa families produce needles capped by relatively small tip complexes. Needles of the Ssa-Esc family on the other hand have a relatively short needle and extended filamentous needle tips. Needle complexes of the Hrp1 family have longer and more flexible needles. These differences most likely reflect specific adaptations in order to function under different conditions, such as the presence of mucus layers or cell walls (Cornelis 2006).

The T3S system is made of more than 20 different proteins. The largest part is the basal body: a complex that traverses the cytosolic membrane, the periplasmic space, and the outer membrane of the bacterium (Fig. 1). The overall structure of the basal body is conserved among the different species. Two inner rings make up the base of the structure, this continues into the neck, and finally the outer ring that crosses the outer membrane (Kosarewicz et al. 2012). On the cytosolic side, the export apparatus forms an assembly platform for the C-ring and an ATPase complex (Portaliou et al. 2015).

The needle extends from the bacterial outer surface and the basal body. The needle itself is a tube-like structure made of multiple copies of a single protein. The needle is capped with a structure made of single proteins. In the Ysc and Inv-Mxi-Spa families, this structure is made of several subunits, while in the Ssa-Esc family the needle tip is an extended structure that is more filamentous (Cornelis 2006). The last component of the T3S system is the translocon. The translocon is made of multiple copies of two different membrane-associating proteins, the so-called translocators. These translocators are secreted and form a pore in the host cell membrane. This pore allows for passage of toxins/effectors into the host cell.

The tip of the needle, the translocators, and associated chaperones are encoded in a single operon (Mueller et al. 2008; Sawa et al. 2014). The needle tip and the translocators are secreted and essential for translocation of effector proteins into the target cell, but not for protein secretion outside the bacterium. Initially it was assumed that the three proteins formed the translocon, and therefore it is not uncommon to find that all three proteins are referred to as translocators in the literature. However, it is now clear that the water-soluble protein is secreted first and forms the needle tip

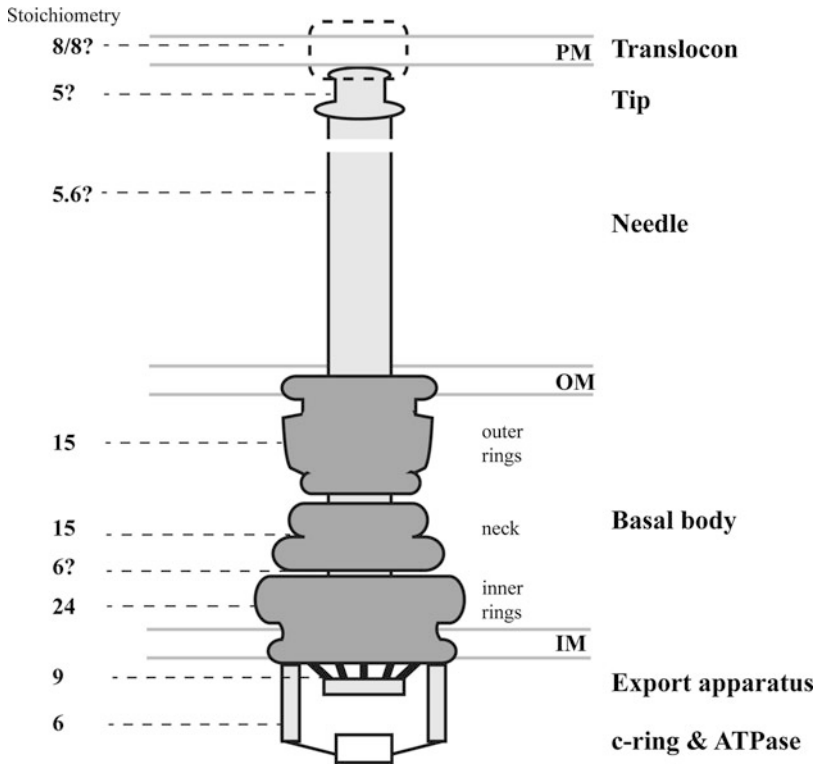

Fig. 1 Schematic overall structure of a T3S injectisome. The basal body is drawn based on reconstitutions from cryo-EM images of isolated *Salmonella* T3S injectisomes (Kosarewicz et al. 2012). *Dash line box* at the *top* represents the translocon complex which has not been visualized yet. The stoichiometry (or fold symmetry) for different T3S structures are shown on the *left*. For the needle the stoichiometry per turn is indicated. The basal body is separated from the tip and translocon to indicate that while the basal body is a schematic of *Salmonella* injectisome, the tip part is adapted from data of the Ysc family. *PM* plasma membrane, *OM* outer membrane, *IM* inner membrane

before translocation takes place (Broz et al. 2007). Therefore, in this chapter for clarity, the term translocator will exclusively be used for the two membrane-associated proteins.

Effectors are proteins that function inside the host cytosol and modulate aspects of the host cell's physiology. Some bacteria can produce up to 30 different effector proteins. Others like *P. aeruginosa* use a relatively small arsenal of three different effectors that can modulate the host cell's cytoskeleton, trigger apoptosis, break down the cell membrane, or alter intracellular cAMP levels.

The assembly and functioning of the T3S system is a tightly regulated process (Büttner 2012; Portaliou et al. 2015). Assembly of the basal body is mediated by Sec dependent translocation. Once the basal body is complete, a substrate switch takes place. Inner rod proteins and needle subunits (the early secretion substrates) are secreted through the T3S system and assemble spontaneously, forming a needle

structure that extends from the inner rod and protrudes from the cell's surface. As the needle reaches a certain length, another switch takes place and the T3S system starts secreting the tip protein and prepares for the secretion of translocators (the middle secretion substrates) and effectors (the late secretion substrates).

Substrate selection and regulation of secretion are important aspects of the T3S system. A major regulator is a gatekeeper protein (PopN in *P. aeruginosa*). Work in *P. aeruginosa* indicates that PopN binds indirectly to the basal body (Lee et al. 2014). Mutants carrying deletions of *popN* secrete effectors (late secretion substrates) prematurely (Lee et al. 2010; Sundin et al. 2004). Precise timing of secretion is further regulated through interactions with several other components of the T3S system. In *S. enterica*, a structure called the sorting platform binds different secretion substrates. In absence of the middle substrates the sorting platform binds late secretion substrates, suggesting that this structure plays an important role in regulating the order of secretion (Lara-Tejero et al. 2011). It is likely that sorting platforms are present in all T3S systems.

Basal bodies containing the needle, and needles capped by the tip, have been observed by negative stain electron microscopy (EM) and cryo-EM; however, little is known about how the needle/tip interacts with the translocon. While the tip proteins may help to seal the needle before secretion starts, it is not clear if they stay attached to the needle when interaction with the translocon occurs. The *P. aeruginosa* PopB and PopD can efficiently replace YopB and YopD in a *Yersinia* mutant lacking these two translocators only if PcrV (the tip protein from *P. aeruginosa*, see Table 1) is also present. This observation suggests that the tip remains attached to the needle and engages with the membrane-assembled translocon during secretion (Bröms et al. 2003). The switch to secretion of late substrates presumably takes place when the translocon pore is formed in the host cell membrane and engages with the needle. After this switch, effector proteins are secreted and injected into the target cell's cytosol. Interestingly, the secretion of late substrates can also be induced by non physiological signals. For example, in the Ysc family depletion of calcium from the medium trigger secretion, and in *S. flexneri* secretion can be induced by adding the Congo red dye to the medium.

It is clear that the final stage of T3S system assembly, the formation of the translocon complex, is essential for toxin injection. Therefore, a precise understanding of the translocon composition, stoichiometry, and structure is critical to develop the much

Table 1 Short names of the tip protein and translocators used in different species

Family	Species	Tip protein	Translocators	
Ysc	*P. aeruginosa*	PcrV	PopB	PopD
	Yersina sp.	LcrV	YopB	YopD
	Aeromonas sp.	AcrV	AopB	AopD
Inv-Mxi-Spa	*Salmonella* sp. *(SPI-1)*	SipD	SipB	SipC
	Shigella sp.	IpaD	IpaB	IpaC
Ssa-Esc	*Enteropathogenic E. coli*	EspA	EspD	EspB
	Salmonella spp. *(SPI-2)*	SseB	SseC	SseD

needed therapeutic agents against these several human pathogens. In the following sections a summary of the current knowledge of the T3S needle tip and translocators will be presented.

The Needle Tip

One T3S secreted protein is responsible for capping the needle at the distal point, before the bacterium makes contact with the target cell (Table 1). In *Yersinia enterocolitica*, LcrV is the only protein present at the tip of the needle (Mueller et al. 2005). Likewise, *P. aeruginosa* PcrV is the cap for the needle tip (Cisz et al. 2008). Based on a model built using the crystal structure of LcrV monomer and the EM density maps, the quaternary structure of the needle tip of *Yersinia* has been proposed to be a pentameric ring. Immunolabeling and crosslinking studies using antibodies against IpaB show that the needle tip of *S. flexneri* contains four IpaD subunits and one IpaB subunit (Olive et al. 2007; Veenendaal et al. 2007). However, these results are controversial since other studies have shown that *S. flexneri*'s IpaB is not present at the tip. In this last model, the recruitment of IpaB to the needle tip occurs only after binding of bile salts (Cheung et al. 2015; Dickenson et al. 2011).

Structure of the Needle Tip

The first high resolution structure of the T3S needle tip protein (LcrV) was solved for *Yersinia pestis* (Derewenda et al. 2004). Since then, more structures became available for the needle tip proteins of other T3S systems (Fig. 2) (Blocker et al. 2008; Mueller et al. 2008; Sato and Frank 2011). The most striking feature of the needle tip structures is the extended coiled-coil at the core of the protein. In LcrV, the central coiled-coil connects two globular domains giving the protein a dumbbell-like shape. One globular domain consists of the N-terminus, while the other is made of a central region of the polypeptide. The structures of IpaD, BipD, and SipD did not show a globular domain at the N-terminus. Instead, the N-terminus forms two antiparallel alpha-helices that fold along the coiled-coil giving the overall structure a more elongated appearance (Blocker et al. 2008).

Only low resolution structures of tip proteins assembled on top of the needle are available. The overall shape of assembled needle tips, as determined by negative stain EM, reflect the differences in their subunit structures (Mueller et al. 2005; Veenendaal et al. 2007). Members of the Inv-Mxi-Spa family with their lack of globular domains assemble in a scepter-like structure (Cheung et al. 2015). Homologs of LcrV on the other hand show a distinct structure at the needle tip that has a shoulder-neck-head organization, which reflects the more globular structure of the needle tip subunits (Mueller et al. 2005; Broz et al. 2007). Mutant strains lacking *lcrV* do not show this structure and instead produce needles that are more scepter like (Mueller et al. 2005). The closely related tip proteins of *Y. enterocolitica*,

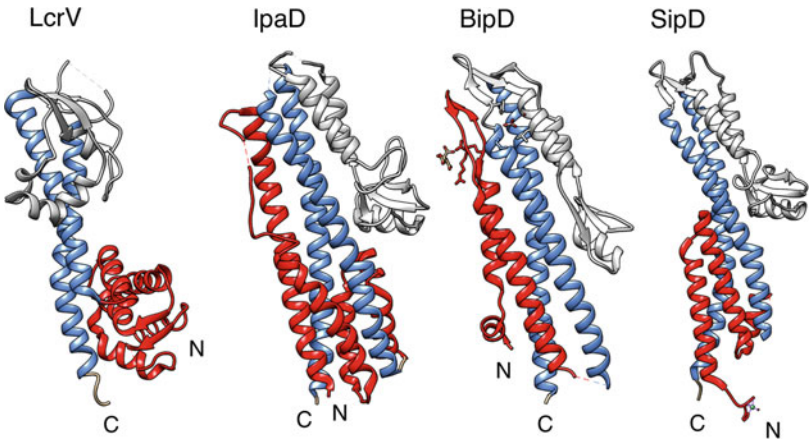

Fig. 2 Resolved structures of the needle tip proteins LcrV (PDB 1R6F), IpaD (PDB 2JAA), BipD (PDB 2J9T), and SipD (PDB 3NZZ). The central coiled-coil is shown in *blue*, the N-terminal domain in *red*, and the central domain in *gray*. LcrV is a member of the Ysc family and IpaD, BipD, and SipD belong to the Inv-Mxi-Spa family (see Table 1). The N-terminal and central domains of LcrV show a globular conformation, whereas in the Inv-Mxi-Spa family members these domains are elongated and packed against the central coiled-coil. The letters *N* and *C* indicate the location of the N-terminus and the C-terminus, respectively

P. aeruginosa PcrV, and *Aeromonas salmonicida* AcrV all readily assemble on a *Yersinia* needle (Broz et al. 2007). These homologs differ in the size of both globular domains, and by comparing dimensions of the tip complex using EM it was established that the shoulder domain at the base of the needle tip consists of the N-terminal domain, while the head consists of the central globular domain (Broz et al. 2007).

Multiple Roles of the Needle Tip Protein

Under physiological conditions, T3S is activated upon host cell contact. The needle tip is postulated to play a central role in host cell-sensing: contact between the host cell and the needle tip protein would trigger a signal that is then transferred through the needle to the bacterial cytosol (Blocker et al. 2008). In the cytosol, this signal triggers a switch in secretion specificity to secretion of effectors (late secretion substrates) and expression of T3S genes. While little is known about this signal transfer in *P. aeruginosa*, it is better studied in the distantly related *S. flexneri* T3S system. In *S. flexneri*, the gatekeeper protein MxiC (PopN in *P. aeruginosa*) plays a key role in preventing effector secretion before induction. Upon induction MxiC is secreted and secretion of effectors is activated. A single point mutation in the needle protein rendered the bacterium unable to secrete effectors (Kenjale et al. 2005). Further analysis showed that this mutation also blocks secretion of MxiC, while still allowing secretion of the translocators IpaD and IpaB (Martinez-Argudo and

Blocker 2010). This suggests that needle subunits undergo a conformational change upon host cell-sensing and that transfer of this signal through the needle is blocked when the single point mutation of the needle protein is present.

In *P. aeruginosa*, host cell contact leads to increased expression of the effector ExoS. Triggering of effector expression is dependent on the presence of translocators PopB and PopD (Cisz et al. 2008). A recent study suggested that interaction between the C-terminus of PopD and the N-terminal globular domain of PcrV plays a key role in triggering effector secretion upon host cell contact (Armentrout and Rietsch 2016). How this signal is then transferred to the cytosol of the bacterium is not known, but it is likely that it travels through the needle to the basal body where the MxiC homologue PopN plays a similar role in substrate selectivity (Lee et al. 2010). Thus, in *P. aeruginosa* not only host cell-contact, but also the assembly of a translocon complex, seems required to initiate effector secretion.

The needle tip may also contribute to preventing secretion of T3S substrates under noninducing conditions (resting state, see Fig. 3). In *P. aeruginosa*, the needle tip protein (PcrV) plays a role in regulating secretion of effectors. Mutants lacking PcrV secrete effectors in the presence of calcium, whereas in wildtype strains this would completely block secretion (Lee et al. 2010; Sato and Frank 2011; Sundin et al. 2004). Deletion of the gene encoding the chaperone PcrG did cause a similar disruption of secretion regulation. A double mutant lacking both PcrG and PcrV showed an even stronger regulation defect, indicating that both proteins play a role in regulation (Lee et al. 2010). In *Yersinia* sp., deletion of *lcrV* disrupts the low calcium response, and mutants secrete effectors in the presence of calcium (DeBord et al. 2001). A stronger deregulated phenotype is seen in *S. flexneri* needle tip mutants, where secretion control is completely lost and Congo red no longer affects induction (Veenendaal et al. 2007). A *pcrV* mutant, in which the export signal was removed, behaved like a Δ*pcrV* mutant and fails to suppress effector secretion in the presence of calcium (Lee et al. 2010). This suggests that physical blocking of the needle by PcrV assembled at the tip plays a role in regulation of secretion. However, this phenotype may also be due to a change in the signaling state of the needle and the regulatory complex at the base of the needle instead. More work will be needed to discern these two possibilities.

In *P. aeruginosa*, PcrG is a binding partner of the tip protein PcrV. In vitro, PcrG forms a heterodimeric complex with PcrV with high affinity ($K_D \approx 16$ nM) (Nanao et al. 2003). In solution, purified PcrG is an unstructured protein that assumes a more helical form upon binding with PcrV (Nanao et al. 2003). PcrG is usually described as a chaperone for PcrV, but while many T3S related proteins are nonfunctional and unstable without chaperone, deletion of *pcrG* does not seem to disrupt PcrV function. *P. aeruginosa pcrG* deletion mutants show normal cytotoxicity to HeLa cells, as shown by cell rounding (Sundin et al. 2004). In the same assay, a mutant lacking the tip protein on the other hand was no longer cytotoxic. Interestingly, a secretion assay did show a reduction in secretion of PcrV in a *pcrG* deletion mutant, while production of PcrV was not reduced (Lee et al. 2010). Thus these studies suggest that it is unlikely that PcrG is required to chaperone PcrV and prevent oligomerization in

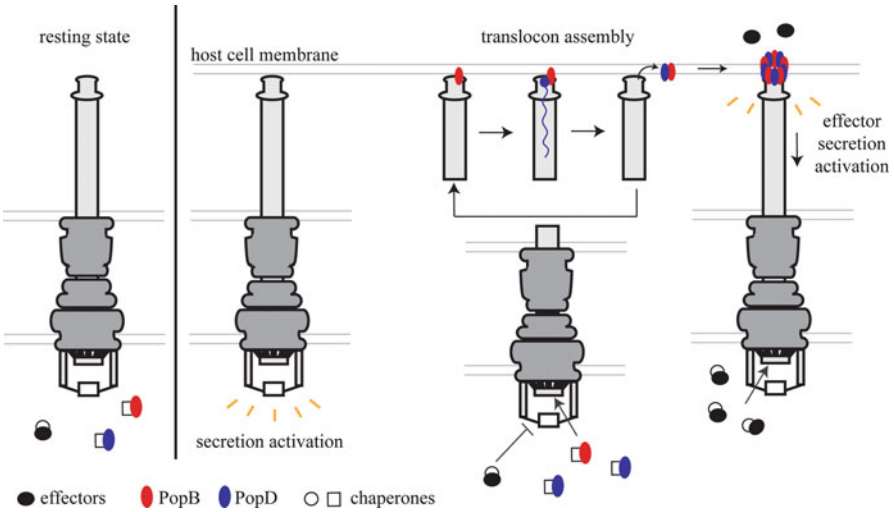

Fig. 3 Hypothetical model for translocon formation in *P. aeruginosa*. In the absence of host cells, only a few T3S injectisomes are pre-assembled on the bacterial surface (resting state). Contact with the host cell membrane initiates the secretion of translocators and activates expression of T3S-related genes. Assembly of a hetero-oligomer, while preventing the formation of homo-oligomers, would require the alternate secretion of PopB and PopD. Alternatively, if the secretion of proteins is stochastic, a model where only one translocator binds to the tip could explain the specific formation of hetero-oligomers. In this figure, PopB was used as the bound translocator; however, a similar mechanism can be envisioned using PopD bound. PopB is secreted and anchored to the needle tip in a way that it cannot bind additional PopB monomers. Additional PopB monomers will be secreted into the membrane, eventually forming nonfunctional homo-oligomers. When PopD emerges from the needle, formation of a heterodimer enables release of the anchored translocator PopB. Subsequently, another PopB anchors to the tip and the cycle repeats. Ultimately, these heterodimers oligomerize into the putative hexadecameric complex as observed during in vitro experiments. The membrane-assembled hetero-oligomer engages the needle, triggering the switch from secretion of translocators to secretion of effectors

the bacterial cytosol. Instead, PcrG may merely keep PcrV in a secretion-competent state that facilitates its secretion.

Recent studies suggest that PcrG may play a role as a regulator of the secretion process. Deletion mutants of *pcrG* show secretion of effectors in the presence of calcium, whereas wildtype cells do not secrete under these conditions (Lee et al. 2010). Deletion of *lcrG* in *Yersinia* also showed secretion of Yop proteins in the presence of calcium, thus disrupting regulation (DeBord et al. 2001). Expression of a mutant form of LcrG that no longer interacts with LcrV blocks secretion of Yops (late secretion substrates) in *Y. pestis* under calcium-depleted conditions (Matson and Nilles 2001). This suggests that titration of LcrG with LcrV removes a blockade at the secretion channel, enabling passage of Yops. A similar mutation in PcrG in *P. aeruginosa* did not significantly change secretion of effectors and translocators, even though interaction with PcrV is disrupted (Lee et al. 2014). Thus, the mechanisms underlying regulation may differ between *Yersinia* spp. and *P. aeruginosa*.

Besides binding PcrV, PcrG also shows interaction with PscO and PcrD, both components of the inner ring of the T3S system (Lee et al. 2014). PcrG and PscO regulate the rate of secretion by modulating proton motif force-dependent activity of the T3S system. The interaction between PcrG and PcrD may play a key role in regulating secretion specificity. Furthermore, pull down assays showed an interaction between PcrD and Pcr1, which is thought to tether the gatekeeper PopN to the T3S apparatus. A PcrD mutant lacking a glutamate that is strongly conserved in T3S systems, but not in flagellar T3S systems, gave rise to a strong deregulation phenotype. A similar deregulation phenotype is seen in strains lacking components of the PopN complex. Thus PcrG plays a role in regulating both specificity and activity of the T3S system. This function may in turn be further regulated by PcrV.

The Translocators

The needle connects with the host cell membrane through a transmembrane protein complex (the translocon) formed by two secreted proteins, the translocators (Fig. 1, Table 1). The two translocators differ in size, with the larger translocator ranging in size from 40 kDa to 60 kDa and the smaller translocator from 30 kDa to 40 kDa. Currently, it is thought that the translocon complex forms the pore through which the secreted effectors are translocated into the host cell. This hypothesis is based in the following experimental observations: (i) absence of either protein resulted in complete loss of translocation, indicating that both translocators must be present to allow injection of effectors; (ii) they are the only T3S secreted proteins found stably associated with host cell membrane after infection; and (iii) both translocators, individually or when added together, form pores in lipid bilayers (Büttner and Bonas 2002).

Maintenance in Bacterial Cytosol

The translocators adopt a molten globular state in aqueous solvents and they contain one or more hydrophobic segments in their primary structure; therefore, it is not unexpected that they are prone to form aggregates when not associated with membranes (Faudry et al. 2007). In the bacterial cytosol, these two proteins are kept in a secretion-competent state by association with a single cognate chaperone. A gene encoding this chaperone is present in the translocon operon. In *P. aeruginosa*, deletion of this gene (*pcrH*) from the *pcrGVHpopBD* operon disrupted secretion of PopB and PopD completely (Bröms et al. 2003). In addition, intracellular levels of PopB and PopD were greatly reduced in this mutant, showing that PcrH is essential for both secretion and stability of PopB and PopD.

A few crystal structures of translocator's chaperones exist, alone or in complex with a short fragment of a translocator (Discola et al. 2014; Job et al. 2010; Lunelli et al. 2009; Schreiner and Niemann 2012). All of these structures show three tandem pairs of tetratricopeptide repeats that give the chaperone a palm-shaped conformation with a concave and a convex side for substrate binding. The concave side forms

a binding site for an approximately nine residue section of the N-terminus of the translocators, the conserved P/VXLXXP motif that is sufficient for binding to the chaperone (Lunelli et al. 2009). More recently a structure of *A. hydrophila* nearly full-length translocator (AopB), in complex with its chaperone AcrH, was solved (Nguyen et al. 2015). In this structure, the conserved motif binds the concave side of the chaperone and the majority of AopB folds over the convex side of AcrH (Nguyen et al. 2015). Interestingly, in this structure three hydrophobic regions fold into two hairpin structures. These two hairpins fold over each other, with the helix containing less hydrophobicity facing outward. Thus, aggregation of the translocator is prevented by burying the hydrophobic regions and shielding those regions from the aqueous solution.

In addition to maintaining translocators in a secretion-competent state, a regulatory function has been proposed for the *Yersinia* LcrH-YopD complex. In *Yersina*, the LcrH-YopD complex negatively regulates synthesis of effectors by modulating mRNA stability (Schiano and Lathem 2012). When T3S is triggered by removing calcium from the medium, YopD is secreted, repression released, and effector expression is initiated. Deletion of YopD or LcrH results in hyper production of effector proteins regardless of calcium level. However, this characteristic of YopD-LcrH complex is not shared among other members of the Ysc family. For example, deletion of PcrH only affects stability of PopB/PopD in the bacterial cytosol but not the calcium-dependent regulation of effector synthesis (Bröms et al. 2003).

Models for Translocation

The currently most widely accepted model, the injectisome model, describes the T3S system as conduit through which unfolded proteins are transported out of the bacterium and into the target cell (Fig. 4). Once loaded onto the base of the injectisome, the ATPase at the basal body induces chaperone release and substrate unfolding (Akeda and Galan 2005). Supporting that different proteins are secreted through the relatively narrow channel of the needle is the evidence of IpaB trapped in an isolated T3S injectisome, in an unfolded form (Dohlich et al. 2014). While the injectisome model seems to be the simplest model that explains the many experimental data obtained to date for T3S, alternative models could also explain some of the experimental observations (Fig. 4). An alternative model has been proposed recently based on the observation that translocators and some effector proteins could be found on the bacterial surface before contacting a target cell (Akopyan et al. 2011). It is not clear in this model what triggers the release of T3S substrates from the surface of the bacterium; however, it was hypothesized that effectors and translocators form a protein complex that is essential for translocation. The translocators would then resemble the B components in AB toxins and transport effectors using a mechanism similar to the one observed for these toxins. This model is supported by the finding that a *yopH* effector deletion in *Y. pseudotuberculosis* could be reverted by coating bacteria with purified YopH prior to host cell contact (Akopyan et al. 2011). No restoration of virulence was

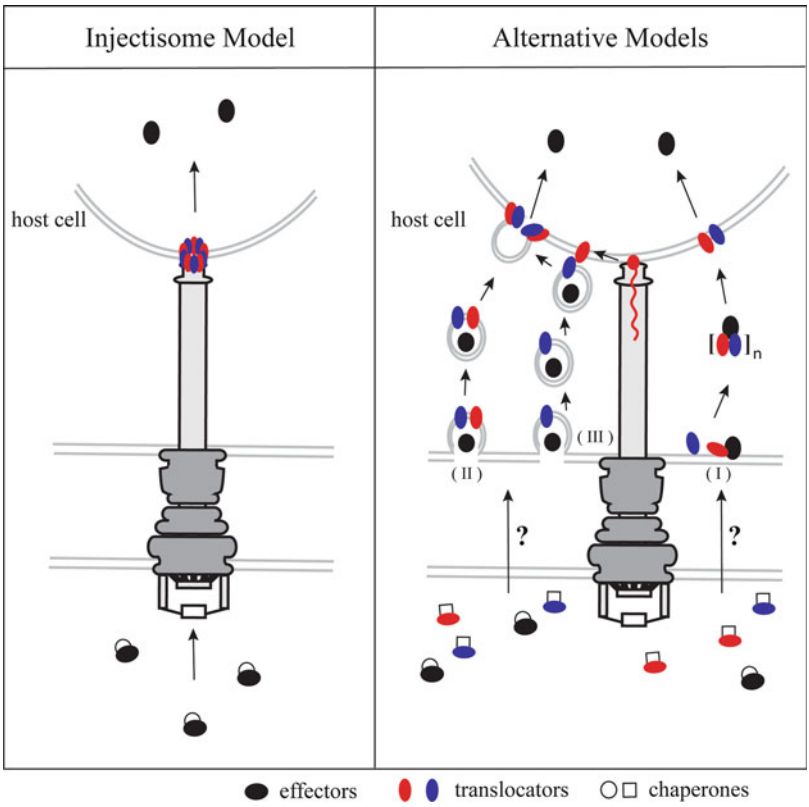

Fig. 4 Schematic view of different models for T3S. In the injectisome model, upon contact-mediated activation, the T3S apparatus injects effectors directly into the target cell through a translocon pore formed by the two translocators. Alternative models are shown on the *right*. (*I*) the T3S injectisome senses the presence of target cell and triggers the release of surface located translocators and effectors. Translocation of effectors would be similar to the one observed for AB toxins; (*II*) sensing of the target cell via the T3S injectisome triggers the release of outer membrane vesicles (*OMVs*) containing the translocators and encapsulated effectors; (*III*) similar to the mechanism described in II, but one translocator is secreted through the needle and acts as a receptor protein for the OMVs in the target cell. Translocator-mediated fusion of the OMVs with the target membrane in turn allows entry of effectors into the host cell

found when translocator-deficient strains were used. Despite the fact that this model gained considerable attention, no other studies supporting this model have been published to date.

Interaction with the Membrane and Formation of the Translocon Complex

Regardless of which mechanism of protein translocation is used to inject effector proteins into the host (Fig. 4), it is clear that the interaction of translocators with

target membrane plays a critical role during T3S. Many attempts to study the interaction of translocators with membranes using purified proteins have been made in the past decade; however, the exact sequence of events leading to translocon assembly remains uncertain. Purified translocators have a tendency to aggregate in aqueous solutions forming heterogeneous water-soluble aggregates, and this has made it difficult to study the interaction between translocators or their interactions with the membrane. For instance, in early studies acidic pH was used to dissociate translocators from their chaperone, but this resulted in the formation of heterogeneous oligomeric aggregates of the proteins (Faudry et al. 2006; Schoehn et al. 2003). Similar heterogeneous aggregates were observed when detergents were used to isolate translocators (Dickenson et al. 2013; Hume et al. 2003; Senerovic et al. 2012).

Since the translocators are secreted through a protein-based needle in an unfolded conformation, later studies used denaturants such as guanidinium chloride or urea to replicate in vitro the initial unfolded state of these proteins (De Geyter et al. 1997; Dey et al. 2012; Faudry et al. 2007; Romano et al. 2011; Wager et al. 2015). These denaturing/refolding protocols have been optimized to allow a more efficient insertion of translocators into model membranes and subsequent biophysical characterization of the membrane-assembled complexes (Romano et al. 2011, 2016).

P. aeruginosa translocators PopB and PopD formed stable and discrete pores in model membranes, and they oligomerized into homo- or hetero-oligomers. Experiments using single molecule photobleaching techniques showed that when individually added to membranes, PopD formed mostly hexamers. PopB, on the other hand, generated a distribution of stoichiometries, with complexes made of 6 or 12 subunits being the most abundant (Romano et al. 2016). Surprisingly, when the translocators were added together, they formed hetero-complexes containing 8 PopB and 8 PopD subunits. These experiments suggested that an early interaction between PopB and PopD guided the assembly of unique hetero-complexes in membranes. Moreover, the formed homo-oligomers were stable and did not dissociate to reassemble into hetero-complexes upon addition of the other translocator. This indicated that the formation of homo-complexes was not reversible (Romano et al. 2016). These findings showed that the translocators can spontaneously form well-defined oligomeric structures on membranes.

While in vivo assembly of the translocators require further investigation, it is worth notice that the molecular mass for the hetero-complex (570 kDa) formed in model membranes is in very good agreement with the molecular mass of 600 +/− 100 kDa roughly estimated for translocons isolated after incubation of *Yersinia* and red blood cells (RBC) (Montagner et al. 2011). Little is known about how the translocators are arranged in the complex and overall shape of the hetero-oligomer. Ring-like structures were observed in electron micrographs of *P. aeruginosa* translocator aggregates incubated with liposomes (Schoehn et al. 2003). Surprisingly, no apparent differences in ring sizes was observed in rings formed with PopB or PopD alone, or mixtures of PopB and PopD in this study. In addition, the composition and stoichiometry of these structures are uncertain as translocators may not have completely dissociated from the chaperones. Thus more work is needed to determine the actual shape of the translocon pore.

The nonreversible association of homo- and hetero-complexes also provided an explanation for the unsuccessful recovery of cytotoxicity when eukaryotic cells were incubated using a mixture of two *P. aeruginosa* strains, each lacking either the *popB* or *popD* gene. Each of these strains was able to secrete proteins, but they were noncytotoxic. Co-infection with both strains does not restore cytotoxicity, suggesting that assembly of a functional translocon requires that the translocators are secreted by the same bacterium (Cisz et al. 2008). Since monomeric translocators form homo-oligomers in membranes, it is clear that these homo-oligomers are nonfunctional for protein translocation. Therefore, co-infecting with mutants expressing a single translocator cannot recover the phenotype, because each translocator assembles into a homo-complex before interacting with the other translocator. These results also suggest that the hierarchy of secretion should play an important role during translocon assembly. If a homodimer is formed, it cannot further interact with the other translocator and vice versa. In contrast, once a heterodimer is formed it could only interact with other heterodimers (Fig. 3) (Romano et al. 2016).

Interaction Between Needle Tip and Translocation Pore

P. aeruginosa strains lacking needle tips are deficient in causing hemolysis, indicating that these strains fail to insert a translocon complex and create a pore in the RBC membranes (Goure et al. 2004). These mutants are also unable to infect cells and cause cell rounding, even though they still secrete effectors (Goure et al. 2004; Sundin et al. 2004). The insertion of translocon complexes requires the presence of a compatible needle tip. When expressed in a *Y. enterocolitica ΔlcrV* strain, PcrV did assemble at the needle tip but was unable to restore hemolytic activity (Broz et al. 2007). In this same assay, YopB was not found associated with the RBC membrane and only reduced amounts of YopD were found associated with the lipid bilayer. Therefore, it seems that for efficient insertion of the translocon, interaction between the translocators and the needle tip is required.

Thus far little physical interaction between PcrV and PopB (or PopD) has been detected. The interaction between PcrV and PopD appears more of a regulatory nature (Armentrout and Rietsch 2016). This interaction may be weak or transient, since PcrV was not associated with RBC membranes after infection with *P. aeruginosa* or with liposomes reconstituted with PopB, PopD, or both (Goure et al. 2004). In addition, PcrV can be found on the bacterial surface, while PopB and PopD are not detected on the surface of *P. aeruginosa* (Cisz et al. 2008; Lee et al. 2014). On the other hand, translocation of T3S effectors into the host cell is efficient and effectors are not detected in the medium when cells were infected with *P. aeruginosa* (Sato and Frank 2011; Sundin et al. 2004). It is therefore likely that the needle and its tip form a tight connection with the translocation pore when forming a conduit that connects the bacterial cytosol with the host cytosol. However, it is not known whether the needle tip remains associated with the needle after translocon

assembly or whether it dissociates. More work will be needed to pinpoint the function of the needle tip in this conduit.

Conclusion and Future Directions

The T3S system is a remarkable nanomachine used by a number of important pathogenic and symbiotic bacteria. Insights gained over the last decade show a machine that is regulated through a number of intricate mechanisms. The essential component of the T3S system, the translocon complex, remains shrouded in mystery. Questions about stoichiometry of the complex have started to be addressed. However, the structure of the translocator complex and how it inserts into the membrane remains largely unknown. What role, if any, the needle tip plays in insertion also needs to be explored. It has been suggested that the needle tip acts as a scaffold, but no data supporting this claim are available.

Another of the big questions in the field is how the timing of secretion is regulated. Several components that regulate the secretion order have been identified, but the specific mechanisms of how they work remains to be explored. Homologs of the recently discovered sorting platform components are found throughout different T3S families, but very few studies have been published that address this structure.

Finally, the role of the needle tip in host cell-sensing and regulation of secretion also needs to be explored in more detail. Several studies have yielded some insight into host cell-sensing, but what the signal is and how this signal is then transferred and converted into an action in the bacterial cytosol still remains a mystery.

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Insights into the Evolution of Bacterial Sphingomyelinases and Phospholipases Associated to Virulence

22

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Abstract

Bacterial sphingomyelinases and phospholipases constitute a structurally heterogeneous group of esterases expressed by a variety of pathogens. These enzymes help bacteria to obtain substrates from the host or to evade the immune response, promoting phagosomal arrest or escape. Therefore, the paradigm regarding their function has been biased toward their roles in pathogenesis in animal and human hosts. However, a comprehensive bioinformatic search of 17 different types of bacterial sphingomyelinases and phospholipases in a database of 7583 complete proteomes shows that several enzyme types have homologues in eukaryotes and one even in archaeas, whereas others, exclusively present in bacteria, are distributed in phylogenetically distant pathogenic and nonpathogenic bacterial lineages. The patchy distribution of some of these enzymes across broad taxonomic boundaries suggests the occurrence of multiple lateral gene transfer events

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among different bacterial phyla, or even between bacteria and species from other kingdoms. The horizontal transference of genes among diverse microorganisms within free-living predator amoebas constitutes a plausible explanation for the patchy distribution of sphingomyelinases and phospholipases among bacteria from phylogenetically divergent phyla. The findings of this work suggest that for some bacterial sphingomyelinases and phospholipases associated to virulence, their roles in pathogenesis during infections of multicellular organisms are coincidental. Thus, these enzymes could have evolved for survival of non-pathogenic bacteria in their natural environments.

Keywords
Bacterial phospholipase • Bacterial sphingomyelinase • Evolution

Contents

Introduction

Membranous structures define cellular life, and a source of membrane-bounded microenvironments on the early earth were essential for the rise of ancestral cells (Lombard et al. 2012). Cellular membranes ensure the individuality and integrity of all cells and mediate their interactions with the surrounding environment (Lombard et al. 2012). Membrane lipids, in addition to their structural role, may serve as metabolic energy reservoirs, regulate membrane protein function, and act as precursors of signaling mediators. In archaea, bacteria, and eukaryotes, the lipid components of cellular membranes are glycerol phosphate phospholipids, fatty acids, and isoprenoids, which suggest that the enzymatic machinery for membrane lipid biosynthesis is ancient (Lombard et al. 2012). However, there are important differences in the membrane lipid composition between organisms of the three domains of

life: archaeal phospholipids are mainly made of glycerol-1-phosphate ether linked to isoprenoid chains, whereas bacteria and eukaryotes have mainly ester-linked fatty acid phospholipids based on glycerol-3-phosphate. On the other hand, inositol-derived phospholipids are present mainly in archaea and eukaryotic membranes, whereas sterols and sphingolipids, such as sphingomyelin, are ubiquitous among eukaryotes. In contrast, these three lipid types (inositol derived, sterols, and sphingolipids) occur only in a few bacterial groups, whereas cardiolipin is abundant in bacterial membrane, as well as in the inner mitochondrial membrane (Michell 2008; Hannich et al. 2011; Tian et al. 2012; Lombard et al. 2012).

According to the most accepted evolution model for the origin of the three domains of life, archaea and bacteria are considered descendants from a shared cenancestor, and Eukarya as having originated secondarily as a chimera derived from the endosymbiosis of the alphaproteobacterial ancestor of mitochondria within a proto-eukaryotic host (Embley and Martin 2006). The cenancestor probably had the enzymatic machinery to synthesize modern phospholipid components including glycerol-1, glycerol-3-phosphate, isoprenoids, and fatty acids (Lombard et al. 2012). Thus, the differences between archaeal and bacterial membranes would have arisen as these two domains diverged by tuning the relative importance of the different components with isoprenoids becoming dominant in archaea and fatty acids in bacteria (Lombard et al. 2012). The fact that eukaryotic cellular membranes have typical bacterial-like phospholipids argues in favor of a bacterium at the origin of the cellular membrane of the proto-eukaryotic host that acquired mitochondria (Lombard et al. 2012).

Although most bacterial membrane lipids are glycerophospholipids that contain two fatty acid chains, the lipidic composition of bacterial membranes is not constant, as it varies considerably in response to changes on environmental conditions such as temperature, osmolarity, salinity, pH, and availability of certain nutrients (Zhang and Rock 2008; Sohlenkamp and Geiger 2015). The capacity to modify their membrane lipid composition allows bacteria to survive environmental changes and unfavorable conditions (Zhang and Rock 2008; Sohlenkamp and Geiger 2015). In some bacteria, phospholipases play a role in environmental fitness in oligotrophic habitats, such as those with phosphorus deficiency, as they scavenge phosphate from the membrane glycerophospholipids, leading to an extensive membrane remodeling (Sohlenkamp and Geiger 2015).

Phospholipases constitute a diverse group of glycerophospholipid-hydrolyzing enzymes which, according to the cleavage site on its substrate, are classified into several groups. Phospholipases A and B are carboxyl ester acyl hydrolases. Phospholipases A release a fatty acid from the glycerol backbone leaving a lysophospholipid. According to their site of ester bond hydrolysis, phospholipases A are classified as phospholipases A_1 [EC 3.1.1.32] when hydrolyzing the fatty acid at the *sn*-1 position of the glycerol moiety, or phospholipases A_2 [3.1.1.4] when removing the fatty acid at the *sn*-2 position. Phospholipases B [EC 3.1.1.5] can hydrolyze both acyl groups at the *sn*-1 or *sn*-2 position of the glycerophospholipid. Phospholipases C [3.1.4.3] and D [EC 3.1.4.4] are phosphoric diester hydrolases which cleave either the glycerol- or alcohol-oriented phosphodiester bond, respectively. Phospholipases C release the phosphorylated head group (e.g., inositol

triphosphate or choline phosphate) and diacyl glycerol, while phospholipases D cleave the terminal phosphodiester bond releasing the head group (e.g., choline or inositol) from a phosphatidic acid.

Sphingomyelins are abundant constituents of animal cell membranes, even though they are also present in some lower eukaryotes and a few groups of bacteria (Hanada 2014; Sohlenkamp and Geiger 2015). Sphingomyelinases are phosphoric diester hydrolases that cleave sphingomyelins. Depending on the cleavage site, there are two different types of sphingomyelinases: sphingomyelinases C (EC 3.1.4.12), which hydrolyze the ester bond between ceramide and phosphorylcholine, and sphingomyelinases D (EC 3.1.4.41), which hydrolyze the phosphodiester bond between ceramide-1-phosphate and choline.

In eukaryotes, phospholipases and sphingomyelinases generate products that play crucial roles in regulating distinct physiological processes including membrane dynamics, cellular signaling, migration, growth, and death (Flores-Díaz et al. 2016). In pathogenic bacteria, 3 types of sphingomyelinases and 14 different types of phospholipases have been described playing roles in virulence for animal and human hosts (Flores-Díaz et al. 2016). However, the distribution of these enzymes within the three domains of life and their evolutionary relationships is not clear. It could have been that the bacterial phospholipases and sphingomyelinases evolved in settings outside of infection if they provide an advantage for survival in their natural environment, and virulence to multicellular organisms could be just coincidental (Flores-Díaz et al. 2016). Thus, the aim of this work was to determine the distribution of protein homologues to bacterial phospholipases and sphingomyelinases associated to virulence within the organisms represented in the reference proteome database, in order to evaluate if their occurrence is restricted to pathogenic bacterial taxa.

Distribution of Homologues to Bacterial Phospholipases and Sphingomyelinases Associated to Virulence

A total of 17 sequences corresponding to bacterial phospholipases or sphingomyelinases associated to virulence (Table 1) (Flores-Díaz et al. 2016) were blasted (Altschul et al. 1990) against 5783 whole proteomes from the reference proteome database of the Universal Protein Resource (UniProt Consortium 2008) (http://www.uniprot.org/), in order to identify homologous sequences. Amino acid sequences with an e-value less than 10^{-3} and more than 25% similarity with the query were extracted to a database and selected for the analysis.

The distribution of the 17 enzyme types across the phylogenetic tree of life is shown in Fig. 1. Even though the bacterial phospholipases and sphingomyelinases used as query in this study have been described as bacterial toxins, there are homologous proteins in the proteomes of several nonpathogenic prokaryotes and eukaryotes. Therefore, it is very likely that they have other biological functions nonrelated to pathogenesis, as previously suggested (Flores-Díaz et al. 2016).

Table 1 Types of bacterial phospholipases and sphingomyelinases and reference protein sequences used in the homologue search analysis (Flores-Díaz et al. 2016)

Enzyme type	Reference protein	UniProt accession code	Acronym of enzyme type
1A. One domain sphingomyelinases C	*Staphylococcus aureus* β-toxin	P09978	SmC-OD
1B. Two domains sphingomyelinases C	*Mycobacterium tuberculosis* outer membrane SMase C	P9WKQ1	SmC-TD
2. Sphingomyelinases D which adopt a TIM barrel structure	*Corynebacterium pseudotuberculosis* SMase D	P20626	SMD-TBS
3. Other sphingomyelinases D	*Photobacterium damselae* damselysin	D1J6Q	Other-SmD
4. Acyl hydrolases which adopt an antiparallel β-barrel structure	*Helicobacter pylori* PldA1	J9SFC6	AH-ABS
5. Surface-associated phospholipases A_2	*Legionella pneumophila* PlaB	D5TDK0	PLA2-SA
6A. One domain acyl hydrolases from the GSNH esterase family	*Salmonella enterica* serovar Typhimurium SseJ	Q9FD10	AH-ODGSNH
6B. Two domain acyl hydrolases from the GSNH esterase family	*Moraxella bovis* PLB	Q939K7	AH-TDGSNH
7. Acyl hydrolases which adopt the α/β hydrolase fold	*Yersinia enterocolitica* YplA	A1JKJ1	AH-αβHF
8. Secreted patatin-like acyl hydrolases	*Pseudomonas aeruginosa* ExoU	O34208	AH-SPL
9. Class XIB phospholipase A_2	Group A *Streptococcus pyogenes* SlaA	Q48SH8	PLA-CXIB
10. Phosphatidylinositol-specific phospholipase C	*Listeria monocytogenes* PlcA	P34024	PLC-PIS
11A. Single domain zinc metallophospholipases C	*Bacillus cereus* PLC	P09598	PLC-ODZ
11B. Two domains zinc metallophospholipases C	*Clostridium perfringens* alpha-toxin	Q0TV31	PLC-TDZ
12. Phospholipases C from the acid phosphatase superfamily	*Pseudomonas aeruginosa* PlcH	P06200	PLC-PacSF
13. Other phospholipases C	*Legionella pneumophila* PlcB	Q5ZVI5	Other-PLC
14. Phospholipases D	*Acinetobacter baumannii* PLD	B0VV34	PLD

Distribution of Homologues to Bacterial Sphingomyelinases and Phospholipases Associated to Virulence within Bacterial Taxa

Distribution of homologues to the bacterial phospholipases and sphingomyelinases associated to virulence within bacterial phyla shows that *Proteobacteria* and Bacteroidetes are the groups with most diverse number of homologues. Inside

Proteobacteria, the *Gammaproteobacteria* and the *Deltaproteobacteria* are the classes with more homologues to different enzyme types, although *Alphaproteobacteria* and *Betaproteobacteria* also include homologue sequences to several phospholipases A, phospholipases C, and one domain sphingomyelinases C. *Epsilonproteobacteria* presents only homologues to phospholipases D and acyl hydrolases which adopt an α/β hydrolase fold. The phyla in which only homologues to one enzyme type were found are *Chlorobi*, *Gemmatimonadetes*, *Planctomyces*, *Verrucomicrobia*, and *Aquificae*.

Archaeal Homologues to Bacterial Sphingomyelinases and Phospholipases Associated to Virulence

Within the archaea domain, only *Euryarchaeota* possess homologues to the zinc-dependent phospholipases C. The small quantity of archaeal proteomes in the database (208) could explain, at least partially, the lack of more homologue protein sequences in archaea.

Eukaryotic Homologues to Bacterial Sphingomyelinases and Phospholipases Associated to Virulence

A high diversity of sequences homologous to bacterial phospholipases and sphingomyelinases associated to virulence, including 8 out of the 17 study groups, is found in eukaryotes. Homologous to one and two domain sphingomyelinases C, several phospholipases A and phospholipases C, as well as phospholipases D homologues, are present. Sphingomyelinases and phospholipases are essential for signal transduction and events related to the upkeep process of membrane lipids in eukaryotes (Ritchie et al. 2016). Considering the similarities in their membrane lipids, the presence of multiple homologues to the bacterial enzymes in eukaryotes is not surprising.

Number and Diversity of Homologous Sequences for Each Enzyme Type

Among bacterial sphingomyelinases and phospholipases, the phospholipases C from the acid phosphate superfamily, phospholipases D, acyl hydrolases that adopt an antiparallel β-barrel structure, and the phosphatidylinositol (PI)-specific phospholipases C (PI-phospholipases C) had the most hits for homologous sequences in the UniProt database (Fig. 2). The phospholipases C from the acid phosphate superfamily got the higher number of hits within the bacteria domain, specifically in the phylum *Proteobacteria*. In contrast, the PI-phospholipase C had most hits (184 out of 246) in the Eukarya domain, with 136 sequences belonging to the fungi phylum

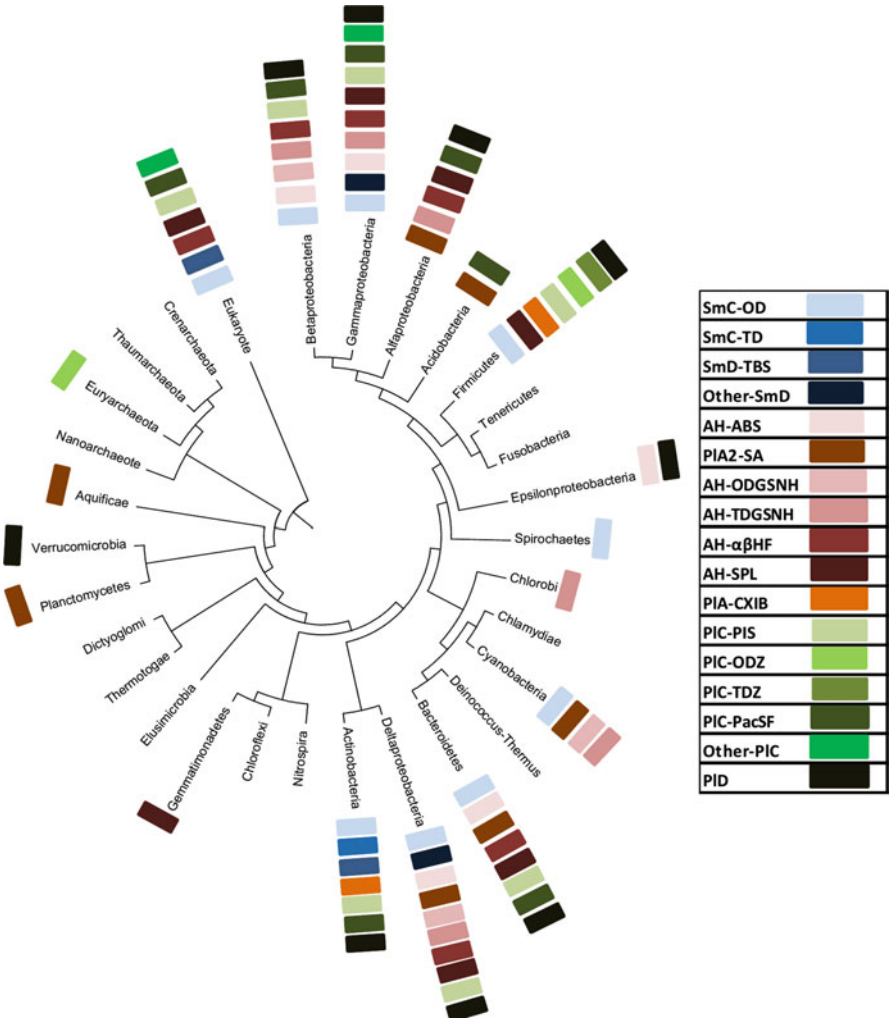

Fig. 1 Distribution of proteins homologous to the bacterial sphingomyelinases and phospholipases associated to virulence among the taxonomic groups, represented in the reference proteome database of UniProt. The phylogenetic tree was constructed based on the sequence of the 16S RNA gene

Ascomycota and 7 to *Basidiomycota*. Similarly, 80% of the hits of the sphingomyelinases D which adopt a TIM barrel structure are found in *Ascomycota*.

The one domain sphingomyelinases C (SmC-OD), despite getting only 85 hits, have the widest distribution with homologues spread across the bacterial phyla *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, *Proteobacteria*, and *Spirochaetes*. Smc-OD homologues are also present in the eukaryotic phyla *Alveolata*, *Amoebozoa*, *Glomeromycetes*, *Parabasalia*, and Cnidaria. Homologues to the

	Bacteria					Archaea	Eukarya		Total
	Actinobacteria (605)	Bacteroidetes (352)	Firmicutes (959)	Proteobacteria (1442)	Other_Bacteria (216)	Euryarchaeota (128)	Opisthokonta (612)	Other_Eukarya (75)	
PLC-PacSF	105	1	0	134	5	0	3	2	250
PLD	1	2	16	227	3	0	1	0	250
PLC-PIS	24	7	16	15	0	0	181	3	246
AH-ABS	0	10	0	171	0	0	0	0	181
SmC-OD	29	4	6	21	5	0	6	14	85
AH-TDGSNH	0	1	0	62	1	0	0	0	64
AH-ODGSNH	0	0	0	29	12	0	0	0	41
SmD-TBS	7	0	0	0	0	0	31	0	38
AH-αβHF	0	2	0	33	0	0	3	0	38
PLC-ODZ	0	0	30	0	0	3	0	0	33
Other-PLC	0	0	0	7	0	0	22	0	29
PLA2_SA	0	1	0	17	4	0	0	0	22
AH-SPL	0	2	1	13	1	0	2	1	20
PLA_CXIB	2	0	3	0	0	0	0	0	5
PLC_TOZ	0	0	5	0	0	0	0	0	5
Other-PLC	0	0	0	4	0	0	0	0	4
SmC-TD	3	0	0	0	0	0	0	0	3

Fig. 2 Heatmap showing the distribution of the hits for each enzyme type among the phyla represented in the reference proteome database of UniProt. Numbers of proteomes available for each phylum in the database are shown under the phyla name in each column

secreted patatin-like acyl hydrolases are also widely distributed, being present in *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Gemmatimonadetes*, as well as in the eukaryotic phyla Chordata and Heterokontophyta. The class XIB phospholipases A_2, the two domains zinc metallophospholipases C, the two domain sphingomyelinases C, and the other sphingomyelinases D have the lower number of hits and narrowest distribution and likely evolved more recently. Homologues of the *Streptococcus pyogenes* SlaA class XIB phospholipases A_2 are present only in two *Firmicutes* and two *Actinobacteria*. Two domains of zinc metallophospholipases C homologues were found only in different species of the *Clostridium* genus. The two-domain sphingomyelinases C have hits only in *Mycobacterium* species and other sphingomyelinases D only in the phylum *Proteobacteria*, specifically in *Pseudoalteromonas* species.

Phylogenetic Relations Between Homologues of Selected Enzyme Types

All sequences were clustered first according to the protein type and then according to the phyla contained in each type. Pairwise and multiple protein sequence alignments of each phyla with three or more sequences were carried out using MEGA7 (Kumar et al. 2016), using the muscle alignment tool and the default parameters. Phylogenetic analyses for each phylum within a protein type were conducted using the maximum likelihood methods with MEGA7, and reliability for internal branching was assessed using the bootstrap method. Graphical representation and edition of the

phylogenetic tree was performed and edited with MEGA6 (Tamura et al. 2013). Once the first series of trees was built, a second set comprising the totality of the sequences per protein group was calculated. The second set of phylogenetic analysis was performed randomly selecting a sequence per branch within each of the first phylogenetic trees of every phylum. The result was a representative set of sequences used to calculate a final tree for each group of proteins.

The sequences for the final trees for each protein group were analyzed using a different strategy. Protein sequences were aligned using GUIDANCE (Penn et al. 2010) employing a MAFFT algorithm and 100 bootstrap repeats, and the final editing of the alignments was done in MEGA6 (Tamura et al. 2013). Phylogenetic analyses were performed through Bayesian inference, using MrBayes 3.2 (Ronquist et al. 2012) mount on the CIPRES interface (Miller et al. 2010). All analyses employed one cold chain and three incrementally heated chains, with a temperature parameter set to 0.1. Four separate Markov Chain Monte Carlo runs were performed, with 1 million generations each, discarding the initial 250 000 generations from each run as burn-in and sampling one every 1000 generations to calculate posterior probabilities for each branch. All trees remaining after burn-in were used to construct a majority rule consensus tree. The final editing of each phylogenetic tree was done in *Archeopteryx* (Han et al. 2009).

A comprehensive analysis of the phylogenetic relations between protein families from the phospholipases and sphingomyelinases revealed that some groups present intra-genus-specific clades, whereas in others, closely related sequences are distributed among different domains. The phylogenetic relationships of the homologue sequences could reveal more clues about the distribution of these protein groups. A tree where all the sequences, regardless of their phylogenetic origin, share common ancestors (eukaryotic and prokaryotic) supports the possibility of a horizontal transfer. In contrast, a well-defined distribution of the homologue sequence clustering according to their phylogenetic origin suggests a possible vertical evolutionary process of that protein.

Homologues to Bacterial One Domain Sphingomyelinases C

Bacterial one domain sphingomyelinases C adopt the DNase I fold as mammalian neutral sphingomyelinases (Clarke et al. 2006). Homologues to bacterial one domain sphingomyelinases C were identified at the UniProt reference proteome database using the *Staphylococcus aureus* β-toxin sequence as query. This enzyme is required for *S. aureus* virulence in different infection models, as it contributes to the phagosomal escape, induces biofilm formation, and is cytotoxic to human keratinocytes, polymorphonuclear leukocytes, monocytes, and T lymphocytes (Flores-Díaz et al. 2016).

Figure 3 shows how the reference sequence of the *S. aureus* β-toxin (P09978) clusters in a well-defined branch with other *Staphylococcus* sequences, within a branch containing the sequences of homologous proteins from *Actinobacteria*, *Firmicutes*, and the anemone *Nematostella vectensis* (Naamati et al. 2009). Other

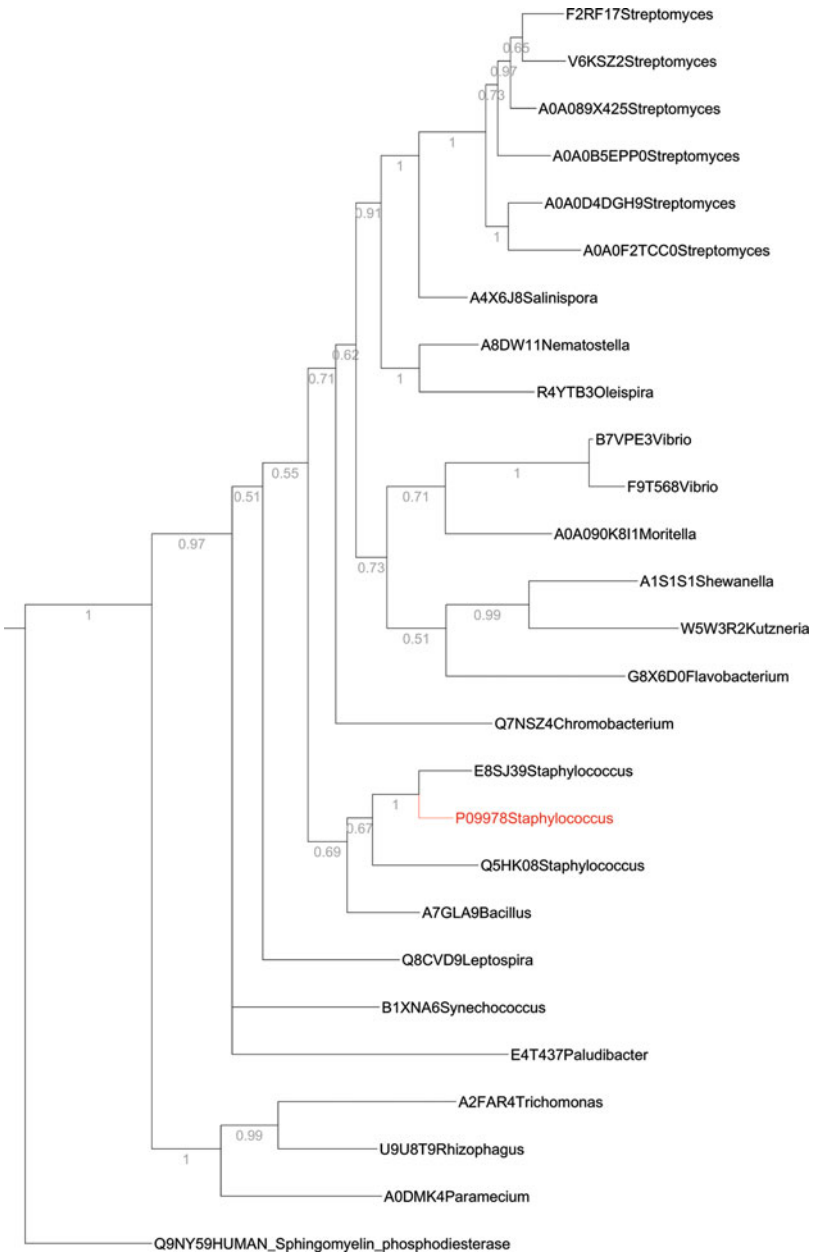

Fig. 3 Consensus phylogenetic tree of homologues to bacterial one domain sphingomyelinases C. Each taxon name indicates the NCBI accession code and genus of each sequence; the numbers in the tree branches correspond to the posterior probabilities calculated by the Bayesian method. The reference protein sequence for this tree is *red*

eukaryotic sequences corresponding to trichomonas, *Paramecium*, and the fungus *Rhizophagus* clustered in a separate branch apart from the reference sequence.

Homologues to Bacterial Sphingomyelinases D Which Adopt a TIM Barrel Structure

Bacterial sphingomyelinases D which adopt a TIM barrel structure are homologues to enzymes from several fungi and from spiders of the Sicariidae family (Dias-Lopes et al. 2013). Homologues to bacterial sphingomyelinases D were identified at the UniProt reference proteome database using the sequence of the *Corynebacterium pseudotuberculosis* enzyme as query. *C. pseudotuberculosis* sphingomyelinase D induces death of infected macrophages and is critical for the establishment and dissemination of the bacterium in caseous lymphadenitis in ruminants (Flores-Díaz et al. 2016).

As shown in Fig. 4, the sequence of *C. pseudotuberculosis* sphingomyelinase D clusters in a branch surrounded by sequences from fungi of the *Ascomycota* phylum. Homologue sequences from other *Actinobacteria* are located in a more derived branch, closer in the tree to sequences from *Coccidioides* and other fungi than to the one including the *C. pseudotuberculosis* enzyme. This result supports a plausible

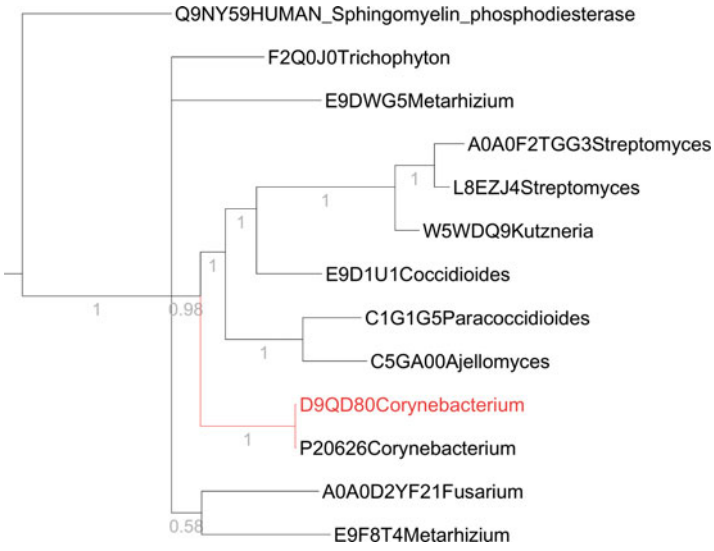

Fig. 4 Consensus phylogenetic tree of homologues to bacterial sphingomyelinases D which adopt a TIM barrel structure. Each taxon name indicates the NCBI accession code and genus of each sequence; the numbers in the tree branches correspond to the posterior probabilities calculated by the Bayesian method. The reference protein sequence for this tree is *red*

horizontal transfer of the corresponding gene between bacteria and fungi, as previously suggested (Dias-Lopes et al. 2013).

Homologues to Bacterial Acyl Hydrolases that Adopt the α/β Hydrolase Fold

Homologues to bacterial acyl hydrolases that adopt the α/β hydrolase fold were identified at the UniProt reference proteome database, using as query the sequence of the *Yersinia enterocolitica* YplA. This enzyme is a secreted phospholipase A_2 which plays a role in colonization of lymphoid tissue and bowel tissue necrosis in a mouse model of infection (Schmiel et al. 1998). Figure 5 shows that the YplA sequence clusters with proteins from other bacteria and is separate from the homologue sequence from *Pyrenophora*, the only fungi sequence in the tree, which suggests an early divergence of the bacterial proteins from the more ancestral fungal protein.

Homologues to Bacterial PI-Specific Phospholipases C

Bacterial PI-phospholipases C adopt a TIM barrel structure and have been described in *Firmicutes*, *Spirochaetes*, and *Actinobacteria* (Flores-Díaz et al. 2016).

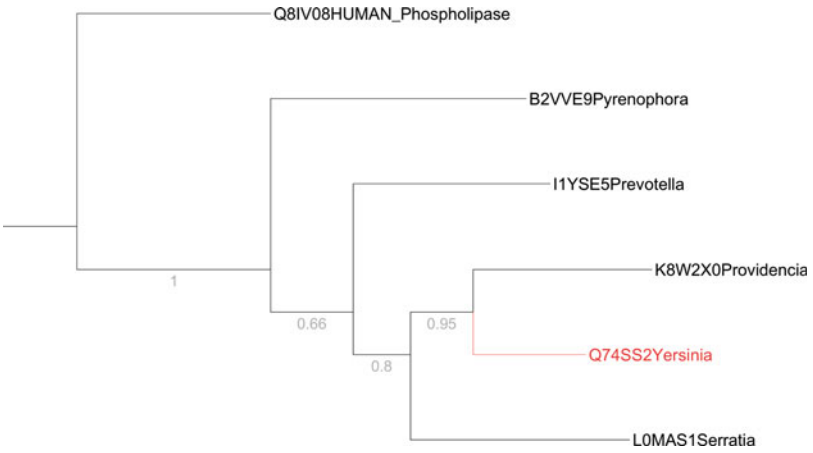

Fig. 5 Consensus phylogenetic tree of homologues to bacterial acyl hydrolases which adopt the α/β hydrolase fold. Each taxon name indicates the NCBI accession code and genus of each sequence; the numbers in the tree branches correspond to the posterior probabilities calculated by the Bayesian method. The reference protein sequence for this tree is *red*

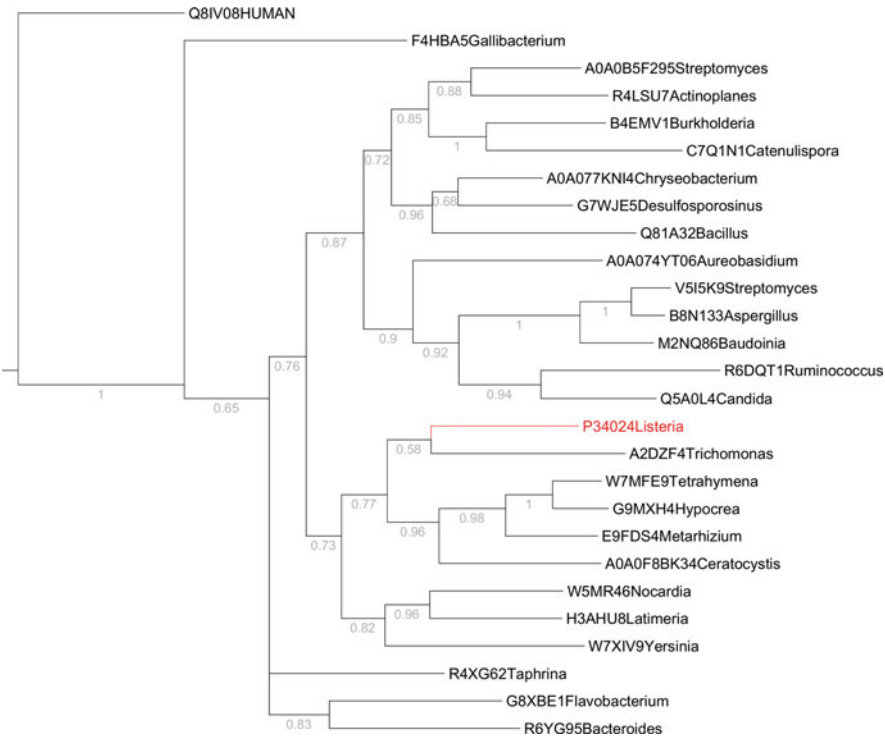

Fig. 6 Consensus phylogenetic tree of homologues to bacterial PI phospholipases C. Each taxon name indicates the NCBI accession code and genus of each sequence; the numbers in the tree branches correspond to the posterior probabilities calculated by the Bayesian method. The reference protein sequence for this tree is *red*

Homologues to bacterial PI-phospholipases C were identified at the UniProt reference proteome database using as query the sequence of the *Listeria monocytogenes* enzyme. *L. monocytogenes* PI-phospholipase C is required for virulence as it contributes to bacterial escape from the phagosome into the cytosol and prevents autophagic flux, thus favoring the escape of cytosolic bacteria from the host autophagic defense (Tattoli et al. 2013).

The phylogenetic tree of proteins homologous to bacterial PI-phospholipase C in Fig. 6 shows the biggest branch divided in two main subbranches. One contains the *L. monocytogenes* reference sequence clustered with sequences from *Actinobacteria*, fungi, and *Parabasalia* and the other one with sequences from yeast, *Actinobacteria*, and *Firmicutes*. A heterogeneous distribution like this suggests that this protein has undergone a horizontal gene transfer process instead of following a vertical evolution, where homologues would be more closely related when the organisms share a common ancestor.

Homologues to Bacterial Phospholipases C from the Acid Phosphatase Superfamily

Homologues to bacterial phospholipases C from the acid phosphatase superfamily were identified at the UniProt reference proteome database using the sequence of the *P. aeruginosa* PlcH/PlcS as query. PlcH/PlcS is hemolytic and cytotoxic to endothelial cells and is required for virulence in systemic experimental infections in mice (Flores-Díaz et al. 2016).

The phylogenetic tree of the PlcH/PlcS homologues (Fig. 7) locates the reference sequence in a separate branch sharing a common ancestor with the three most ancient nodes of the tree, which could be explained by an early divergence of the protein compared to other homologues. Regarding the main branches of the tree, the first one

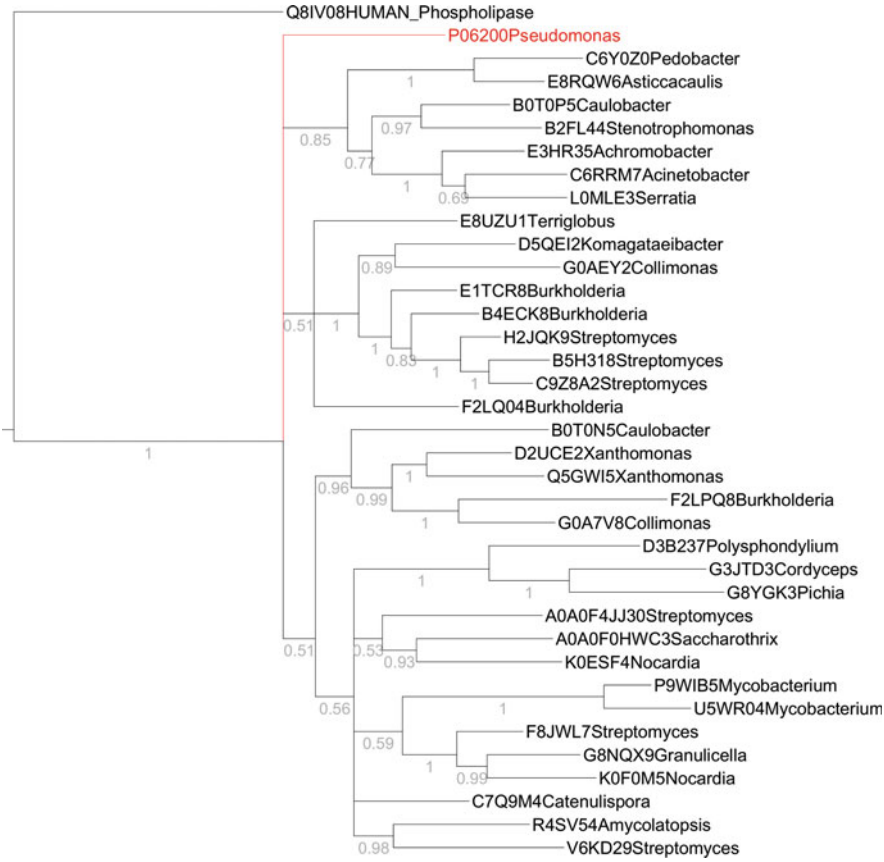

Fig. 7 Consensus phylogenetic tree of phospholipase C from the acid phosphatase superfamily. Each taxon name indicates the NCBI accession code and genus of each sequence; the numbers in the tree branches correspond to the posterior probabilities calculated by the Bayesian method. The reference protein sequence for this tree is *red*

includes sequences from *Proteobacteria*; the second combines sequences from *Actinobacteria*, *Acidobacteria*, and *Proteobacteria*; and the third comprises sequences from *Actinobacteria*, *Proteobacteria*, and eukaryotic sequences from the *Ascomycota* and *Amoebozoa* phyla.

Concluding Remarks

The phylogenetic analysis performed in this study shows the presence of proteins homologous to bacterial sphingomyelinases and phospholipases, associated to virulence, in several nonpathogenic bacteria and eukaryotes including fungi and protozoa.

Most bacterial sphingomyelinases and phospholipases are present in proteomes of at least two different bacterial phyla, and about half of the enzyme types are present in eukaryote proteomes as well. Several types of bacterial sphingomyelinases and phospholipases associated with virulence show a patchy distribution across broad taxonomic boundaries, which suggest the occurrence of horizontal gene transfer events. Lateral gene transfer is recognized as playing a substantial role in the adaptive expansion of many prokaryotic protein families (Dagan et al. 2008; Treangen and Rocha 2011).

The presence of homologues to bacterial sphingomyelinases and phospholipases associated to virulence in eukaryotes suggests either an ancient origin or the occurrence of horizontal gene transfer events. Lateral gene transfer plays a role in the evolution of the pangenome of different species (Soucy et al. 2015), being documented to occur even between a prokaryote and a multicellular organism. A good example is the case of some insect species and the endosymbiont bacteria *Wolbachia pipientis* (Hotopp et al. 2007). In this case, insect genes have been detected within the *Wolbachia* genome, and on the other hand, genes from the bacterium have also been found inserted in the genetic material of infected insects (Hotopp et al. 2007).

The presence of homologues to bacterial sphingomyelinases and phospholipases associated to virulence in nonpathogenic bacteria supports the possibility of additional roles, not related to pathogenesis. Some bacterial phospholipases are known to contribute to some aspects of the lifestyles of bacteria, including membrane remodeling and competition with other microorganisms for survival in their natural environments. Some phospholipases A which adopt the α/β hydrolase fold play a role in antagonistic intra- and interspecies bacterial interactions, after being injected by a type VI secretion system into the periplasmic space of adjacent target cells, and are referred to as type VI lipase effectors (Tle) (Russell et al. 2013). In *Vibrio cholerae*, the Tle effector Tle2VC/VC1418/TseL contributes to colonization of the upper intestinal mucosa during an experimental infection in rabbits (Fu et al. 2013) and furthermore is also required to escape predation by amoeba (Dong et al. 2013). Other enzymes, such as PlcH/PlcS, which play a critical role in pathogenesis of infections by *P. aeruginosa*, also have a role in killing *Candida albicans* filaments, and this antagonism could have contributed to its evolution and maintenance (Hogan

and Kolter 2002). Thus, some bacterial phospholipases which play roles in virulence exert deleterious effects on a broad array of competitors and could have evolved as a strategy to compete for space and resources with other microorganisms in their natural environments outside of infection settings (Russell et al. 2014).

Several types of bacterial sphingomyelinases and phospholipases associated to virulence serve bacteria in phagosomal escape or induce phagosomal arrest. On the other hand, several of those enzyme types (including acyl hydrolases that adopt the α/β hydrolase fold, secreted patatin-like acyl hydrolases, and phospholipases C from the acid phosphatase family) play roles in avoiding amoeba predation in different bacterial species. Bacterial survival in diverse ecosystems has been constrained for more than 800 million years by the pressure of predation by amoeba and other heterotrophic protists, and this is considered a major force in the selection of traits serving as antipredator adaptations (Matz and Kjelleberg 2005; Cavalier-Smith 2009). Phagocytosis evolved in an ancestral unicellular eukaryote as a way to acquire food by predation of other microorganisms and was then maintained as a core function of professional phagocytes in metazoans (Greub and Raoult 2004; Cavalier-Smith 2009; Smith and May 2013; Weiss and Schaible 2015).

Phagocytosed bacteria are killed by both amoeba and phagocytes by similar mechanisms activated after extensive remodeling of the phagosomal membrane and a phagosome maturation process, which requires a sequence of fusion events with multiple components of the endocytic pathway (Levin et al. 2015). Due to the striking similarities between the phagocytic and microbicide molecular machineries of amoeba and macrophages, the same mechanisms are effective for escape or survival within amoeba or macrophages (Siddiqui and Khan 2012). Thus, it is possible that some obligate and facultative intracellular bacterial pathogens of animals and humans evolved after surviving phagocytosis by free-living amoeba and adapting in them to an intracellular lifestyle (Harb et al. 2000; Greub and Raoult 2004; Casadevall and Pirofski 2007; Martínez 2013; Erken et al. 2013).

Since the metabolism of phagosomal membrane lipids controls a remarkable number of events during phagocytosis, the acquisition of bacterial sphingomyelinases and phospholipases which contribute to phagosomal escape or arrest could have been favored by the selective pressure of fighting predator amoebae (Molmeret et al. 2005). Thus, bacterial predation by amoeba could have served as a selection force during the evolution of bacterial sphingomyelinases and phospholipases associated with virulence in some pathogens. Those enzymes might have arisen before the emergence of multicellularity, as adaptations for intra-amoebal survival and multiplication, and then by chance providing enhanced fitness for pathogenicity in metazoans.

Certain ecosystems with a vast availability of nutrients and a high density of cells, such as the gastrointestinal tract of multicellular organisms, rhizospheres, and manure soil, are known to facilitate horizontal gene transfer between diverse bacteria (Aminov 2011; Huddleston 2014). The cytoplasm of free-living amoebas, which brings together diverse microorganisms, also provides ideal conditions for the occurrence of horizontal gene transfer among diverse microorganisms and with the amoebal host itself (Bertelli and Greub 2012; Scheid 2014). Free-living amoebas are

a heterogeneous group within *Amoebozoa*, commonly found in humid environments, which feed on bacteria and other microorganisms. During biofilm synthesis, free-living amoebas undergo constant interactions with many microorganisms, some of which manage to escape degradation of the intracellular amoeba machinery and eventually adapt to on intracellular life.

After more than 800 million years of evolution, several bacterial species from different phylogenetic groups have developed the ability to persist and thrive within amoeba (Greub and Raoult 2004; Molmeret et al. 2005). Among the human pathogenic bacteria known to be amoeba resistant are species within the genera *Chlamydia*, *Bacillus*, *Staphylococcus*, *Listeria*, *Mycobacterium*, *Helicobacter*, *Rickettsia*, *Pseudomonas*, *Yersinia*, *Legionella*, *Salmonella*, *Francisella*, *Acinetobacter*, *Vibrio*, *Aeromonas*, and *Burkholderia* (Greub and Raoult 2004; Scheid 2014). Furthermore, several fungi species, like *Cryptococcus neoformans*, also survive inside different free-living amoeba species (Greub and Raoult 2004; Scheid 2014). Genes encoding sphingomyelinases and phospholipases, which contribute to intracellular survival, could have been horizontally transferred among diverse microorganisms with a sympatric lifestyle within free-living amoebae.

Conclusion

The wide distribution of bacterial sphingomyelinases and phospholipases described in this work points to the fact that probably more functions of these proteins remain to be described. The capacity of these enzymes to modify lipid membranes suggests that their acquisition confers higher competence in the environment. Factors that pressure lateral gene transfer events like harsh environments, cell to cell contact, and host immune response create the opportunity for the transference of genes encoding those enzymes between organisms of different lineages. Further work would be necessary to clarify the extent of the functions performed by these lipid hydrolyzing enzymes and the mechanisms underlying the transference of their genes between different species.

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Mode of Action and Toxicity of Major Cyanobacterial Toxins and Corresponding Chemical Variants

23

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Abstract

Toxic cyanobacterial blooms are often found in aquatic ecosystems, spanning from fresh to brackish waters and marine waters, and may reflect the increased eutrophication of these environments and alterations in climate. Cyanobacterial toxins (cyanotoxins) are secondary metabolites, with very different chemical structures, and highly reactive to various biological molecules. Scientists and public health and environmental agencies have recognized that contamination by cyanobacterial toxins is a global and serious environmental and health threat. Notwithstanding, it is notorious the efforts done so far from the scientific community that resulted in the isolation, purification, identification, structure elucidation of several of these groups of toxins from many ecosystems worldwide as well as the identification of respective molecular targets and biological activities. The chemical variability of cyanotoxins is a critical determinant of the biological activity leading to a need to classify the different groups but also to distinguish the several chemical variants within each group. Cyanotoxins are thus currently classified on the basis of their chemical composition and toxic activity. This chapter aims to review and summarize key information concerning this class of natural compounds produced by cyanobacteria, as perceived by the authors being

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critical for understanding the impact of these compounds in the environment, and thus necessary for carrying out and validating risk assessment studies.

Keywords
Harmful algal blooms • Cyanobacterial toxins • Chemodiversity • Mode of action • LC_{50}

Contents

Introduction

The intensification of agricultural and industrial activities in recent decades has caused several environmental problems such as the eutrophication of marine, brackish, and fresh waters that can lead to excessive development of cyanobacterial blooms (O'Neil et al. 2012). The frequency and intensity of these blooms, which may contain strains that produce toxins, known as cyanotoxins, increased in the last decades limiting the use of the available water resources and representing a risk to human health (O'Neil et al. 2012). These toxins are secondary metabolites that can accumulate in the food chain and contaminate drinking waters, thus posing a potential threat to the health of humans and aquatic organisms (Chorus and Bartram 1999).

The cyanobacterial toxins can be classified into five functional groups: hepatotoxins, neurotoxins, cytotoxins, dermatotoxins, and irritant toxins (lipopolysaccharides) (Chorus and Bartram 1999). Moreover, cyanotoxins are structurally diverse chemicals with different modes of action and toxicity. Until now, more than 100 different molecules have been described, with microcystins constituting the most diverse group of toxins. With reference to the chemical structure, cyanotoxins fall into several groups: cyclic peptides (microcystin and nodularin), alkaloids (e.g., anatoxin-a, cylindrospermopsin, saxitoxin, palytoxin, lyngbiatoxin), amino acids (e.g., β-methylamino-L-alanine), phosphate esters (anatoxin-a(s)), lipopolysaccharides, and lipopeptides (jamaicamides, antillatoxins).

This review reports the chemical structure of the major cyanotoxins from fresh, brackish, and marine habitats and their main mechanisms of toxicity. This information is presented in the subsequent sections and Table 1. Particular emphasis is given to the differential toxicity of the known chemical variants, with this being a factor of critical importance to assess the fate of this group of natural compounds in the environment and human health.

Table 1 Chemodiversity and toxicity of major cyanobacterial toxins

Toxin name	Group	General chemical formula	Molecular target	Cyanotoxin variant	Toxicity (LD50)	References
Microcystins	Hepatotoxin	$C_{49}H_74N_{10}O_{12}$	Protein phosphatase 1 and 2A	Microcystin-LR (MC-LR)	50–100 μg/kg (mice)	Rinehart et al. 1994
				6Z-Adda-MC-LR	>1200 μg/kg (mice)	
				Microcystin-RR (MC-RR)	500–800 μg/kg (mice)	Rinehart et al. 1994 Watanabe et al. 1988
				Microcystin-LA (MC-LA)	50 μg/kg (mice)	Chorus and Bartram 1999
				Microcystin-LF (MC-LF)	250 μg/kg (mice)	Chorus and Bartram 1999
				Microcystin-YR (MC-YR)	150–200 μg/kg (mice)	
				Microcystin-YM MC-YM)	56–110 μg/kg (mice)	
				Microcystin-LY (MC-LY)	90 μg/kg (mice)	
Nodularins	Hepatotoxin	$C_{41}H_60N_8O_{10}$	Protein phosphatase 1 and 2	NOD-R	50 μg/kg (mice)	Eriksson et al. 1988
				[D-Asp1]NOD	75 μg/kg (mice)	Namikoshi et al. 1994
				[DMAdda3]NOD	150 μg/kg (mice)	
				[(6Z)-Adda3]NOD	>2000 μg/kg (mice)	

(*continued*)

Table 1 (continued)

Toxin name	Group	General chemical formula	Molecular target	Cyanotoxin variant	Toxicity (LD50)	References
Anatoxin-a	Neurotoxin	$C_{10}H_{15}NO$	Nicotinic acetylcholine receptor (NAChR)	Anatoxin-a (ATX-a)	375 μg/kg (mouse)	Chorus and Bartram 1999
				Homoanatoxin-a (HTX-a)	250 μg/kg (mouse)	
				Dihydroanatoxin-a (dhATX)	2 mg/kg (mouse)	Mann et al. 2012
				Dihydrohomoanatoxin-a (dhHTX)		
Saxitoxins	Neurotoxin	$C_{10}H_{17}N_7O_4$	K, Na, Ca voltage-gated channels	Saxitoxin (SXT)	10 μg/kg (mice)	Halstead and Schantz, 1994
				NeoSaxitoxin (NeoSXT)	65 μg/kg (mice)	Wolf and Frank 2002
Lyngbyatoxins	Neurotoxin	$C_{27}H_{39}N_3O_2$	Protein kinase C	Lyngbyatoxin A	250 μg/kg (mice)	Ito et al. 2002
				Lyngbyatoxin B	–	Aimi et al. 1990
				Lyngbyatoxin C	–	
Palytoxins	Neurotoxin	$C_{129}H_{223}N_3O_{54}$	Na/K pump	Palytoxin (PLTX)	767 μg/kg (mice)	Tubaro et al. 2011
				42-hydroxypalytoxin (42-OH-PLTX)	651 μg/kg (mice)	
β-methylamino-L-alanine	Neurotoxin	$C_4H_{10}N_2O_2$	Multiple mechanisms of action	β-methylamino-L-alanine (BMAA)	3 mg/g (mice)	Al-Sammak 2012
Anatoxin-a(s)	Neurotoxin	CHN_4O_4	Acetylcholinesterase (AChE)	Anatoxin-a(s) (ATX-a(s))	40–60 μg/kg (mice)	Mamood and Carmichael 1986

Jamaicamides	Neurotoxin	$C_{27}H_{36}BrClN_2O_4$	Voltage-gated sodium channel	Jamaicamide A	17.62 nmol (100% lethality) (goldfish)	Edwards et al. 2004
				Jamaicamide B	10.22 nmol (100% lethality) (goldfish)	
				Jamaicamide C	20.36 nmol (100% lethality) (goldfish)	
Antillatoxins	Neurotoxin	$C_{28}H_{45}N_3O_5$	Voltage-gated sodium channel	Antillatoxin	0.1 μM[a] (goldfish) 0.05 μg/ml (goldfish)	Nogle et al. 2001
				Antillatoxin B or 8-demethyl-antillatoxin	10 μM[a] (goldfish)	
Cylindrospermopsin	Cytotoxin	$C_{15}H_{21}N_5O_7S$	Glutathione and protein synthesis	Cylindrospermopsin (CYN)	2100 μg/kg (mice)	Banker et al. 2001
				7-epicylindrospermopsin (7-epi-CYN)	–	
				7-deoxycylindrospermopsin (7-deoxy-CYN)	–	

[a]Toxicity value refers to LC50

Hepatotoxins

Microcystins

Microcystins (MCs) were firstly described as being produced by *Microcystis aeruginosa*, but the number of reports showing that MCs may be produced by many other genera and species of cyanobacteria is increasing (Chorus and Bartram 1999). MCs are probably the most widespread and frequently occurring cyanotoxins (Dawson 1998; Chorus and Bartram 1999).

MCs (MW 900–1200 Da) are small cyclic peptides with the following general structure: cyclo-(D-alanine-X-DMeAsp-Z-Adda-D-glutamate-Mdha), in which X and Z are variable L-amino acids, D-MeAsp is D-erythro-b-methylaspartic acid, Mdha is *N*-methyldehydroalanine, and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Dawson 1998). A two-letter suffix (XY) nomenclature indicates the two variable amino acids in the structure of this molecule. Differences in amino acid composition and methylation patterns, and other less common chemical modifications, give rise to a diverse number of MC variant/congener molecules. At least 100 different MC congeners have been characterized and new ones are continuously being discovered (Puddick et al. 2014; Qi et al. 2015). The substitutions of the variable L-amino acids, in positions X and Z of this molecule, lead to some of the most common MC chemical variants, with leucine, arginine, or tyrosine in position X and arginine, alanine, or methionine in position Z (Rinehart et al. 1994).

MCs are known to be potent inhibitors of protein phosphatase 1 and 2A (PP1 and 2A), establishing a covalent bond with these enzymes. The liver is the main target organ of this group of toxins that specifically accumulate in hepatocyte cells via organic-anion membrane transporting system (OATP) (Dawson 1998; Fischer et al. 2005). The inhibition of PP1 and PP2A starts with the noncovalent binding of the amino acid Adda in the active center of the enzymes, this is responsible for the main inhibitory effects of the toxin (MacKintosh et al. 1990). This interaction becomes definitive with the covalent binding between the carboxyl group of Mdha residue of the toxin and the cysteine-273 of the catalytic subunit of PP1A or the cysteine-266 of the catalytic subunit of PP2A (MacKintosh et al. 1990). This, and also other proposed mechanisms, can modulate the expression of several oncogenes, proto-oncogenes, cytokines, and the tumor necrosis factor α (TNFα), which in turn affects cell division, cell survival, and induces apoptosis (IARC 2010). Several studies have also reported that MCs induce oxidative stress (Zegura et al. 2011). MC-LR is a potential tumor promoter, being classified by IARC (International Agency for Research on Cancer) as possibly carcinogenic to humans (Group 2B) (IARC 2010). In relation to the structure of this toxin, modifications of either Adda (e.g., isomerization of its diene group from 6(E) to 6(Z)) or D-glutamate (e.g., acetylation or esterification) dramatically decrease or abolish the toxicity of MCs in mice (Harada et al. 1990; Rinehart et al. 1994). Acute toxicity studies demonstrated that MC-LR ($C_{49}H_{74}N_{10}O_{12}$, MW 995.17 Da, Fig. 1) is one of the most potent cyanobacterial toxins. The toxicity within this group of molecules can vary greatly, from the highly toxic MC-LR with a median lethal dose (LD_{50}) of 50 μg/kg

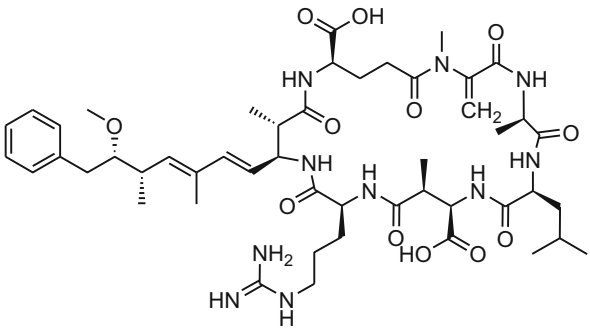


Fig. 1 Structure of microcystin-LR

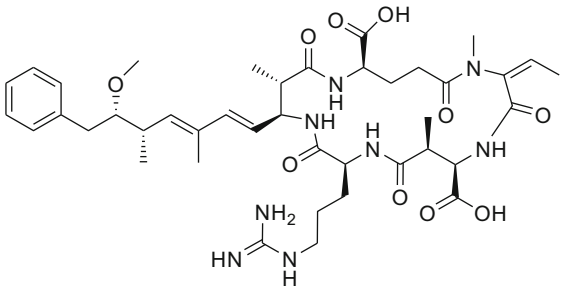
to the nontoxic variant 6Z-Adda5-MC-LR with $LD_{50} > 1200$ µg/kg, as assessed by mouse bioassay (Rinehart et al. 1994). Other chemical MC variants show a similar LD_{50} range such as MC-LR, MC-LA, MC-YR, and MC-YM (Rinehart et al. 1994; Chorus and Bartram 1999), while for MC-RR a significantly higher dose of 600 µg (about ten times higher) is needed to produce the same lethal effect (Watanabe et al. 1988). Surprisingly, the inhibitory activity of MC-LR and MC-RR on PP2A is in the same range: 1.6 and 3.4 nM, respectively (Yoshizawa et al. 1990). Moreover, the hydrophilic properties of MCs have apparently no significant effect on protein phosphatase inhibition as the more lipophilic MCs, namely MC-LW and MC-LF, and the more hydrophilic MC-LR and MC-RR inhibit ser/thrPP activities at approximately equimolar concentrations (Fischer et al. 2010). This observation leads to the assumption that the differences in toxicity of the known MC variants are also the result of their individual toxicokinetics (absorption and distribution), in addition to the toxicodynamics (PP-inhibition).

Nodularins

Nodularin (NOD) (Fig. 2) is produced mainly by *Nodularia spumigena*, a cyanobacterium that usually forms toxic blooms in brackish and estuarine environments (Chorus and Bartram 1999).

NOD ($C_{41}H_{60}N_8O_{10}$, MW 824.96 Da) is structurally similar to MC, being a cyclic nonribosomal pentapeptide with a reference structure constituted of Adda as well as D-glutamic acid (D-Glu), *N*-methyldehydrobutyrine (MeDhb), D-erythro-β-methylaspartic acid (D-MeAsp), and L-arginine (L-Arg) (NOD-R) (Rinehart et al. 1988). The structural differences between MC and NOD are the absence of two core amino acids in NOD (D-Alanine and the variable amino acid in position X), and the substitution of the MC residue, *N*-methyldehydroalanine, by *N*-methyldehydrobutyrine in NOD (Rinehart et al. 1988). Having a similar structure to MC, NOD also inhibits serine/threonine PP1 and PP2A being a potent hepatotoxin. However, contrary to MC, NOD does not bind covalently to PP1 and 2A (Bagu et al. 1997). Like MC, NOD is a potent tumor promoter that may also act as a carcinogen or tumor initiator of liver cell division (Ohta et al. 1994). However, and in contrast to MC-LR, NOD has been placed into Group 3 (substances not

Fig. 2 Structure of nodularin



classifiable as to its carcinogenicity to humans) of IARC, probably due to the lack of sufficient experimental data on carcinogenicity to humans (IARC 2010).

NOD chemical variants can emerge at every amino acid position of the peptide; taking NOD-R as the reference molecule, the following chemical variants have been identified: linear NOD, [D-Asp1]NOD, [DMAdda3]NOD, [6(Z) Adda3]NOD, [Glu4(OMe)]NOD, [MeAdda]NOD, [dhb5]NOD, [L-Har2]NOD, and [L-Val2]NOD (Har = homoarginine) (Mazur-Marzec et al. 2006). NOD-R has an LD_{50} of 50 μg/kg (intra-peritoneal route of injection) in mice (Eriksson et al. 1988). The linear NOD is evidently the precursor of this cyclic peptide (Choi et al. 1993) and may have no biological activity, as the cyclic conformation seems to be necessary for the molecule to interact with its molecular targets (Rinehart et al. 1994). There are three demethylated variants described until now: [D-Asp1]NOD with D-Asp1 instead of D-MeAsp1 (Namikoshi et al. 1994), [DMAdda3]NOD with DMAdda3 instead of Adda3 (Namikoshi et al. 1994), and a demethylated dehydrobutyric acid [dhb5]NOD (Mazur-Marzec et al. 2006). The LD_{50} values in mice (i.p.) for [D-Asp1]NOD and [DMAdda3] NOD were 75 and 150 μg/kg, respectively (Namikoshi et al. 1994). There are also two variants that have an additional methyl group: [MeAdda]NOD (Namikoshi et al. 1994) and [Glu4(OMe)]NOD (Rinehart et al. 1994). Additionally, the L-Arg residue of NOD may be replaced by a homoarginine (nodularin-Har: [L-Har2] NOD) (Saito et al. 2001) or valine (motuporin: [L-Val2]NOD) (de Silva et al. 1992). The L-Val residue is responsible for additional cytotoxicity of motuporin against cancer cell lines. In contrast, the 6Z-stereoisomer of Adda3 [6(Z)Adda3]NOD seems to reduce the toxicity of NOD. This variant was not toxic to mice at 2 mg/kg (Namikoshi et al. 1994).

Neurotoxins

Anatoxin-a

Anatoxin-a (ATX-a) (Fig. 3) is a potent naturally occurring alkaloid neurotoxin, produced worldwide by several genera of cyanobacteria such as *Anabaena* (particularly *Anabaena flos-aquae*), *Aphanizomenon, Microcystis, Planktothrix*, and *Oscillatoria* (Viaggiu et al. 2004).

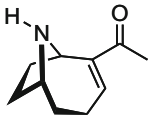


Fig. 3 Structure of anatoxin- a

ATX-a exists as two enantiomers, but only (+)ATX-a is found in nature. Chemically, ATX-a is an alkaloid described as a semi-rigid bicyclic aliphatic secondary amine, with an α,β-enone ketone moiety and molecular formula $C_{10}H_{15}NO$ (MW = 165.26 Da) (Devlin et al. 1977). The molecule suffers rapid hydrolyses in water and is photo-unstable; therefore, it does not persist for long periods in the environment and provokes mainly acute, rather than chronic, toxicity. This alkaloid mimics the neurotransmitter acetylcholine (Ach) and irreversibly binds the nicotinic acetylcholine receptor (NAChR) which depolarizes the cell membrane (Wonnacott and Gallagher 2006). In addition, ATX-a, which seems to be more efficient in stimulating muscles than Ach itself, is not degraded by acetylcholinesterase (Osswald et al. 2007). Thus, the toxin affects signal transmission between neurons and muscles, eventually leading to muscle paralysis, respiratory arrest, and death within a few minutes (Osswald et al. 2007). It is thought to be the bicyclic structure and ketone group of ATX-a that enables this molecule to mimic neurotransmitter Ach and to bind strongly to the NAChR in neurons and at neuromuscular junctions. ATX-a can also trigger dopamine and noradrenaline release (Barik and Wonnacott 2006).

Several analogs of ATX-a have been detected in field samples or in cultures of cyanobacteria, but the most commonly found are homoanatoxin-a (HTX-a), dihydroanatoxin-a (dhATX), and dihydrohomoanatoxin-a (dhHTX) (Wonnacott et al. 1992; Mann et al. 2012). HTX-a is a methyl derivative of ATX-a, with molecular formula $C_{11}H_{17}NO$ (MW = 179 Da) that has a propionyl group at C-2 instead of the acetyl group present in ATX-a (Wonnacott et al. 1992). In addition to being a nicotinic agonist, HTX-a can increase the release of Ach from peripheral cholinergic nerves through opening of endogenous voltage-dependent neuronal L-type Ca^{2+} channels (Wonnacott et al. 1992). In mouse bioassays, the LD_{50} of ATX-a and HTX-a is 375 and 250 μg/kg, respectively (Chorus and Bartram 1999). While HTX-a is a neurotoxin as potent as ATX-a, dhATX, and dhHTX, which are respective derivatives of ATX-a and HTX-a, have an approximate tenfold reduction in binding affinity for nicotinic acetylcholine binding sites with a LD_{50} of 2 mg/kg (Mann et al. 2012). It has been suggested that dhATX and dhHTX are degradation products of ATX-a and HTX-a, respectively (Stevens and Krieger 1991), although the exact chemical reactions and enzymes responsible for these modifications were not identified yet (Mann et al. 2012).

Saxitoxins

In fresh waters, saxitoxins (STXs) are mainly produced by *Anabaena circinalis* and *Aphanizomenon flos-aquae* (Chorus and Bartram 1999), but they have also been

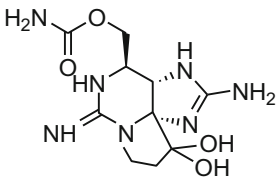


Fig. 4 Structure of saxitoxin

associated with *Lyngbya wollei* and *Cylindrospermopsis raciborskii* (Nicholson et al. 2003). STXs are part of a wider group of toxins designated as paralytic shellfish poisoning (PSPs), which are produced by dinoflagellates in a marine environment.

STXs are a family of trialkyl tetrahydropurine, with the NH_2 groups at positions C2 and C8 forming the two permanent guanidinium moieties ranging from 241 to 491 Da (Shimizu et al. 1981). Chemical variants arise from the presence or absence of an *O*-sulfate group and/or an *N*-sulfate carbamoyl group (Shimizu 1996). The following STX chemical groups can be distinguished: nonsulfated (STX), singly sulfated (gonyautoxins – GTX), or doubly sulfated (C-toxins) (Nicholson et al. 2003). Decarbamoyl derivatives (Dc) and several new toxins (*Lyngbya wollei* toxins, LWTXs) have also been identified (Chorus and Bartram 1999). Structurally, STXs are divided into two categories: the saxitoxin and neosaxitoxin (neoSTXs) series. STXs and neoSTXs possess a unique tricyclic structure with hydropurine rings and guanidine subunits. In each series, structural variants arise from the differential stereochemistry of the sulfate groups at C-11 and the occurrence of *N*-sulfate carbamoyl groups at C-17 (Shimizu 1986).

STXs are potent neurotoxins and specific inhibitors of voltage-gated sodium, potassium, and calcium channels (Su et al. 2004). Animals treated with STXs show typical neurological effects including nervousness, jumping, convulsions, and paralysis. All STXs act in the same way but significantly differ in toxicity, with STX ($C_{10}H_{17}N_7O_4$, MW 299.99 Da, Fig. 4), being the most toxic molecule in this group (Funari and Testai 2008). The toxicity of STXs seems to be dependent on the specific interaction between the guanidinium ions of the toxin and its protein target (Strichartz 1984). Carbamate toxins are 10–100 times more potent than the *N*-sulfo-carbamoyl derivatives (Hall et al. 1990). *N*-sulfo-carbamoyl analogs are, however, labile and easily converted to the more toxic carbamate derivatives (Cembella et al. 1994). STX is a highly potent phycotoxin with an intraperitoneal (i.p.) LD_{50} of 10 μg/kg body weight in mice (Halstead and Schantz 1994), whereas neoSTXs are relatively less potent with an LD_{50} of 65 μg/kg (Wolf and Frank 2002).

Lyngbyatoxins

Lyngbyatoxins are produced by a filamentous blue-green algae *Lyngbya majuscula* (Cardellina et al. 1979) and *Moorea producens*, a cyanobacterium found in estuarine and coastal waters in tropical and subtropical climates (Engene et al. 2012).

Lyngbyatoxin ($C_{27}H_{39}N_3O_2$ MW 437.62DA, Fig. 5) and analogs are terpenoid indole alkaloids characterized by an indolactam ring and contains prenyl side chains

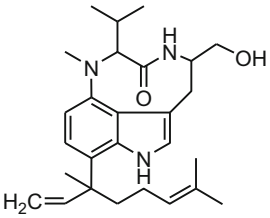


Fig. 5 Structure of Lyngbyatoxin

closely resembling teleocidins (teleocidin A-1 is equivalent to lyngbyatoxin and teleocidin A-2 is the C-19 epimer of lyngbyatoxin A), a group of toxins produced by *Streptomyces* bacteria (Cardellina et al. 1979).

Lyngbyatoxins are potent skin irritants that cause a blistering dermatitis called "swimmers itch," frequently reported by surfers in Hawaii (Cardellina et al. 1979). The toxin is highly inflammatory and vesicatory. Lyngbyatoxins are potent tumor promoters that operate by competitive binding to protein kinase C (PKC) (Jeffrey and Liskamp 1986). Lyngbyatoxin A was the first chemical variant to be discovered (Cardellina et al. 1979) and shows an LD_{50} toxicity of 250 μg/kg by mouse bioassay (i.p.) (Ito et al. 2002). Meanwhile two other congeners, lyngbyatoxins B and C, from the same cyanobacterium were discovered by Aimi and co-workers (1990). Lyngbyatoxins B and C share the indolactam structure with lyngbyatoxin A, but have modified (hydroxylated) lynalyl moieties. Lyngbyatoxins B and C show different biological activities versus lyngbyatoxin A. The 50% inhibition for specific binding of 3H-12-*O*-tetradecanoylphorbol-13-acetate (TPA) binding to PKC occurred at a median effective dose (ED_{50}) of 2.2 and 0.2 mM for lyngbyatoxin B and lyngbyatoxin C, respectively, which in turn corresponds to 1/200 and 1/20 the activity of lyngbyatoxin A (Aimi et al. 1990). Lyngbyatoxins B and C both possess a positive response in the mouse ear irritant test (Aimi et al. 1990).

Palytoxins

Palytoxin (PLTX) (Fig. 6) is a nonprotein marine toxin identified in *Palythoa* and zoanthid corals, *Ostreopsis* dinoflagellates, as well as *Trichodesmium* cyanobacteria. PLTX and various analogs have also been detected in a variety of marine organisms including mussels, sea urchins, crustaceans, gastropods, cephalopods, sea anemone, polychaete worm, dinoflagellates, algae, crab, and fish (Biré et al. 2015).

PLTX is a large, very complex molecule ($C_{129}H_{223}N_3O_{54}$; MW 2680.14, Fig. 6) composed of an aliphatic backbone containing cyclic ethers, 64 chiral centers, 40–42 hydroxyl and three nitrogen atoms, present as one primary amino group and two amide moieties (Ramos and Vasconcelos 2010). The chiral centers of this molecule and the 8 double bonds exhibiting *cis*/*trans*-isomerism leads to a hypothetical number of 1021 stereoisomers (Ramos and Vasconcelos 2010). PLTX is a super-carbon-chain compound, with the longest chain of continuous carbon atoms in any known natural compound (Inuzuka et al. 2008). The hydrophilic and hydrophobic domains might lead to micelle formation at high concentrations of the toxin in water,

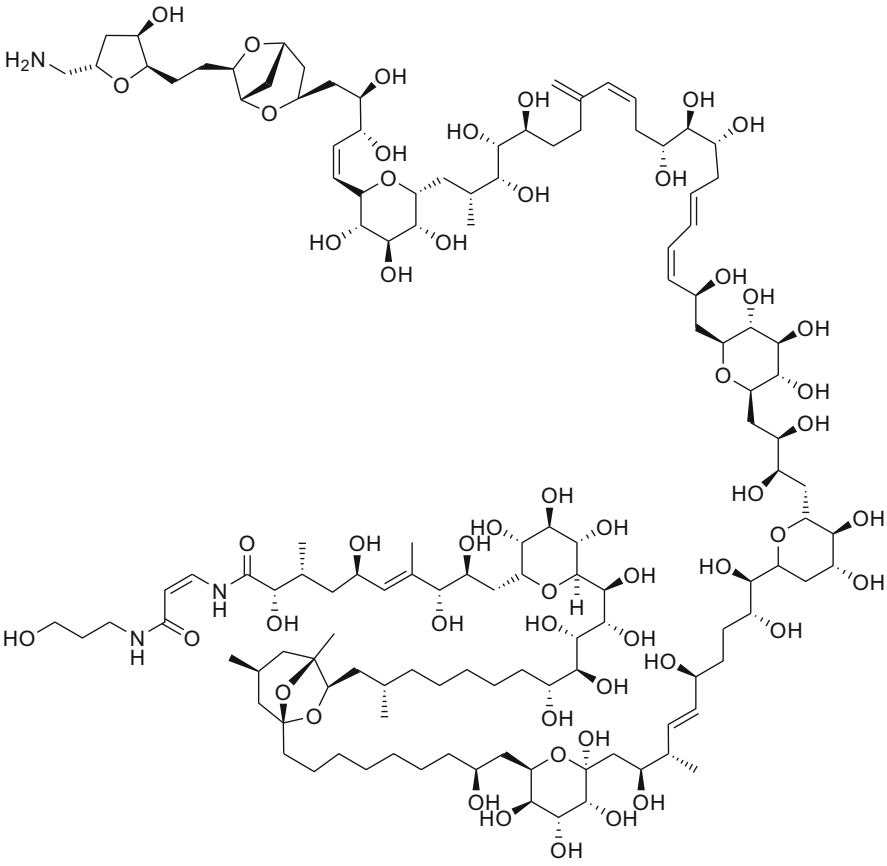


Fig. 6 Structure of palytoxin

affecting the mode in which PLTX interacts with targeted organisms (Rossini and Hess 2010). Over a dozen members of this group of natural products are currently known: PLTX, ostreocin, ovatoxins, mascarenotoxins, homopalytoxin, bishomopalyloxin, neopalytoxin, deoxypalytoxin, and 42-hydroxypalytoxin (Ciminiello et al. 2011), the most recent being ovatoxin-f (Ciminiello et al. 2010). *Trichodesmium* cyanobacteria only seem to be capable to produce PLTX and one of its analogs, 42-hydroxy-palytoxin (42-OH-PLTX) (Kerbrat et al. 2011).

It is suggested that PLTX binds to the Na,K-ATPase (sodium pump), opens a cation pathway through the pump, and transforms it into a nonspecific ion channel for monovalent cations (Hilgemann 2003). The toxin stimulates sodium influx and potassium efflux, leading to membrane depolarization in a wide range of organisms and to the massive increase of Ca2+ in the cytosol that interferes with some vital cell functions (Rossini and Bigiani 2011). These processes could contribute to, and could explain several, toxic effects attributed to PLTX such as violent contraction of

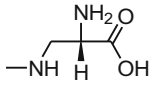


Fig. 7 Structure of β-methylamino-L-alanine

skeletal, smooth, and cardiac muscles, cardiovascular effects, hemolysis, histamine, prostaglandin and norepinephrine release, platelet aggregation, bone resorption, and inhibition of sperm motility (Ramos and Vasconcelos 2010). Regarding the toxicity of PLTX analogs, 42-OH-PLTX showed a toxicity profile comparable with that of the parent compound PLTX in mice (LD_{50} = 651 and 767 μg/kg, respectively) (Tubaro et al. 2011).

β-methylamino-L-alanine

β-methylamino-L-alanine (BMAA) (Fig. 7) is a cyanobacterial, nonproteinogenic methylated amino acid. The cyanobacterium *Nostoc* sp., a symbiont of Guam cycad (*Cycas micronesica*) roots, was suggested as the primary source of this toxic compound. Recently, it was reported that a large number of cyanobacteria strains such as those of the genera *Nodularia* and *Aphanizomenon*, that dominate massive blooms in the Baltic Sea, could produce BMAA either as symbionts or as free-living species in marine, brackish, or fresh water biota (Cox et al. 2005; Banack et al. 2007; Jonasson et al. 2010).

BMAA ($C_4H_{10}N_2O_2$, MW 118.13 Da, Fig. 7), also known as α-amino-β-methylaminopropionic acid (MeDAP), is a derivative of the amino acid alanine with an added methylated amino group on the side chain. BMAA contains a carboxyl group, a primary amine, and a secondary amine that can be present not only as a free form but also in a protein-bound fraction functioning as an endogenous reservoir, being transferred and accumulated between trophic levels (Jonasson et al. 2010). When ingested, BMAA is incorporated into proteins within the body, substituting L-serine during protein synthesis (Dunlop et al. 2013). It has been speculated that the incorporation of BMAA can cause protein misfolding and protein truncation (Banack et al. 2007). On the other hand, the release of free BMAA by protein catabolism is a slow process that can take years (Murch et al. 2004).

Toxicology of BMAA is based on the formation of carbamate adducts (BMAA-α-NCO_2, BMAA-β-NCO_2) in exposed organisms. These adducts can act as low affinity full agonists of metabotropic glutamate receptors (Myers and Nelson 1990). In addition, it could also cause intraneuronal protein misfolding, one of the processes that could evolve into neurodegeneration (Banack et al. 2010). With this regard, the implications of BMAA in various neurodegenerative diseases (i.e., Alzheimer's) and amyotrophic lateral sclerosis (ALS)/parkinsonism dementia complex (ALS/PDC) observed in local Chamorro people that use cycad (*Cycas circinalis*) flour as a staple food have been speculated (Banack et al. 2010; Murch et al. 2004). The neurotoxicity of BMAA was analyzed in vitro and in vivo; however, an animal model for BMAA induced ALS is still lacking (Banack et al. 2010). Moreover, Al-Sammak (2012) in a 14-day BMAA exposure (i.p.) test on mice

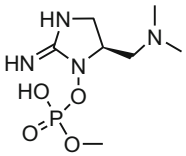


Fig. 8 Structure of anatoxin-a(s)

observed a LD_{50} of 3 mg/g and symptoms like dyspnea, urinary and fecal incontinence, and uncoordinated limb and body movement. Recently, α-,γ-diamino butyric acid (DAB, $C_4H_8O_2$, MW 88.11 Da), a structural isomer of BMAA, was detected with BMAA in a cyanobacterium (Rosen and Hellenäs 2008). Like BMAA, DAB is neurotoxic, although primarily hepatotoxic (O'Neal et al. 1968).

Anatoxin-a(s)

Anatoxin-a(s) (ATX-a(s)) is a neurotoxin, and the only naturally occurring organophosphate anticholinesterase inhibitor found in nature. The toxin has been described only in *Anabaena* strains, but the biosynthesis is not totally known (Hyde and Carmichael 1991).

ATX-a(s), with a molecular formula of CHN_4O_4, is a unique *N*-hydroxyguanidine methylphosphate ester (MW = 252 Da) structurally related to organophosphorous insecticides (Fig. 8). Despite the similar nomenclatures ATX-a(s) is structurally unrelated to ATX-a, being both toxins chemically and biologically different. Contrary to ATX-a, ATX-a(s) does not directly stimulate muscles, instead it blocks acetylcholine degradation through acetylcholinesterase (AChE) inhibition (Molica et al. 2005). The toxin acts in a similar way as organophosphorous and carbamate insecticides that include paraoxon, physostigmine, and pyridostigmine (Cook et al. 1988). The toxin binds on the active site serine of AChE, leading to the quasi-irreversible inhibition of the enzyme, thereby affecting signal transduction in neurons and thus triggering serious nervous dysfunction, potentially ending in the death of the exposed organism (Matsunaga et al. 1989). The toxicity of ATX-a(s) is extremely high, with a murine LD_{50} of 40–60 μg/kg (i.p.) (Mamood and Carmichael 1986). Structural variants of ATX(S) have not been detected so far.

Jamaicamides

The jamaicamides were isolated from a dark green strain of cyanobacterium *Lyngbya majuscula* in Hector's Bay in Jamaica (Edwards et al. 2004). Jamaicamides are a family of lipopeptide neurotoxins containing unusual structural features originated from halogenation reactions. Mechanistically, these molecules act as sodium channel blockers and exhibit fish toxicity as well as cytotoxicity to both H-460 human lung and Neuro-2a mouse neuroblastoma cell lines (Edwards et al. 2004).

The jamaicamides variants (A-C) arise from modifications at the terminal end of the polyketide aliphatic chain. Jamaicamide A (Fig. 9) is a highly functionalized lipopeptide containing an alkynyl bromide, vinyl chloride, β-methoxy eneone

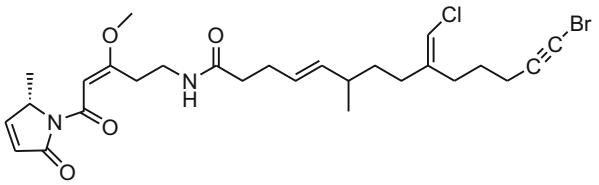


Fig. 9 Structure of jamaicamide A

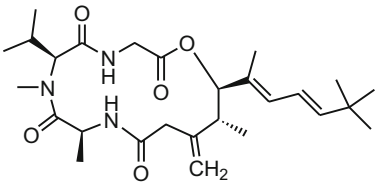


Fig. 10 Structure of antillatoxin

chemical groups, and a pyrrolinone ring. Jamaicamide B is a debromo analog of jamaicamide A and is slightly more polar than jamaicamide A. In jamaicamide C, which also lacks the bromine atom, a terminal olefin replaces the terminal alkyne resulting in it being slightly more hydrophobic than either jamaicamide A or B. The median lethal concentrations (LC_{50}) for all three variants were estimated at approximately 15 μM to both H-460 human lung and Neuro-2a mouse neuroblastoma cell lines. All three variants also exhibited sodium channel blocking activity at 5 μM, but none exhibited sodium channel activating activity (Edwards et al. 2004). However, in a goldfish toxicity assay, jamaicamide B was the most toxic (100% lethality at 10.22 nmol after 90 min) followed by jamaicamide C (100% lethality at 20.36 nmol after 90 min), while jamaicamide A was the least active fish toxin (sublethal toxicity at 17.62 nmol after 90 min) (Edwards et al. 2004).

Antillatoxins

Antillatoxins (ANTX) are produced by marine cyanobacteria such as *Lyngbya majuscule* and was first reported in 1995 (Orjala et al. 1995). ANTX ($C_{28}H_{45}N_3O_5$, MW = 504.34 Da) is a structurally remarkable lipopeptide, presenting a high degree of methylation. The molecule carries a conjugated diene that contains a tert-butyl group and a terminal olefin. In addition, a *cis* amide bond between alanine and *N*-methylvaline appears in the cyclic carbon skeleton as a single rotational isomer (Fig. 10). ANTX is among the most ichthyotoxic metabolites isolated (LD_{50} = 0.05 μg/ml in a goldfish toxicity assay) that is only exceeded in potency by brevetoxins (Orjala et al. 1995). The studies performed so far to determine the mechanism of action of ANTX showed that it activates the mammalian voltage-gated sodium channel at a pharmacological site that is distinct from any previously described toxin (Li et al. 2001).

There are two variants of ANTX already described, ANTX B also known as 8-demethyl-antillatoxin and 8-demethyl-8,9-dihydro-antillatoxin (DH-ANTX). Chemically, ANTX B is an unusual *N*-methyl homophenylalanine analog of ANTX. Moreover, Nogle et al. (2001) using High Resolution Fast Atom Bombardment found that several signals associated with the *N*-methyl valine moiety were absent and replaced by two midfield methylenes and a mono-substituted aromatic ring. The two variants were less potent than ANTX in producing toxicity in neuro-2a mouse neuroblastoma cells. The half maximal effective concentration (EC_{50}) values in mouse neuro-2a neuroblastoma cells were found to be 0.18 and 1.77 μM, for ANTX and ANTX B, respectively. Additionally, ANTX B is strongly ichthyotoxic to goldfish, with an LC_{50} of 1.0 μM (ANTX LC_{50} = 0.1 μM) (Nogle et al. 2001). Thus, ANTX B's biological activity appears to be tenfold decreased in potency versus ANTX. Nogle et al. (2001) hypothesized that probably the substitution of a larger *N*-methyl homophenylalanine residue for an *N*-methyl valine residue accounts for this decrease in activity.

Cytotoxins

Cylindrospermopsin

Cylindrospermopsin (CYN) is an emerging threat worldwide due to the progressive distribution of its main producer, the cyanobacteria *Cylindrospermopsis raciborskii*. Nevertheless, other cyanobacterial species such as *Anabaena bergeii, Aphanizomenon ovalisporum, Raphiodiopsis curvata, Umezakia natans, Aphanizomenon flos-aquae, Anabaena lapponica*, and *Lygnbya wollei* have been described also as CYN producers (Rzymsk and Poniedziałek 2014).

CYN is a stable tricyclic alkaloid with low molecular weight ($C_{15}H_{21}N_5O_7S$, MW 415.42), and structurally composed of a tricyclic guanidine unit and an uracil moiety (Fig. 11) which seems to be linked with biological activity (Banker et al. 2001). Chemically, CYN is a zwitterionic molecule and thus highly water soluble. Interestingly, for more than a decade, only two additional analogs were described: 7-epicylindrospermopsin (7-epi-CYN) (Banker et al. 2000) and 7-deoxycylindrospermopsin (7-deoxy-CYN) (Norris et al. 1999). While 7-epi-CYN has an OH orientation (epimer at the hydroxyl bridge) different from CYN (Banker et al. 2000), 7-deoxy-CYN lacks this OH group (Norris et al. 1999). Recently,

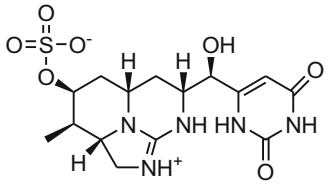


Fig. 11 Structure of cylindrospermopsin

Wimmer et al. (2014) found two additional new analogs: 7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin.

The toxicity of CYN is linked to the ribosomes, resulting in inhibition of glutathione and protein synthesis. Other possible mechanisms of action of CYN include the inhibition of cytochrome P450 and the direct interaction of the molecule with DNA (Runnegar et al. 1995; Froscio et al. 2003).

After ingestion, the toxin accumulates in the liver and affects mainly this organ via the above mechanisms. The uptake of this toxin is relatively fast and the irreversible block of protein synthesis occurs after 1 h incubation (Froscio et al. 2003). In mammals, CYN can cause oxidative stress, genotoxicity, immunosuppression, and abnormal function of hepatocytes leading to liver, kidney, thymus, and heart damage (Humpage et al. 2000; Zegura et al. 2011; Poniedziałek et al. 2014a, b).

7-epi-CYN reportedly has similar toxic potency as CYN (Banker et al. 2000), whereas the toxicological studies on 7-deoxy-CYN are less consistent. 7-deoxy-CYN analog was initially thought to be non-toxic (Runnegar et al. 2002); however, subsequent studies have shown that the 7-epi-CYN does indeed inhibit protein synthesis with potency similar to that of CYN, and likely has corresponding toxicity in vivo (Looper et al. 2005). Regarding the most recently discovered CYN variants, 7-deoxy-desulfo-CYN and 7-deoxy-desulfo-12-acetyl-CYN, they can also carry significant biological activity as assessed by their chemical structure (Wimmer et al. 2014).

Irritant Toxins

Lipopolysaccharides

Lipopolysaccharides (LPS) are part of the outer membrane of gram-negative bacteria, including cyanobacteria. Although basically similar, bacterial and cyanobacterial LPS differ in both their chemical and biological characteristics. LPS were first isolated from *Anacystis nidulans* and since this initial finding, endotoxins were found in several species of cyanobacteria (Keleti and Sykora 1982). Fig. 12 shows the structure of LPS from the cyanobacterium *Synechococcus* (Snyder et al. 2009).

LPS are generally classified as endotoxins, located within or at the surface of the outer cell layer, and generally consists of three essential structural parts: (1) a glycan with an O-specific polysaccharide, (2) a glycolipid anchor lipid A, and (3) a connecting polysaccharide core region (Stewart et al. 2006). This complex chemical structure leads to considerable diversity among LPS and complex chemical profiling. Indeed, LPS chemical profile is specific of the producer species and will carry a specific toxicity (Durai et al. 2015). It has been determined that lipid-A is responsible for LPS toxic action (Rietschel et al. 1993).

In contrast to other bacteria, LPS from cyanobacteria are quite diverse regarding the long chain unsaturated fatty acids, hydroxyl fatty acids, and the lack of phosphate (Wiegand and Pflugmacher 2005). Also, cyanobacterial LPS seem to be more toxic than the bacterial chemical congeners (Bernardová et al. 2008).

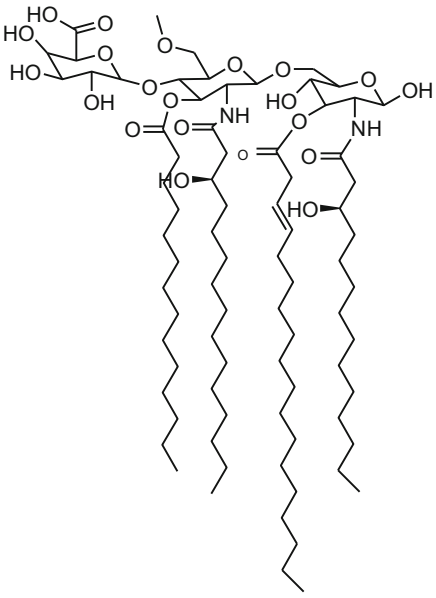


Fig. 12 Structure of the lipopolysaccharide glucose x5 of *Synechococcus*

LPS are related with a range of pathological effects in humans, from gastrointestinal illness, cutaneous signs and symptoms, allergy, respiratory disease, headache and fever. It is accepted that LPS cause fever in mammals and are involved in septic shock syndrome. Besides their action on the immune system, LPS from bacteria and cyanobacterial origin also affect the detoxification system of different organisms (Wiegand and Pflugmacher 2005). Inhalation of LPS might contribute in cyanobacteria related adverse effects known as "flu-like symptoms," characterized by cough, chills, and sore throat. However, these associations are poorly understood and additional research on health implications of cyanobacterial LPS in humans is required (Stewart et al. 2006).

Other Cyanotoxins

Cyanobacteria are a potential source of several other toxic secondary metabolites such as kalkitoxin, aplysiatoxin, debromoaplysiatoxin, besarhanamides, curacin, barbamide, microviridins, microginins, cyanopeptolides, etc. Particularly, the marine *Lyngbya majuscula* is highly toxic (Taylor et al. 2014). It was already described that this species produces more than 70 biologically-active components, many of them with toxic properties (detailed in Osborne et al. 2001).

It is also important to notice that although they can cause severe damage, especially when they are present in high concentrations, cyanotoxins are a rich source of natural cytotoxic compounds with a wide range of pharmacological, toxicological, and biological activities (Rastogi and Sinha 2009; Zanchet and Oliveira-Filho 2013).

Conclusion and Future Directions

Toxic cyanobacterial blooms and cyanotoxin contamination are increasing in many aquatic ecosystems worldwide due primarily to two factors, water eutrophication and climate change. The profile of toxins produced by cyanobacteria and encountered in aquatic environments is complex and dynamic, perhaps reflecting the adaptive activity and metabolism of cyanobacteria in adverse growth conditions. Extreme environmental and climatic conditions can lead to the proliferation of more virulent/toxic cyanobacterial strains. In view of the possibility of the occurrence of new toxins and highly toxic strains, and knowing the susceptibility of most animals including mammals and humans to cyanotoxins, it becomes imperative for worldwide monitoring of cyanobacterial blooms. Surveillance programs should be flexible and complete to enable identification and quantification of the different chemical/functional groups of cyanotoxins, and the discovery of new bioactive molecules that raise potential health risks. This is, by all means, one of the major challenges addressed to the scientific community on this thematic. Future directions aim at the improvement of cyanobacteria toxicity assessment through the systematic analysis of the bioactive compounds produced by each species/strain and the characterization of new metabolites and respective biological activities. More research is also needed with regard to the functional analysis of cyanotoxins and health effects upon humans, which includes necessarily the identification of distinct biological targets and respective mechanisms of cellular internalization and toxicity. This knowledge will directly contribute towards more concrete and effective mitigation strategies to improve cyanotoxin surveillance and reduce human exposure.

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Fusarium Mycotoxin: Toxicity and Detection

24

M. Venkataramana, G. Selvakumar, and S. Chandranayaka

Abstract

Mycotoxins are a group of secondary metabolites produced by filamentous fungi, which may cause health hazards to animals and human beings. These mycotoxins are diverse in their structure and biological activity, and can be produced on cereals and grains during their growing stage or harvesting and postharvesting stages. When mycotoxin-contaminated grains are consumed, they can cause severe ill-health effects in humans and animals including livestock. Among the mycotoxin-producing fungal species, *Fusarium* species are one of the most considerable ones due to their incidence, diverse toxigenic nature, and its health effects ranging from acute skin lesions to cancers. The purpose of the present review is to provide an update on the toxigenic *Fusarium* species, their harmful effects, and toxicity of fusarialtoxins as well as their detection from food and feed samples.

Keywords

Fusarium toxins • Trichothecenes • Fumonisins • Zearalenone • Toxicity • Detection

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Contents

Introduction

Mycotoxins and Mycotoxicosis

Mycotoxins are by-products of fungal metabolism, that have been implicated as causative agents of adverse health effects in humans and animals that have consumed fungus-infected agricultural products (Chandra Nayaka et al. 2008). Consequently, fungi that produce mycotoxins, as well as the mycotoxins themselves, are potential problems from both public health and economic perspectives. Various genera of toxigenic fungi are capable of producing such diverse mycotoxins as the aflatoxins, trichothecenes, satratoxins, and fumonisins (Chandra Nayaka et al. 2013; Ramana et al. 2013, 2014; Priyanka et al. 2014).

Mycotoxin elaboration is an important concern of mold infestation due to its implications in a range of human and animal diseases. The disease caused by consumption of moldy food dates back to ergotism, reported back in the eighteenth century (Chandra Nayaka et al. 2011). Worldwide attention to mold infestation and mycotoxins was further drawn together after the outbreak of Turkey-X disease in England in 1960, which led to the death of 100,000 poults due to the consumption of moldy Brazilian peanuts (Bullerman 1979). The causative agent was identified as *Aspergillus flavus* and the metabolite produced by the fungus was identified as aflatoxin. Mycotoxins are diverse in their structure and biological activity.

Mycotoxin contamination of crops may cause economic losses at all levels of food and feed production, including crop and animal production. The occurrence of mycotoxins varies from commodity to commodity, year to year, and region to region. Although the prevention of mycotoxin contamination of grain is the main goal of food and agricultural industries throughout the world, under certain environmental conditions the contamination of various cereal grains with *Fusarium* fungi and mycotoxins is unavoidable for grain producers. The most prevalent *Fusarium* mycotoxins are mainly associated with cereal crops, in particular corn, wheat, barley, rye, rice, and oats throughout the world (Ramana et al. 2014; Priyanka et al. 2014). Due to their deletorius effects to humans and animals, fumonisins and trichothecenes are classified by International Agency of Research on Cancer as possible human carcinogens (IARC 1993).

Review

The Genus *Fusarium*

The name of *Fusarium* comes from Latin *fusus*, meaning a spindle. *Fusarium* is a large genus of filamentous fungi widely distributed in soil in association with plants. Some species of *Fusarium* produce mycotoxins in cereal crops that can affect human and animal health if they enter the food chain. The main toxins produced by these *Fusarium* species are trichothecenes, zearalenone, and fumonisins.

General Characteristics of *Fusarium* Species

Fusarium species may produce three types of spores called macroconidia, microconidia, and chlamydospores (Nelson et al. 1983). Some species produce all three types of spores, while other species do not. The macroconidia are produced in a specialized structure called a sporodochium in which the spore mass is supported by a superficial, cushion-like mass of short monophialides bearing the macroconidia (Hawksworth et al. 1983). The sporodochium sometimes may be encased in slime. Macroconidia may also be produced on monophialides and polyphialides in the aerial mycelium. A monophialide is a conidiophore with only one opening or pore through which endoconidia are extruded, while a polyphialide has two or more such openings or pores. Some conidia are intermediate in size and shape, and these have been referred to as both macroconidia (Nelson et al. 1983) and mesoconidia. These intermediate conidia are found primarily in *F. semitectum, F. avenaceum*, and *F. subglutinans*. Until more cultures of each species are studied thoroughly, the use of the term mesoconidia is questionable. Microconidia are produced in the aerial mycelium but not in sporodochia. They may be produced in false heads only, or in false heads and chains on either monophialides or polyphialides. False heads occur when a drop of moisture forms on the tip of the conidiophore and contains the endoconidia as they are produced. Microconidia are of various shapes and sizes and those produced in chains have a truncate base. The third type of spore formed by *Fusarium* species is a chlamydospore, which is a thick-walled spore filled with lipid-like material that serves to carry the fungus over winter in soil when a suitable host is not available. The chlamydospores may be borne singly, in pairs, in clumps, or in chains, and the outer wall may be smooth or rough.

Occurrence of Toxigenic *Fusarium*

The *Fusarium* genus contains some of the most important toxigenic plant pathogenic fungi. Their infection of important crops such as wheat, barley, oats, rice, and maize pose a serious problem as infection leads to yield loss through lowered growth rate, reduction of grain size (*Fusarium* Head Blight/tombstone/scab (http://www.Plantmanagementnetwork.org/pub/php/research/2003/fhb/), and weakening of the straw (Foot rot) (Ramana et al. 2014; Samson et al. 2000). Apart from the field infections, *Fusarium* is also known to cause storage rot of sugar beet, potatoes, and

apples. Storage rot of cereals is typically a secondary effect of the primary field infection (Goswami and Kistler 2004).

Important Groups of *Fusarium* Toxins

Chemical analysis has shown that *Fusarium* sp. are capable of producing a wide range of secondary metabolites, including trichothecenes (T-2 toxin, deoxynivalenol (DON/vomitoxin), nivalenol (NIV), zearalenone (ZEN), fumonisin (B1, B2, B3, B4), and diacetoxyscirpenol (DAS)), moniliformin, enniatin, fusaric acid, fusarin C, fusaproliferin, aurofusarin, fuscofusarin, and their respectable derivatives (Chandra Nayaka et al. 2010; Ramana et al. 2011, 2012, 2014; Desjardins 2003).

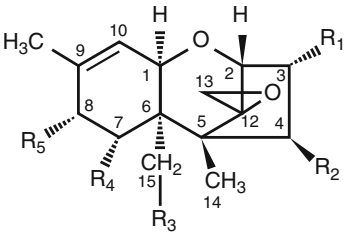
Trichothecenes

Trichothecenes are sesquiterpenoid mycotoxins which share the 12, 13-epoxytrichothecene skeleton as the common structural feature. Presence or absence of an 8-keto moiety leads to differentiation of group B and group A trichothecenes, respectively, the latter of which have a valerianyl-, acetyl-, or hydroxyl moiety in that position. Furthermore, group C trichothecenes (macrocyclic trichothecenes) are differentiated by the presence of a macrolide ring system attached at position 4β and 15 of the trichothecene verrucarol (Grove 1993). *Fusarium* spp. produce the widest variety of different trichothecene compounds among which the type B-trichothecenes deoxynivalenol (DON) and nivalenol (NIV), as well as the type-A-trichothecenes T-2 toxin, HT-2 toxin, neosolaniol (NEOS), and diacetoxyscirpenol (DAS) are the most common and/or toxic compounds isolated from natural sources (Fig. 1).

Fumonisins

Fumonisins are a group of mycotoxins produced by species within the *Gibberella fujikuroi* complex of species. The major producers are *F. verticillioides, F. proliferatum, F. subglutinans*, and *F. nygamai*. Fumonisins B1 and B2 are of toxicological significance, while the others (B3, B4, A1, and A2) occur in very low concentrations and are less toxic (Ramana et al. 2012, 2014). In India, a single outbreak of acute food-borne disease possibly caused by fumonisin B1 has been reported (Bhat et al. 1997). In this episode, several villages were involved, and the individuals affected were from the poorest social strata, who had consumed maize and sorghum harvested and left in the fields during unseasonable rains. The main features of the disease were transient abdominal pain, borborygmus, and diarrhea, which began shortly following consumption of unleavened bread prepared from moldy sorghum or moldy maize. Patients recovered fully when the exposure ceased, and there were no fatalities. Fumonisin B1 was found in much higher concentrations in the maize and sorghum from the affected households than from the controls. Fumonisins typically only exerts low levels of acute toxicity; however, horses and pigs are more susceptible than other production animals and ingestion can cause

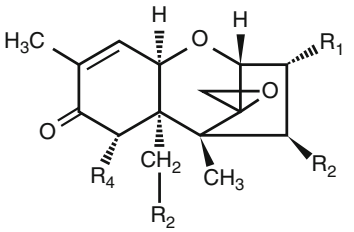
a



Group A Trichothecenes

	MW	R_1	R_2	R_3	R_4	R_5
Neosolaniol	382	OH	OAc	OAc	H	OH
HT-2 Toxin	424	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 Toxin	466	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
T-2 Triol	382	OH	OH	OH	H	$OCOCH_2CH(CH_3)_2$
T-2 Tetraol	298	OH	OH	OH	H	OH
Scirpentriol	282	OH	OH	OH	H	H
15-Monoacetoxyscirpenol (MAS)	324	OH	OH	OAc	H	H
4,15-Diacetoxyscirpenol (DAS)	366	OH	OAc	OAc	H	H

b



Group B Trichothecenes

	MW	R_1	R_2	R_3	R_4
Deoxynivalenol (DON)	294	OH	H	OH	OH
3-Acetyl-DON	338	OAc	H	OH	OH
15-Acetyl-DON	338	OH	H	OAc	OH
Nivalenol	312	OH	OH	OH	OH
Fusarenon-X (4-Acetyl-nivalenol)	354	OH	OAc	OH	OH

Fig. 1 (continued)

c

Naturally occurring macrocyclic trichothecene mycotoxins.

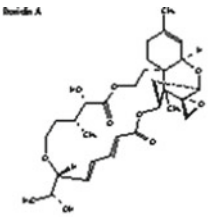
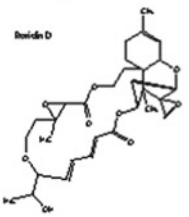
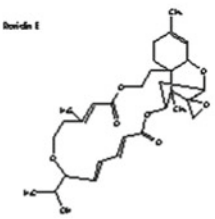
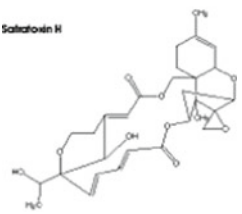
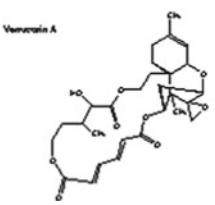
Name	Structure	Source organism
Roridin A		*Myrothecium verrucaria, M. roridum*
Roridin D		*Myrothecium verrucaria*
Roridin E		*Myrothecium verrucaria, Stachybotrys chartarum*
Satratoxin H		*Myrothecium verrucaria, Stachybotrys chartarum*
Verrucarin A		*Myrothecium roridum*

Fig. 1 (continued)

Naturally occurring macrocyclic trichothecene mycotoxins.

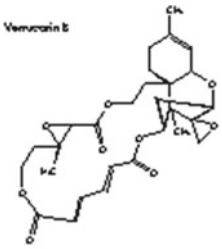
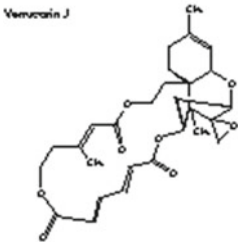
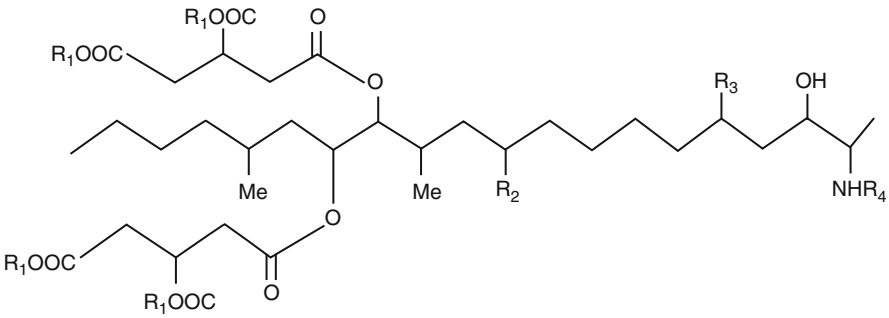
Name	Structure	Source organism
Verrucarin B		*Myrothecium verrucaria, M. roridum*
Verrucarin J		*Myrothecium verrucaria,*

Fig. 1 (**a**) Type A trichothecenes. (**b**) Type B trichothecenes. (**c**) Other trichothecene Mycotoxins (Grove 1993)

equine leukoencephalomalacia and porcine pulmonary edema syndrome (Marasas et al. 1988). Besides various animal diseases, they are suspected to be responsible for certain forms of human esophageal cancer observed in South Africa, China, and Northeast Italy (Peraica et al. 1999). Moreover, fumonisin B1 has been associated with neural tube defects in experimental animals and may therefore be involved in cases of spina bifida in humans (Hendricks 1999). The toxin has been assigned a 2B carcinogen (possibly carcinogenic to humans) status by the IARC (IARC 1993; Fig. 2).

Zearalenone

Zearalenone (ZEN) previously known as F-2 toxin is produced mainly by *Fusarium graminearum* and related species, principally in wheat and maize but also in sorghum, barley, and compounded feeds. Zearalenone is a nonsteroidal oestrogenic mycotoxin that induces hyperoestrogenic responses in mammals and can result in reproductive disorders in farm animals. Zearalenone has also been shown to be immunotoxic, mutagenic, hemotoxic, and hepatotoxic; however, the mechanisms of toxicity are not fully understood (Zinedine et al. 2007). The biotransformation of ZEA in animals involves the formation of two metabolites α-zearalenole and β-zearanol, which are subsequently conjugated to glucuronic acid. Recently

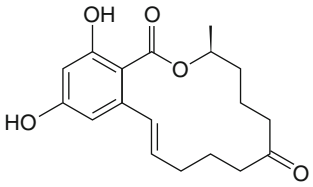


	R_1	R_2	R_3	R_4
Fumonisin B_1	H	OH	OH	H
Fumonisin B_2	H	H	OH	H
Fumonisin B_3	H	OH	H	H
Fumonisin A_1	H	OH	OH	$COCH_3$

Structure of some Fumonisins

Fig. 2 Fumonisins (Proctor et al. 1999)

Fig. 3 Structure of zearalenone (Kim et al. 2005)



Venkataramana et al. 2014a reported the neurotoxic potential of zearalenone in in vitro cell line models (Fig. 3).

Toxicity of *Fusarium* Mycotoxins

Fusarium toxins can cause diverse health effects on humans and other farm animals. Table 1 shows the important groups of toxins and their effects.

Carcinogenicity

The International Agency for Research on Cancer (1993) has grouped the main *Fusarium* mycotoxins on the basis of the fungal species producing them, as it represents the best way to identify the real situation through which humans become exposed to these naturally occurring toxins. Therefore, the most important mycotoxins, in terms of natural occurrence and toxicology, have been grouped into toxins derived from *F. sporotrichioides* (T-2 toxin and related trichothecenes); toxins derived from *F. graminearum, F. culmorum*, and *F. crookwellense* (Deoxynivalenol, Nivalenol, Fusarenone X, and Zearalenone); and toxins derived from *F. moniliforme*

Table 1 Major *Fusarium* toxins and their effects on humans and other animals

Toxin	Produced by	Disease/syndrome	Target
T-2 toxin	*F. sporotrichioides, F. poae*	Alimentary toxic aleukia (ATA) (e)	Human
		Hemorrhagic syndrome (d)	Cattle, Swine
DON	*F. graminearum*	Feed refusal and Emetic syndromes (b)	Swine
Zearalenone	*F. sporotrichioides, F. poae, F. graminearum*	Estrogenic syndrome (a)	
Fumonisin B1, B2	*F. moniliforme*	Pulmonary syndrome (c)	
		Equine-leukoencephalomalacia(c)	Horses
Aurofusarin	*F. graminearum, F. culmorum*	Altered fatty acid composition in egg yolks (g)	Poultry
Furanoterpenoide	*F. solani*	Moldy sweet potato toxicosis (d)	Cattle

Note: (a) (Department of crop sciences University of Illinois at Urbana-Champaign August, 1997), (b) (Forsyth et al. 1977), (c) (Marasas et al. 1988), (d) (Nelson et al. 1994), (e) (Chasseur et al. 2001) and (g) (Dvorska et al. 2003)

(fumonisins and fusarin C). Only the group of toxins derived from *F. moniliforme* were identified as group 2B, i.e., possible carcinogenic to humans and with sufficient evidence of carcinogenicity towards experimental animals, whereas the data relevant to the other groups of toxins or to individual toxins were not sufficient (or adequate) to make them classifiable as to their carcinogenicity to humans (group 3). Fumonisin B1 induces hepatocellular carcinoma, cholangiofibrosis, and cholangiocarcinoma in rats and it is shown to be a strong tumor promoter, but only a weak initiator (similar effects have also been observed for FB_2 and FB_3). The summary of the IARC evaluation of *Fusarium* mycotoxins, their toxic effects, and contaminated food sources (IARC 1993) is shown in Table 2.

Cytotoxic Effects of *Fusarium* Toxins

Fusarium mycotoxins may predispose livestock to infectious disease, and this might result in feed refusal and decreased productivity. T-2 toxin is the most toxic of the *Fusarium* trichothecenes, though less widely distributed than deoxynivalenol. T-2 toxin is a known mycotoxin that causes immunosuppressive effects especially in livestock (Li et al. 2011; Sokolovic et al. 2008). In pigs, clinical signs of T-2 toxicosis include emesis, posterior paresis, lethargy, and frequent defecation (Meissonnier et al. 2008). At natural levels of contamination in the diet, T-2 toxin causes reduced feed intake and animal performance (Pandey et al. 2006). At high concentrations in the diet it produces diarrhea, emesis, and feed refusal. T-2 toxicosis in poultry causes oral lesions, reduced feed consumption and growth rate in young animals, and reduced egg production in laying hens (Pandey et al. 2006; Sklan et al. 2003). The impact on poultry production becomes important at dietary

Table 2 Fungal infestation leading to toxicity outbreaks in human/animal populations

S No	Toxic effects	Fungi	Toxins	Regions	Symptoms and organs effected	Food grains
1	Fusarium toxicosis[a]	*Fusarium* species	DON	China	Nausea, abdominal pain, diarrhea	wheat
2	DON toxicosis in India[a]	*Fusarium* species	DON	India, Kashmir valley	Abdominal pain, diarrhea, vomiting	Wheat
3	Red mold disease in Japan[a]	*Fusarium* species	Trichothecene, DON	Japan and Korea	Nausea, vomiting, diarrhea	Wheat and maize
4	Esophageal cancer[a]	*Fusarium* species	Fumonisins	South Africa, Iran and China	Esophageal cancer	Cereals and Millets
5	Kashmir Beck Disease[a]	*Fusarium* species	T-2 toxin	Russia, China, Korea, Sweden Holland	Symmetric stiffness and swelling in finger and wrist joints	Moldy wheat and maize
6	Onyala[a]	*Alternaria* and *Phoma* species	Unknown	Southern Sahara, European countries	Hemorrhagic bullae in oral cavity and skin, hematuria	Mold millet and sorghum
7	Ergotism[a]	*Claviceps purpurea*	Ergotamine Ergot alkaloids	Russia, India, Central Europe	Gangrene and convulsions	Millets, Maize
8	Mseleni joint Disease[a]	*Fusarium* species	Unknown	Southern Africa	Osteoarthritis, Disorder in hip, knee and ankle joints	Maize and groundnut
9	Alimentary Toxic Aleukia[a]	*Alternaria, Fusarium* species	T-2 toxin	U.S.S.R	Leucopenia, agranulocytosis, Hemorrhage	Cereals
10	Aflatoxicosis[a]	*Aspergillus* flavus	Aflatoxin	Gujarat India, 1974	Cancer, cirrhosis, Swollen abdomen, Liver	Moldy maize
11	Fusarium Toxicosis[a]	*Fusarium* species	DON	China	Nausea, abdominal pain, diarrhea, dizziness	Moldy Wheat
13	Balkan Endemic Nephropathy[a]	*Penicillium verrucossum, Aspergillus ochraceus*	Ochratoxin A	Balkan states Romania, Bulgaria, Yugoslavia, Tunisia	Renal impairment, proteinuria, creatininuria, anemia,	Moldy Cereals

[a]Beardall and Miller 1994

concentrations above 2 mg/kg. In ruminants, T-2 toxicosis results in a wide range of responses, such as feed refusal, leucopenia, depression, diarrhea, coagulopathy, enteritis, and posterior ataxia (Pandey et al. 2006; Sklan et al. 2003). Reduction of humoral immunity is a common effect for pigs, poultry, and ruminants exposed to low concentrations of T-2 toxin in the diet (Meissonnier et al. 2008; Pandey et al. 2006).

Deoxynivalenol (vomitoxin) is the most important trichothecenes because of its high incidence in cereals, including maize, wheat, barley, and oats, but it is not one of the most acutely toxic of this group of mycotoxins (Ramana et al. 2011, 2014). At the cellular level, the main toxic effect is inhibition of protein synthesis via binding to ribosomes (Pestka 2010). In animals, the main overt effect at low dietary concentrations appears to be a reduction in food consumption (anorexia) and weight gain, while higher doses induce feed refusal, diarrhea, and vomiting (Bonnet et al. 2012; Sobrova et al. 2010). Deoxynivalenol is known to alter brain neurochemicals, and the serotonergic system appears to play a role in mediation of the feeding behavior and emetic response (Bonnet et al. 2012). Animals fed low doses of toxins are able to recover from initial weight losses, while higher doses induce more long-term changes in feeding behavior (Pestka 2007). Swine are more sensitive than other livestock species to the presence of deoxynivalenol in their feed. Extensive degradation of deoxynivalenol to secondary metabolites in the rumen has been reported, and this may explain the higher tolerance to the toxin by ruminants with respect to poultry and pigs (Pestka 2007). The capacity of deoxynivalenol to alter normal immune function is particularly important. There is an extensive evidence that deoxynivalenol can be immunosuppressive or immunostimulatory, depending upon the dose and the duration of exposure (Solcan et al. 2012). While immunosuppression can be explained by the capacity of inhibiting protein synthesis, immunostimulation can be related to interference with normal regulatory mechanisms.

Zearalenone contamination is common in cereal crops grown in cooler and moist regions worldwide. Zearalenone and related metabolites possess strong estrogenic activity and can result in severe reproductive and infertility problems, when they are fed to domestic animals in sufficient amounts (Zinedine et al. 2007). Swine appears to be the most sensitive of the domestic animal species, and therefore the most frequently reported with problems caused by zearalenone, which include enlargement or swelling and reddening of the vulva in gilts and sows (vulvovaginitis), swelling of the mammary glands, and atrophy of the ovaries, vaginal, and rectal prolapses. Effects in other species are much less pronounced (Tessari et al. 2006). High concentrations of zearalenone have been associated with infertility and development of atypical secondary sexual characteristics in heifers (Choi et al. 2012). Zearalenone and its analogues are capable of inhibiting mitogen-stimulated lymphocyte proliferation and can induce thymic atrophy and macrophage activation (Choi et al. 2012; Tessari et al. 2006).

Fumonisins are the major toxic compounds present in maize and maize-based human foodstuffs and animal feeds in several countries throughout the world

Table 3 CD50 values of type-A trichothecenes on animal cell line models (Visconti et al. 1991)

Trichothecenes	Substituents					CD_{50} (μg/ml)	
	C-3 (R_1)	C-4 (R_2)	C-15 (R_3)	C-7 (R_4)	C-8 (R_5)	MIN-GLI	K-562
Type A							
T-2 toxin	OH	OAc	OAc	H	O-Isoval	0.0003	0.001
HT-2 toxin	OH	OH	OAc	H	O-Isoval	0.0025	0.02
Acetyl T-2 toxin	OAc	OAc	OAc	H	O-Isoval	0.025	0.03
T-2 triol	OH	OH	OH	H	O-Isoval	0.040	0.09
T-2 tetraol	OH	OH	OH	H	OH	0.200	0.28
8-acetoxyneosolaniol	OH	OAc	OAc	H	OAc	0.003	0.002
Acuminatin	OH	OH	OAc	H	OAc	0.0045	0.01
T-2 tetraol	OAc	OAc	OAc	H	OAc	0.022	0.04
Neosolaniol	OH	OAc	OAc	H	OH	0.05	0.15
Diacetoxy T-2 tetraol	OH	Oac	OH	H	OAc	0.08	0.10
Diacetoxyscirpenol	OH	OAc	OAc	H	H	0.001	0.001
Type B							
Fusarenon-X	OH	OAc	OH	OH	=O	0.06	0.15
Nivalenol	OH	OH	OH	OH	=O	0.20	0.30
Deoxynivalenol	OH	H	OH	OH	=O	0.40	0.30
15-AC-DON	OH	H	OAc	OH	=O	2.00	0.40
3 AC-DON	OAc	H	OH	OH	=O	7.00	2.00

(Visconti 1996). Commercially available maize products for human consumption are generally contaminated at levels below 1 μg/g FB_1, although individual products in certain countries can reach far higher levels (Doko and Visconti 1994). Fumonisins (particularly FB_1 and in some cases FB_2) have been shown to cause fatal toxicoses in animals (Voss et al. 2007). The ingestion of fumonisin-contaminated maize has been associated with spontaneous outbreaks of equine leukoencephalomalacia, a neurological syndrome characterized by focal, often extensive, liquefactive necrosis of the white matter of the cerebrum, and with acute pulmonary edema in pigs, swelling of the lungs and thoracic cavity (Voss et al. 2007). Several studies have shown altered immune response in poultry fed diets containing fumonisins at relatively high levels (Tessari et al. 2006).

The CD_{50} values of various type-A trichothecene mycotoxins against different cell line models are represented in Table 3 (Visconti et al. 1991).

Methods for Identification of *Fusarium* Species and their Toxins

Conventional Culture Based Methods for Identification of *Fusarium* Species

Identification of *Fusarium* species was carried out using morphological and microscopic characters under specific growth media. Table 4 represents the growth characteristics of different *Fusarium* species on corn steep liquor agar (CLA) growth media.

Table 4 Cultural and morphological characters of mycotoxigenic species of *Fusarium* (Nelson et al. 1983)

Name of the species	Culture Pigmentation	Microconidia	Macroconidia	Chlamydospores
Fusarium avenaceum	Rose, Red with White	1–3 Septate 8–50 × 3.5–4.5 μm	Primary macroconidia polyblastic, secondary macroconidia from phialides, 4–7 septate, 40–80 × 3.5–4.5 μm	Absent, rarely formed in chains
F.acuminatum	Carmine to red	Not observed	3–7 septate, 30–70 × 3.5–5 μm curved elongation of the apical cell produced from phialids	Globose, Intercalary in knots or chains
F. culmorum	Red becoming Reddish-brown	Not observed	Uniform in shape, Borne simple phialides, borne on complex, loosely branched conidiophores, 3-septate	Oval to globose intercalary, singly or in chains, smooth to rough wall
F. equiseti	Peach usually changing to vellaneous and finally becoming buff-brown	Not observed	Conidia produced from single solitary or grouped phialides, conidia 4–7 septate, 20–60 × 3.5–9 μm. Often with elongated apical cell	Globose intercalary in solitary chains or clumps
F. graminearum	Grayish, yellow or white, rose, coral becoming vinaceous with brown tinge	Not observed	Single, lateral, sickle-shaped or falcate, 3–7 septate, simple lateral phialides may or may not be grouped on branched conidiophores	Absent or rare if present intercalary
F. lateritium	Variable peach to deep orange, vinaceous to reddish-brown, greenish-yellow to bluish black	Absent	Straight, beaked at the apex produced in sporodochia,	Sparsely oval to globose, intercalary

(*continued*)

Table 4 (continued)

Name of the species	Culture Pigmentation	Microconidia	Macroconidia	Chlamydospores
			phialides simple 7–10 × 7–8 µm	
F. moniliforme	Vinaceous, purple to violet, peach salmon	Fusiform to clavate, 0–1 septate, conidia from subulate, lateral philides, in chain, 5–12 × 1.5–2.5 µm	Elongated, sharply curved, equilaterally fusoid, 25–60 × 2.5–4 µm	Absent but globose stromatic initial cells may be present
F. nivale	White to pale-peach	Absent	Curved broadly falcate 1 to 3 septate, 10–30 × 2.5–5 µm	Absent
F. oxysporum	White, peach, salmon, vinaceous gray to purple, violet	Cylindrical to curved, oval ellipsoid, produced from simple, short lateral phialides, 5–12 × 2.2–3.5 µm	Hooked apex & pedicellate base, 3–7 septate 27–66 × 3.5 µm	Globose, solitary or in pair. Intercalary formed on short lateral branch
F. poae	White to yellow, salmon to livid-red, vinaceous	Globose, single spore or small globes to obclavate phialides, 8–12 × 7–10 µm, O-septate	Single spore curve, 3-septate 20–40 × 3–4.5 µm	Globose, formed sparsely, single or in chains in old cultures
F. sambucinum	Peach to orange or ochreous, white in some isolates vinaceous to bay.	Absent	Strongly dorsiventral with beaked apical cell, curved pointed apex, marked foot cell, 3–5 septate 30–55 × 4–5.5 µm	Formed sparsely globose, single or chains or knots intercalary

F. semitectum	Peach changing to buff-brown	Not observed	Two types 1. Primary conidia 0–5 septate, blastospores, polyblastic. 2. Secondary conidia 3–7 septate, typically heeled foot cell	Sparse, globose, intercalary, single or in chains
F.solani	Grayish-white to blue or bluish-brown	Single, cylindrical to ovate, 1-septate, borne on lateral phialides	Curved, 1–9 septate inequilaterally fusoid	Globose, singly or in pairs on short lateral branches or intercalary
F. sporotrichioides	Surface mycelium white and red agar livid-red becoming with brown	Clavate to cymbiform, 0-septate, blastic spores from pegs	An incurved, 37 septate 3-0–7.0 × 3.5–5 μm	Globose intercalary chains
F. sulphureum	Cream or light-brown or reddish-brown	Not observed	Curved pointed apical cell pedicellate foot cell, 3–5 septate, 14–15 × 3.5 μm	Globose short chain or single formed sparsely
F. proliferatum	Heavy white aerial mycelial that may become tinged with purple	Single celled with flattened base in short or varying length change or in false head for polyphialides	Abundantly, 3–5 septa slightly curve to almost straight basal cell foot shaped teleomorph *Gibberella fujikuroi*	No Chlamydospores

Molecular Methods for Identification of Toxigenic *Fusarium* Species

Detection of Trichothecene Producing *Fusarium* Species

The genetics and regulation of trichothecene biosynthesis have been elucidated in *F. sporotrichioides* by Hohn et al. (1993) and in *F. graminearum* by Kimura et al. (2003). Sequencing of parts of the trichothecene gene cluster was done for species in the *F. graminearum* group, i.e., *F. crookwellense*, *F. culmorum*, *F. lunulosporum*, and *F. pseudograminearum*. The *Tri5* gene, which codes for trichodiene synthase catalyzing the first specific step in the biosynthesis of all trichothecene-producing fungi, was particularly well characterized in *Fusarium* spp. and in *Stachybotrys chartarum* (Strauss and Wong 1998). Schilling et al. (1996) were among the first authors to report on the use of a PCR-based detection systems for *Fusarium* species. Niessen and Vogel (1998) aligned *Tri5* gene sequences of several *Fusarium* spp. to find two highly conserved regions within the gene. Primers designed for those regions amplified a 658 bp product from 20 different species and varieties within *Fusarium.* Based on the same gene, Schnerr et al. (2002) designed primers which resulted in a smaller PCR product detecting essentially the same set of species as in Niessen and Vogel (1998). The schematic representation of trichothecene gene cluster is shown in Fig. 4.

Primers developed by Niessen and Vogel (1998) were used in follow-up studies to detect the *Tri5* gene in pure cultures and in sample materials by conventional PCR (Demeke et al. 2005). Available sequence information was used to set up species-specific assays for identification, detection, and quantification of typical trichothecene producers *Fusarium* species.

Figure 5 represents the trichothecene biosynthetic pathway (Kimura et al. 2003). Niessen et al. (2004) developed *Tri5* gene-based primers to set up species-specific detection assays for the newly described *F. langsethiae, F. kyushuense, F. poae,* and *F. sporotrichioides.* The detection systems used the forward primer described by Niessen and Vogel (1998), which was combined with reverse primers binding specifically to the intron region of the *Tri5* gene in the respective species. Using the same gene, Strausbaugh et al. (2005) set up a TaqMan™ quantitative real-time PCR assay for quantification of *F. culmorum* in infected wheat and barley crops.

A group-specific PCR assay for the detection of trichothecene-producing *Fusarium* spp., involving primers binding to the *Tri6* gene (transcription factor), was set up by Ramana et al. 2011. The same primers further used to develop a multiplex PCR assay for simultaneous detection of toxic *Fusarium* species by Ramana et al. 2011. Ramana et al. (2012) demonstrated the usefulness of the method for detection of trichothecene producers, as well as *F. culmorum* and *F. graminearum*, in contaminated cereal samples. Most authors used variable regions present within the internal

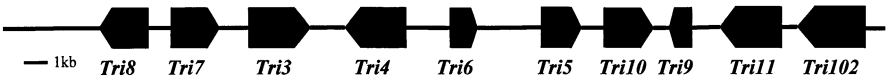


Fig. 4 Schematic representation of trichothecene metabolic pathway gene cluster

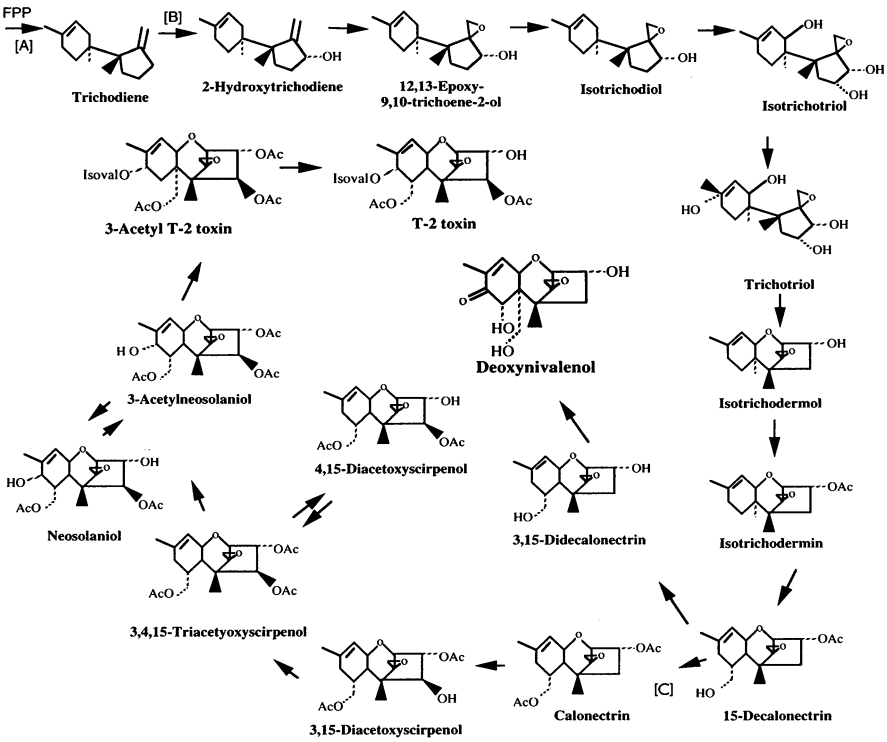


Fig. 5 Trichothecene biosynthetic pathway

transcribed spacers separating the genes coding for 18S rRNA and 5.8S rRNA (ITS1), and between the latter gene and the gene coding for 28S rRNA (ITS2). Mishra et al. (2003) used the ITS region in the rRNA genes of *F. avenaceum, F. culmorum, F. equiseti, F. oxysporum,* and *F. sambucinum* to identify the target organisms in a four-color fluorescent assay. An assay method published by Kulik et al. (2004) was based on the ITS2 region of the rRNA gene, which is useful for identification of *F. sporotrichioides* pure cultures, but also for detection of the fungus in plant tissue. Nicholson et al. (1998) used RAPD-based primers to set up competitive quantitative PCR assays for *F. graminearum* and *F. culmorum*, in which internal competitor fragments were added in defined concentrations to compete for primer binding with the target DNA, thus enabling accurate quantification.

Molecular Methods for Detection of Fumonisin Producing *Fusarium* Species

As in other mycotoxin producers, genes involved in the biosynthesis and regulation of fumonisins are organized in clusters within the genome. Waalwijk et al. (2004) studied the fumonisin gene cluster of *F. proliferatum* and found 19 genes to be involved in biosynthesis and regulation of toxin metabolism.

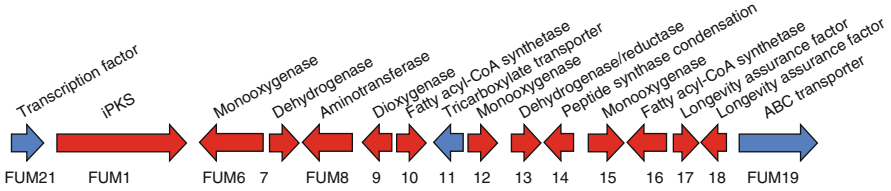


Fig. 6 The *FUM* gene cluster of *F. moniliformis* (Proctor et al. 1999)

Elucidation of the genetic basis for fumonisin biosynthesis was initiated with a classical linkage analysis in 1996, followed by studies in 1999 and 2001, which identified a cluster of five genes required for synthesis (Desjardins et al. 1996; Proctor et al. 1999). Subsequent mapping and characterization of the surrounding genes resulted in the identification of 16 coexpressed genes (Fig. 6), responsible for a highly complex biosynthetic pathway, which included a *iPKS*, two fatty acid synthases and numerous different classes of modifying enzymes, including monooxygenases, dehydrogenases, aminotransferase, and dioxygenase (Proctor et al. 1999).

Identification of this gene cluster allowed for the formulation of a biosynthetic model (Fig. 7) which also offered an explanation for the different types of reported fumonisins (B1, B2, B3, and B4). Presently, only *Fum*1 has been linked to formation of the polyketide backbone and *Fum9* to hydroxylation of the C5 position, meaning the majority of the suggested steps remain to be experimentally proven (Butchko et al. 2003).

Based on this metabolic pathway elucidation, several researchers developed several PCR and DNA probe-based methods for rapid and sensitive detection of fumonisin-producing *Fusarium* species from contaminated cereal grains (Ramana et al. 2011, 2012). Zheng and Ploetz (2002) developed a RAPD-based PCR which they applied to the screening of *F. subglutinans* isolates from *Mangifera indica*, where the fungus caused a destructive disease called mango malformation. Testing various isolates of different species within the *G. fujikuroi* complex showed that the system was specific for *F. subglutinans* and *F. nygamai*.

Patino et al. (2004) set up a highly sensitive PCR system for toxigenic isolates of *F. verticillioides* from different geographical regions and hosts. Grimm and Geisen (1998) compared nucleotide sequences of the ITS1 region within the rRNA genes of typical fumonisin-producing *Fusarium* species, with those of species that do not typically produce the toxin. A pair of PCR primers and a biotinylated probe were designed which used sequence differences to specifically amplify a 108 bp fragment from fumonisin producers. Grimm and Geisen (1998) used a microplate-based enzyme-linked oligosorbent assay (ELOSA) with a biotinylated capture probe and detection of bound PCR fragments via binding of enzyme-linked anti-DIG (digoxigenin) antibodies to DIG incorporated into the PCR product of fumonisin-producing *F. verticillioides*. The assay was specific for detection of fumonisin-producing isolates of *F. verticillioides, F. proliferatum, F. nygamai,* and *F. napiforme*. Recently, two PCR-based assays were published which used sequence

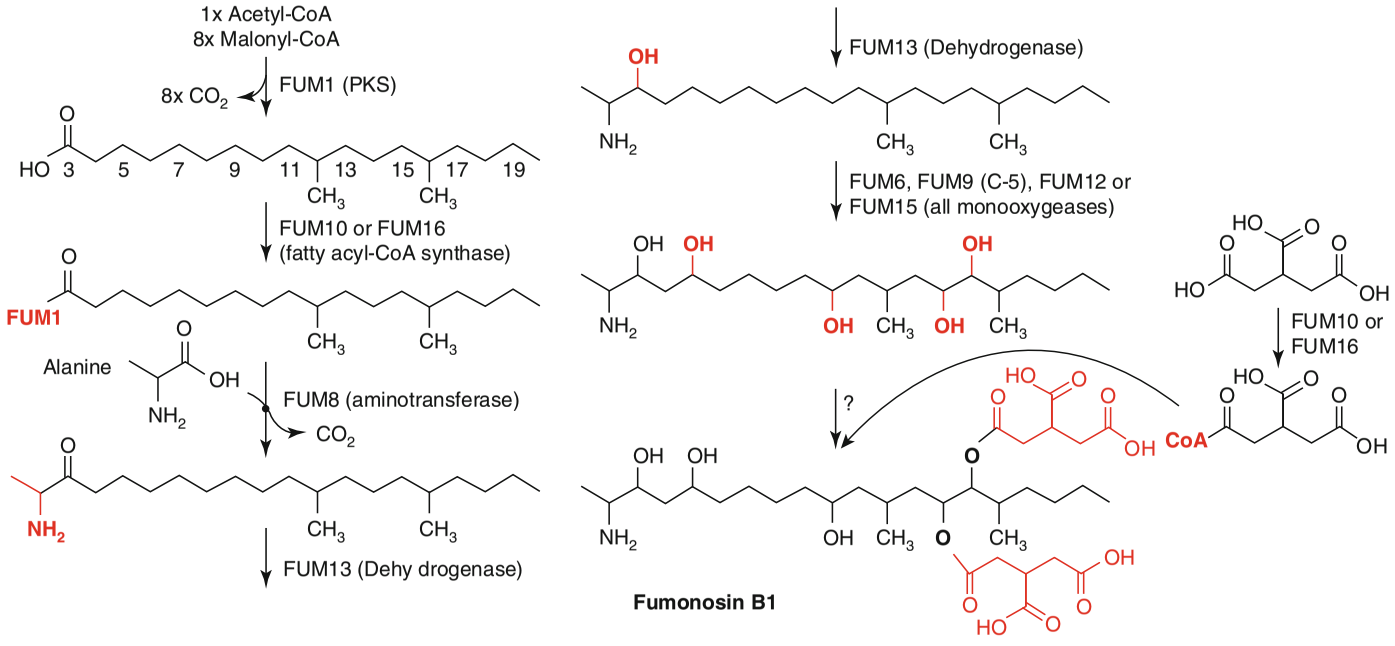


Fig. 7 Proposed biosynthetic pathway of fumonisin biosynthesis in *F. moniliformis* (Waalwijk et al. 2004). Note: The order of reactions following the formation of the linear polyketide chain is not known; however, the hydroxylation of position 14 and 15 has to occur prior to addition of the two branched fatty acid side chains

information from genes involved in the biosynthesis of fumonisin for the selective identification of toxigenic isolates of *F. verticillioides*.

Gonzalez-Jaen et al. (2004) demonstrated that genes *Fum1 (=Fum5), Fum6,* and *Fum8* were only present in *F. verticillioides, F. proliferatum, F. fujikuroi,* and *F. nygamai*, which represent the principle producers of fumonisins within the *G. fujikuroi* complex. Primers were derived from the sequence representing the β-ketoacyl reductase domain within the *Fum1* gene. Primers appeared to be highly specific for *F. verticillioides* isolates which produced fumonisin in vitro, and it was assumed that the nonproducing isolates must have lost the *Fum1* gene or at least the part of it where PCR primers hybridize in fumonisin producers. Sanchez-Rangel et al. (2005) reported similar results with a different pair of primers for the same part of the *Fum1* gene. It was speculated that the principle ability of a *F. verticillioides* isolate to produce fumonisin will depend on the presence or absence of the *Fum1* gene, but additional factors may be necessary which regulate the concentrations of fumonisin finally produced. Another group-specific assay for fumonisin producers was published by Ramana et al. (2011). The system was based on the *Fum1* and *Fum13* gene sequences of *F. proliferatum* and *F. verticillioides* and was applied to the detection of these fungi in artificially contaminated cornmeal in a multiplex PCR assay. Potential producers of fumonisins were detected together with potential trichothecene producers (*Tri6* gene).

Molecular Methods for Detection of Zearalenone Producing *Fusarium* Species

The compound zearalenone is synthesized by the combined action of two PKSs and an isoamyl alcohol oxidase (Kim et al. 2005; Lysoe et al. 2006). The involved genes are encoded by a 39 kb large gene cluster consisting of four genes, *FgPKS13*, *FgPKS4*, *ZEB1*, and *ZEB2* (Fig. 8).

The ZEB1 protein contains a basic-region leucine zipper (bZIP) domain and functions as a cluster specific transcription factor that controls expression of the cluster (Kim et al. 2005). The current model for zearalenone biosynthesis is shown in Fig. 9, based on Kim et al. (2005) and Gaffoor and Trail (2006). The biosynthetic pathway is initiated by Fg*PKS4*, a reducing PKS, which catalyze the condensation of one acetyl-CoA and five malonyl-CoA units, resulting in a hexaketide.

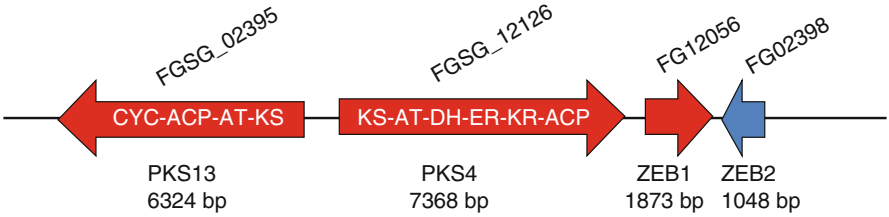


Fig. 8 Zearalenone genes cluster (Kim et al. 2005). Note: PKS4 does not contain a thioesterse domain which normally is responsible for releasing the final product from the PKS

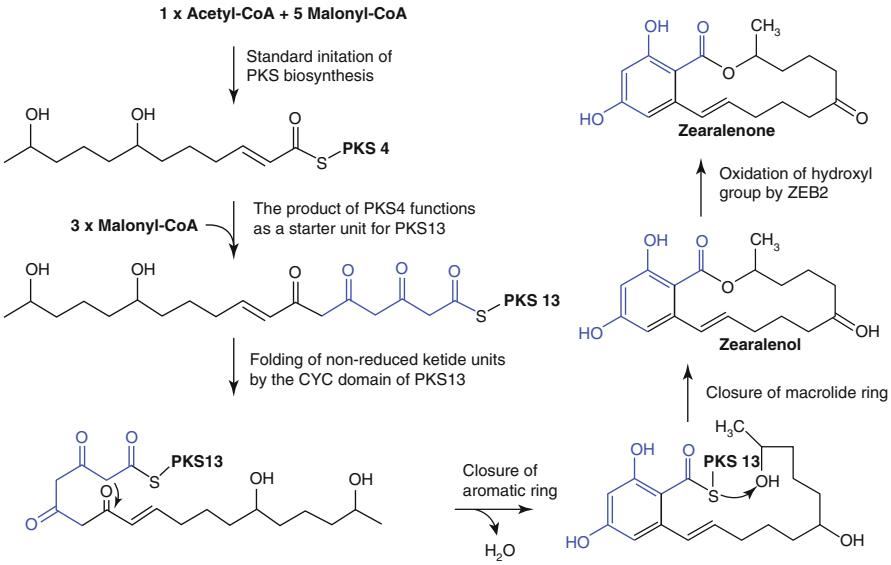


Fig. 9 Model of zearalenone biosynthetic pathway (Waalwijk et al. 2004)

The three reducing domains of *PKS4* are deployed to various degrees during the different cycles of synthesis, ketide no-6 remains unreduced, ketide *no-1* and 3 are processed by the KR domain resulting in hydroxyl groups, and ketide no-5 is processed by the KR and DH domains resulting in an enoyl group, while ketide no-2 and 4 are reduced completely to alkyls by the KR, DH, and ER domains. The formed hexaketide is then passed onto the nonreducing Fg*PKS13*, functioning as a starter unit for further extension of the polyketide chain. Fg *PKS13* completes three iterations, extending the chain by three additional ketide units and resulting in a nonaketide. The nonaketide then undergoes two rounds of intramolecular cyclication reactions, resulting in formation of an aromatic ring and a macrolide ring structure with a lactone bond. The final conversion of the formed zearalenol to zearalenone is catalyzed by the isoamyl alcohol oxidase, ZEB1, which converts the macrolide bound hydroxyl group to a ketone.

Targeted replacement of Fg*PKS4* results in downregulation of the *PKS13* gene, suggesting the existence of a regulatory feedback loop similar to that described for bikaverin biosynthesis in *F. fujikuroi* (Lysoe et al. 2006).

Traditional Technologies for Detection/Quantification of Mycotoxins

Recently, there has been an increasing interest in the potential use of molecularly imprinted polymers (MIPs) as adsorbents for SPE due to their low costs, easy preparation, high chemical stability, and long shelf-life. MIPs are cross-linked polymers that are thermally, photochemically, or electrochemically synthesized by the reaction of a monomer and a cross-linker in the presence of an analyte (mycotoxin), used as a template. After polymerization, the analyte is removed leaving specific

recognition sites inside the polymer. MIPs provide biomimetic recognition elements capable of selective binding/rebinding to the analyte with efficiencies comparable to those of antibody-antigen interactions. The synthesis of MIPs with high affinity for DON, ZEN, and Ochratoxin A (OTA) was already reported (Pascale et al. 2008). These polymers have been used as a stationary phase in chromatographic applications or for the preparation of solid-phase extraction (SPE) columns that are to be used in sample cleanup, although, in a few cases, nonimprinted polymers, i.e., polymers synthesized without a mycotoxin template, performed similarly to molecularly imprinted polymers. Recently, itaconic acid has been identified by molecular modeling and computational design as a functional monomer with high affinity towards DON. Itaconic acid polymers, synthesized without the template (i.e. DON), were successfully used as adsorbents for SPE cleanup and preconcentration of DON from contaminated pasta extracts prior to the HPLC analysis (Pascale et al. 2008).

Gas chromatographic methods based on FID (Flame Ionization Detector), ECD (Electron Capture Detector), and MS (Mass Spectra) detection are the most widely used methods for quantitative simultaneous determination of trichothecenes (mainly type A) in cereals and cereal-based products (Krska et al. 2001). These methods require a preliminary cleanup of extracts, generally by MycoSep® columns, and precolumn derivatization of the purified extract with specific reagents. Mass Spectrometry (MS), or tandem mass spectrometry (MS/MS), offers an advantage in confirming the identity of a chromatographic peak. The main problems associated to GC analysis include increased trichothecene responses, nonlinearity of calibration curves, drifting responses, carry over or memory effects from previous samples and high variation in terms of reproducibility and repeatability (Petterson and Langseth 2002). HPLC coupled with UV, diode array (DAD), or fluorescence detector (FD) is currently the most widely used technique for the analysis of major mycotoxins occurring in cereals. AFs, OTA, FBs, and ZEN are routinely analyzed by HPLC-FD [Florescent Detector] and DON by HPLC-UV (DAD) with good accuracy and precision. HPLC-FD is highly sensitive, selective, and repeatable, so specific labeling reagents have been developed for the derivatization of nonfluorescent mycotoxins to form fluorescent derivatives. Either precolumn derivatization with trifluoroacetic acid (TFA) or postcolumn derivatization with Kobra Cell® (electrochemical bromination cell) or photochemical reactor (UV irradiation) can be used to enhance fluorescence of aflatoxins B1 and G1, whereas precolumn derivatization with OPA reagent is required for the detection of fumonisins B1, B2, and B3, after purification of the extracts with immunoaffinity columns, solid-phase extraction, or MycoSep® columns.

In previous studies, 1-anthroylnitrile (1-AN), 2-naphthoyl chloride (2-NC), and pyrene-1-carbonyl cyanide (PCC) have been used as fluorescent labeling reagents for T-2 and HT-2 detection by HPLC-FD (Lippolis et al. 2008). The derivatization reaction was used to develop a sensitive, reproducible, and accurate method for the simultaneous determination of T-2 and HT-2 after IAC cleanup in naturally

contaminated cereal grains (wheat, maize, oats, and barley). Several HPLC methods for identifying various mycotoxins in a number of cereals and cereal-based products have been validated by collaborative studies, and their performance characteristics, such as accuracy, repeatability, reproducibility, detection, and quantification limits, were established. These methods have been adopted as official or standard methods by the AOAC [**Association of Analytical Communities**] International, or the European Standardization Committee (ESC).

In addition, HPLC/IAC methods have been validated for the measurement of DON in cereals and cereal products, and ZEA in barley, maize, wheat flour, polenta, and maize-based baby food (Macdonald et al. 2005). Liquid chromatography coupled with mass spectrometry (LC-MS) has been used for many years, mainly as a technique for mycotoxin confirmation. At the present time, LC-MS and LC-MS/MS are the most promising techniques for the simultaneous screening, identifying, and measuring of a large number of mycotoxins. Advances and recent trends in mycotoxin detection by LC-MS have been recently reviewed for detection of patulin, aflatoxins, ochratoxin A, zearalenone and its metabolites, trichothecenes, and fumonisins (Songsermsakul and Razzazi-Fazeli 2008). LC-MS/MS and Atmospheric Pressure Chemical Ionization (APCI) or Electro-Spray Ionization (ESI) interface was used for the simultaneous determination of the major type A- and type B-trichothecenes and ZEN in cereals and cereal-based products at trace levels. LC-APCI-MS/MS method using reversed phase SPE Oasis® HLB columns for extract cleanup has been recently developed for the simultaneous determination of NIV, DON, T-2, and HT-2 in cereals and cereal-based products (Lattanzio et al. 2008). The LC-ESI-MS/MS method has been recently developed for simultaneous determination of major *Fusarium* toxins (DON, T-2, HT-2, FB1, FB2, ZEN) together with aflatoxins (AFB1, AFB2, AFG1, AFG2) and OTA in maize, based on the use of new multitoxin immunoaffinity columns (Lattanzio et al. 2007). HPLC-MS/MS is also proven to be a powerful technique for the determination of masked mycotoxins. Accuracy, precision, and sensitivity of LC-MS methods may vary depending on the mycotoxin, matrix, and instrument with the sensitivity of the method, depending on the ionization technique. Quantitative measurement of mycotoxins by LC-MS is often unsatisfactory due to matrix effects and ion suppression. Purification of extracts by MycoSep® or IACs is generally needed prior to MS detection (Lattanzio et al. 2009). Advantages and disadvantages of different methods are discussed in Table 4.

Immuno Methods for Detection of *Fusarium* Toxins

Immunoassay techniques using polyclonal and monoclonal antibodies (mAb) have been developed to detect fumonisins, DON, and other mycotoxins within grain, food, feed, and animal serum (Venkataramana et al. 2014b; Sinha et al. 1995). These systems typically use antimycotoxin mAbs from hybridoma cell lines producing murine immunoglobulins. Although these tests provide robust and precise results, mAb development requires highly specialized equipment and labor-intensive

procedures to select, culture, and maintain optimal hybridoma cell lines (Venkataramana et al. 2015).

Immunological assays, such as enzyme-linked immunosorbent assays (ELISA), have become very popular in mycotoxin screening since the late 1970s. In general, ELISA does not require cleanup procedures and the extract containing the mycotoxin is analyzed directly. Even though they often lack accuracy at very low concentrations and are limited in the range of matrices examined, immunoassays provide fast, inexpensive screening assays. However, matrix interference or the presence of structurally related mycotoxins can interfere with the binding of conjugate and antibody, leading to mistakes in quantitative measurements of mycotoxins. ELISA kits should be used routinely only for the analysis of matrices that are extensively tested.

Several ELISA kits that use monoclonal or polyclonal antibodies against mycotoxins have been commercially developed for qualitative, semiquantitative, or quantitative analyses of the main known mycotoxins in cereal-based matrices. Over the past years, rapid immunoassay-based tests have increasingly been used for the analyses of mycotoxins in cereals and cereal-based foods (Goryacheva et al. 2007). Lateral flow devices (LFDs) are rapid immunoassay instruments that have been developed for the most prevalent mycotoxins in cereals and are commercially available for the determination of AFs and FBs in maize, DON in wheat, and OTA, ZEN, T-2, and HT-2 in cereal grains. Portable photometric strip readers allow quantitative or semiquantitative analysis in the field.

Fluorescence polarization immunoassay (FPIA) is a simple technique that measures interactions between a fluorescently labeled antigen and a specific antibody in solution. FPIAs had been developed for rapid determination of AFs, ZEN, FBs, and DON, although low accuracy and sensitivity were observed when these assays were used with cereal samples (Chun et al. 2009). Pascale et al. (2008) optimized FPIA for rapid determination of DON in durum and common wheat, semolina, and pasta. The assay showed better accuracy and precision, with respect to a widely used HPLC-IAC method, in the range of 100–2,000 mg/kg. Fourier transformed near-infrared (FT-NIR) spectroscopy and principal component analysis (PCA) have been recently used for the determination of DON content in durum and common wheat (Table 5).

The advantages of these methods compared to other methods are the ease of operations, rapidity of analysis, and nondestruction of samples. Immunochemical biosensors that use surface plasmon resonance (SPR) or screen-printed carbon electrodes have been described for the detection of mycotoxins in cereals. Competitive SPR-based immunoassays have been recently described for the determination of DON in wheat, with or without extract cleanup, showing a good correlation between DON concentration measured with biosensor and GC-MS, or HPLC-UV and HPLC-MS/MS as reference methods (Prieto-Simon et al. 2007). Competitive electrochemical ELISAs based on disposable, screen-printed carbon electrodes have been developed for quantitative determination of ochratoxin A in wheat. The results

Table 5 Advantages and disadvantages of traditional and emerging methods for mycotoxin analysis (Pascale 2009)

Method	Advantages	Disadvantages
GC	Simultaneous analysis of mycotoxins, good sensitivity, may be automated (autosampler), provides confirmation (MS detector)	Expensive equipment, specialist expertise required, derivatization required, matrix interference problems, nonlinear calibration curve, drifting response, carry-over effects from previous sample, variation in reproducibility and repeatability
HPLC	Good sensitivity, good selectivity, good repeatability, may be automated (autosampler), short analysis times, official methods available	Expensive equipment, specialist expertise required, may require derivatization
LC/MS	Simultaneous analysis of mycotoxins, good sensitivity (LC/MS/MS), provides confirmation, no derivatization required	Very expensive, specialist expertise required, sensitivity relies on ionization technique, matrix assisted calibration curve (for quantitative analysis), lack of internal standards
ELISA	Simple sample preparation, inexpensive equipment, high sensitivity, simultaneous analysis of multiple samples, suitable for screening, limited use of organic solvents	Cross-reactivity with related mycotoxins, matrix interference problems, possible false positive/ negative results, confirmatory LC analysis required
LFD	Rapid, no cleanup, no expensive equipment, easy to use, no specific training required	Semiquantitative (visual assessment), cross-reactivity with related mycotoxins, validation required for additional matrices
FPIA	Rapid, no cleanup required, validated for DON in wheat	Inconsistent with ELISA or HPLC analyses (except for DON), poor sensitivity in some cases, cross-reactivity with related mycotoxins, matrix interference problems
IR spectroscopy	Rapid, nondestructive measurement, no extraction or cleanup, easy operation	Expensive equipment, calibration model must be validated, knowledge of statistical methods, poor sensitivity
Biosensors	Rapid, no cleanup procedure	Cross-reactivity with related mycotoxins, extract cleanup needed to improve sensitivity, variation in reproducibility and repeatability
MIP	Low cost, stable, reusable	Poor selectivity

Note: *GC* Gas Chromatography, *HPLC* High Performance Liquid Chromatography, *LC/MS* Liquid Chromatography/Mass Spectrometry, *ELISA* Enzyme-Linked Immunosorbent Assay, *LFD* Lateral Flow Device, *FPIA* Fluorescence Polarization Immunoassay, *IR* Infrared, *MIP* Molecularly Imprinted Polymer

from screen-printed carbon electrodes and HPLC/IAC cleanup methods for naturally contaminated wheat samples showed good correlation (Alarcon et al. 2006).

Conclusions and Future Directions

The risk of contamination by mycotoxins is an important food safety concern for grains and other field crops. *Fusarium* mycotoxins are very health hazardous, natural chemical compounds to animals and humans. Hence, there is a need to study the structures of toxins, toxicity patterns, their detection, and its control thus to keep away from the harmful health effects to humans and animals. Fusarium mycotoxicoses cause severe problems to humans and animals. Therefore, there is an immediate need to develop strict regulatory policies to combat the issues with toxigenic molds and mycotoxin in food and feed industries as well as in agriculture. This chapter discusses the potential health effects of *Fusarium* species and its toxins to humans and animals, its structure and functional relationship, its metabolism, as well as its detection from food and feed matrices intended for consumption. The prevention of mycotoxin contamination in agriculture is important by development of resistant variety of plants and use of fungicides to prevent growth of toxigenic molds. Although there have been several recent reports in toxicology and detection of fusarial toxins, new methods are still required to achieve higher sensitivity and address the other challenges that are posed by these toxins. Future trends should focus on onsite, rapid detection methods and tools that would assess multiple mycotoxins from a single matrix that would be useful in developing and underdeveloped countries, where *Fusarium* toxin contamination still pose a great challenge.

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