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Virulence Factor Secretion by *Bordetella* Species

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Summary

Here we review the *Bordetella* virulence secretome with an emphasis on factors that translocate into target cells. Recent advances in understanding the functions of adenylate cyclase toxin, a type I secretion system (T1SS) substrate, and pertussis toxin, a type IV secretion system (T4SS) substrate, are briefly described and a compilation of additional secretion systems and secreted factors is provided. Particular attention is devoted to the Bsc type III secretion system (T3SS) and controversies surrounding it. Efforts to identify effector proteins, characterize *in vitro* and *in vivo* phenotypes, and the potential role of type III secretion during human infections are discussed.

Introduction

Bordetella bronchiseptica colonizes respiratory epithelia in a broad range of mammals, while *B. pertussis* and *B. parapertussis* are exclusively human pathogens [1]. *B. pertussis* causes severe respiratory infections that manifest by paroxysmal coughing with potentially fatal complications. *B. parapertussis* is associated with milder, pertussis-like disease. Despite extensive immunization, pertussis remains a leading cause of vaccine preventable deaths worldwide [2]. In contrast, infections with *B. bronchiseptica* are usually asymptomatic, although acute and chronic disease in domesticated animals is often reported [1,3]. *B. bronchiseptica* is the evolutionary progenitor of *B. pertussis* and *B. parapertussis*, and genomic data suggests that human-restriction and the propensity for causing acute disease evolved relatively recently [4].

As listed in Table 1, *Bordetella* species are well endowed with export pathways of relevance to pathogenesis. Remarkably, only three substrates secreted by canonical systems are known to enter target cells. Two prominent intracellular effectors, adenylate cyclase toxin and pertussis toxin, gain entry by intrinsic mechanisms and have been studied for decades, whereas the third, BteA, is a recently discovered T3SS substrate. The apparent simplicity of the translocated portion of the virulence secretome is balanced by the complexity of phenotypes attributed to intracellular effectors and the challenges inherent in understanding how they function together and with factors that modulate host cells without crossing plasma membranes.

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Adenylate Cyclase Toxin

Adenylate cyclase toxin (ACT), encoded by *cyaA*, is a highly potent RTX (repeats in toxin) family toxin and is the substrate of a T1SS encoded by *cyaBDE* [5]. ACT consists of two functional modules: an adenylate cyclase (AC) domain which binds calmodulin and catalyzes unregulated conversion of ATP to cAMP, and an RTX hemolytic domain which is responsible for binding to target cells and translocation of the AC domain into the cytosol by cation-selective pore formation [6]. Independently of its catalytic activities, ACT also increases cytosolic calcium concentrations [7]. A recent crystal structure suggests that the extraordinarily high catalytic activity of the toxin results from unusually extensive interactions between the AC domain and calcium-loaded calmodulin [8]. ACT is cytotoxic *in vitro*, and both cAMP accumulation/ATP depletion and transmembrane pore formation contribute to this effect [9]. Although ACT will intoxicate many cell types, it binds with high affinity to the highly N-glycosylated $\alpha_M\beta_2$ integrin CD11b/CD18 (Mac-1, CR3) present on macrophages, neutrophils and dendritic cells [10]. The RTX hemolytic domain recognizes the CD11b receptor through N-linked oligosaccharides and inhibiting glycosylation ablates ACT binding and penetration [11].

In macrophages, ACT-mediated elevation of intracellular cAMP inactivates RhoA, resulting in unproductive membrane ruffling and a near-complete block of complement-dependent phagocytosis [12]. ACT also induces anti-inflammatory IL-10 expression through MAPK activation and downregulates IL-12p70 production by inhibiting IRF-1 and IRF-8 expression, thereby directing dendritic cells towards a suppressive phenotype [13]. IL-6 secretion by tracheal epithelial cells is upregulated by ACT, suggesting that it may influence immune responses indirectly [14]. Both antigen receptor and chemokine receptor signaling are inhibited by ACT via a cAMP/PKA-dependent pathway, effectively suppressing T-cell activation and chemotaxis *in vitro* [15], but the significance of these observations is unclear since it is unknown whether the toxin acts systemically during infection. Interestingly, a recent report shows that ACT and TLRs act synergistically on macrophages to induce expression of the pro-inflammatory enzyme cyclooxygenase 2 by a protein kinase A (PKA) and CREB-dependent pathway [16]. Thus, in addition to inhibitory and anti-inflammatory effects, ACT may also have pro-inflammatory activities. Although considerable evidence suggests that ACT modulates immune responses to infection, a coherent picture that reconciles *in vitro* and *in vivo* observations has yet to emerge.

Pertussis Toxin

Pertussis toxin (PT), an AB toxin discovered nearly a century ago, is the archetypal *B. pertussis* virulence factor and an important immunogen in acellular vaccines. The active subunit, S1, sits on top of a ring-like binding subunit which consists of the S2-S5 proteins arranged in a 1:1:2:1 molar ratio [17]. Holotoxin assembly occurs in the periplasm and export is mediated by the products of the *ptl* (pertussis toxin liberation) locus that encode a T4SS [18]. In contrast to most other T4SSs involved in pathogenesis, the Ptl machinery seems to be dedicated to export only a single effector and its substrate is secreted into the extracellular milieu as opposed to being injected across host-cell membranes. Although the Ptl apparatus has not been associated with an external secretion conduit, PtlA may form a modified pilus-like structure that acts like a piston to push assembled toxin molecules out of the periplasm and across the outer membrane [19]. *In vitro*, PT secretion occurs at a rate of about 3 molecules per minute [20].

PT holotoxin binds to almost any sialic acid containing glycoprotein and multiple receptors have been implicated *in vitro* [17]. Following binding and endocytosis, PT transits from the Golgi apparatus to the endoplasmic reticulum (ER) by retrograde transport, as demonstrated

by recent studies in which S1 and S4 were tagged with peptide target sites for tyrosine sulfation (a *trans*-Golgi specific activity) or N-glycosylation (an ER-specific activity) [21]. Toxin subunits disassociate in the ER and S1 enters the cytoplasm, most likely through the ER-associated degradation (ERAD) pathway via the Sec61 translocon [22]. An absence of lysine residues allows S1 to avoid ubiquitination and subsequent proteosomal degradation [23]. Cytosolic S1 covalently transfers ADP-ribose from NAD⁺ to conserved cysteine residues located near the C-termini of heterotrimeric G protein α subunits, which include isoforms of $G\alpha_i$, $G\alpha_o$ and $G\alpha_t$ [17]. This inhibits GDP release and blocks an array of agonist-induced cellular responses including cAMP production by host-cell adenylyl cyclase. Although the effects of intoxication vary dramatically depending on the particular cell type, PT is a potent inhibitor of migration for neutrophils, monocytes and lymphocytes that express G protein-coupled chemokine receptors.

ptx and *ptl* loci are present in *B. bronchiseptica* and *B. parapertussis* but they are only expressed to yield functional toxin in *B. pertussis* [17]. In humans, PT is responsible for pronounced leukocytosis, which results from increased release of cells from extravascular sites and continued recirculation without emigration from the bloodstream [1]. In mice, PT facilitates early lung colonization and inhibits neutrophil recruitment to sites of infection [24,25]. Although PT can have direct effects on neutrophil signaling *in vitro*, its *in vivo* effects appear to result from the inhibition of neutrophil-attracting chemokines such as KC, LIX and MIP-2 by alveolar macrophages and other cells present in lung tissue [26,27]. As with ACT, studies in murine models support the hypothesis that PT targets innate immune responses that normally protect the lower respiratory tract from bacterial invaders, but the mechanisms responsible remain unresolved.

Type III secretion

The Bsc apparatus

For reasons of experimental tractability, most studies of type III secretion in *Bordetella* have focused on *B. bronchiseptica*, however, the *bsc* T3SS locus is highly conserved in *B. pertussis* and *B. parapertussis* [28,29]. 22 contiguous genes encode components of the secretion machinery, associated chaperones and regulatory factors. Most have close homologs in other T3SSs with the notable exception of *bsp22*, which encodes the most abundantly secreted substrate *in vitro* [28]. A recent study provides evidence that Bsp22 self-polymerizes to form a filamentous tip complex that forms a flexible conduit connecting the T3SS needle to the pore-forming translocation apparatus [30].

Antibodies against Bsp22 protect epithelial cells from T3SS-dependent killing *in vitro*, and immunization with Bsp22 provides protection in murine models of respiratory infection, presumably by preventing formation of a productive connection between the needle and translocation pore [30].

BteA

The most prominent feature of Bsc activity *in vitro* is the efficient induction of cytotoxicity in a broad range of cell types by a mechanism that is complex and neither fully necrotic nor fully apoptotic [29,31,32]. Efforts to understand cytotoxicity have, until recently, been hampered by the lack of identified effectors. Since sequenced *Bordetella* genomes do not contain easily recognized homologs of known effectors, an *in silico* screen was devised to identify T3SS-associated class I chaperones and their effector pairs. This computational approach, based on shared biophysical characteristics of class I chaperones, conserved tertiary structures, and the frequent colocalization of chaperone loci with cognate effector genes, led to the identification of the BteA chaperone and its cognate effector, BteA [32].

BteA is translocated into target cells in a Bsc-dependent manner and is necessary and sufficient for cell death [32,33]. Remarkably, cytotoxicity is so efficient that death occurs before BteA can even be detected in transfected cells [32]. The carboxyl-terminal segment of the 72 kDa BteA protein bears the cytotoxicity determinant and an aggregation domain that mediates assembly into fibrils of variable length (Panina EM, unpublished). The amino-terminus drives protein localization to ezrin-rich lipid rafts at the cell surface, where BteA co-localizes with sites of *Bordetella* adherence. Localization of BteA at the point of bacteria-host cell contact may position it to alter initial cellular responses to *Bordetella* attachment. Although the mechanisms responsible for the potent cytotoxicity of BteA are under investigation, the relevant pathways must be highly conserved since BteA expression is also lethal for *Saccharomyces cerevisiae* (Panina EM, unpublished).

Extensive proteomic and bioinformatic analyses have failed to reveal Bsc-secreted effectors other than BteA [32,33]. Furthermore, a null mutation in *bteA* confers the same phenotype *in vitro* as mutations that eliminate the BscN ATPase required for T3SS activity. Although the hunt for additional effectors continues, the implausible possibility exists that the Bsc apparatus is dedicated to secreting BteA as its only translocated substrate.

***In vivo* phenotypes**

Null mutations in *bscN*, *bsp22* or *bteA* abrogate persistence in the lower respiratory tract following intranasal inoculation of Wistar rats, C57BL/6 mice, or BALB/c mice with *B. bronchiseptica* [28,29] (Ahuja U and Richards DM, unpublished). Furthermore, infections with T3SS-defective mutants result in elevated antibody responses in comparison to wild type controls [29]. Although recent studies support the notion that type III secretion has an immunomodulatory role, our current understanding is at a phenomenological stage. Infection of mice with wild type *B. bronchiseptica* results in decreased IFN- γ production by restimulated splenocytes and increased IL-10 production compared to animals infected with $\Delta bscN$ strains [34,35]. Furthermore, IFN- γ $-/-$ mice clear *B. bronchiseptica* from the lower respiratory tract with delayed kinetics in comparison to wild type animals, whereas IL-10 $-/-$ mice show faster clearance [34,35]. These data are consistent with the idea that the T3SS alters the balance between immunostimulatory and immunosuppressive cytokine production. Although initially characterized as an altered Th1 phenotype, infection may actually skew the immune response towards a Th17 profile as defined by the production of IL-17 [36]. Th17 responses are often associated with chronic inflammatory conditions [37], and IL-17 expression appears to be stimulated by a synergistic interaction between the Bsc T3SS and ACT. Interestingly, $\Delta bscN$, $\Delta cyaA$ *B. bronchiseptica* mutants are highly attenuated yet capable of conferring potent immunity against wild type strains [38].

Pertussis

Perhaps the most pressing question regarding the Bsc system relates to its role during human infection. Initial reports indicated that T3SS-associated phenotypes were not observed with prototype strains of *B. pertussis* [28,39]. This was surprising for several reasons. First, T3SS genes are intact, highly conserved, transcribed and regulated in *B. pertussis* [39]. Second, the vast majority of nucleotide substitutions observed between *B. bronchiseptica* and *B. pertussis* *bsc* loci are silent or result in conservative amino acid substitutions, implying positive selection for function. Third, the BteA effector protein is conserved and functionally interchangeable between *B. pertussis* and *B. bronchiseptica* [32] (Yeh S, unpublished). A recent analysis begins to shed light on these issues [40]. T3SS activity was not detected in common laboratory strains, but Bsc-mediated secretion of Bsp22 was readily observed in low-passage strains and clinical *B. pertussis* isolates. Although BteA effector secretion was not examined and there was no indication of T3SS-mediated cytotoxicity *in vitro*, eliminating T3SS activity did result in

elevated production of proinflammatory cytokines and accelerated clearance from the lungs of aerosol-infected mice [40].

Conclusions

ACT is highly conserved by *B. bronchiseptica* and its evolutionary descendents, yet PT is only expressed by *B. pertussis* and major differences in Bsc activity are apparent between subspecies. The significance of differential expression of translocated effectors is presently unclear. Although ACT and PT are highly pleiotropic and their effects are complex, the Bsc T3SS seems particularly enigmatic. BteA clearly plays a central role in Bsc-dependent phenotypes, but is it really the only effector? What mechanisms account for its ability to induce such a dramatic collapse of cellular functions? BteA will kill virtually any cell type tested, yet most *B. bronchiseptica* infections are asymptomatic and occur without tissue destruction. Is cytotoxicity a consequence of dysregulation or inappropriate targeting *in vitro*? Perhaps the most important challenge is to understand the roles of type III secretion during human infection. Clinical isolates of *B. pertussis* secrete Bsp22 and ablation of T3SS activity alters the course of infection in mice, yet cytotoxicity is mysteriously absent despite the conservation of BteA. The regulatory circuitry that controls Bsc activity is known to be remarkably complex [39, 41]. Could differential regulation explain differences observed between subspecies? Clarifying these issues could have practical applications. Bsp22 is highly conserved and protective against *B. bronchiseptica* infections in mice, suggesting that T3SS apparatus components should be considered as potential antigens for next-generation vaccines against pertussis.

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Table 1

Secreted Factors Implicated in *Bordetella* Virulence

Substrate	Secretion Pathway	Genome Location	Regulation	Function	Homologies
Adenylate cyclase toxin (ACT)	T1SS	<i>cyaA</i> (encodes ACT) and <i>cyaBDE</i> (encode T1SS) are proximal to one another but contain two transcription start sites [42]	BvgAS regulated [43]	Self-translocates across plasma membrane, activated by calmodulin, catalyzes production of cAMP in host cells; essential virulence factor in <i>B. pertussis</i> [6]	Member of RTX family of toxins
Fimbriae	T2SS export fimbrial subunits to periplasm; Chaperone/usher pathway allows anchoring to outer membrane	<i>fim2</i> , <i>fim3</i> fimbrial subunit genes unlinked to <i>fimBCD</i> biogenesis genes	BvgAS regulated	Adherence, required for tracheal persistence <i>in vivo</i> ; co-ordinates with FHA for colonization in mice; activates CD11b/CD18 which enhances attachment in a protein tyrosine kinase- dependent manner [1,44]	Type 1 and P pili in uropathogenic <i>E. coli</i>
BteA	T3SS	<i>bteA</i> is unlinked to the <i>bse</i> T3SS apparatus locus	BvgAS, BtrS regulated [39]	Required for cytotoxicity <i>in vitro</i> and persistence <i>in vivo</i> [32]	No homology to characterized proteins
Pertussis toxin (PT)	T4SS	<i>ptxA-1</i> (encode T4SS) are directly adjacent to and co-transcribed with <i>ptxA-E</i> (encode PT subunits S1-S5). Genes present but not expressed in <i>B. parapertussis</i> and <i>B. bronchiseptica</i>	BvgAS regulated	Protective antigen; facilitates colonization <i>in vivo</i> ; inhibits agonist-induced GDP release from target G proteins to block agonist-induced responses [17]	PT is a member of the ADP- ribosylating toxin family; Ptl T4SS components are homologous to VtrB proteins in <i>Agrobacterium tumefaciens</i> ,
BrkA	Autotransporter (T5SS)	Divergently transcribed to <i>brkB</i> (required for serum resistance activity)	BvgAS regulated	Resistance to serum killing; involved in adherence and invasion [44,45]	C-terminal domain is homologous to pertactin and other autotransporters
Filamentous hemagglutinin (FHA)	Two-partner secretion (T5SS)	<i>fhaB</i> (encodes FHA) located upstream of <i>fimBCD</i> and <i>fhaC</i> , which is required for secretion	BvgAS regulated	Major adhesin <i>in vitro</i> , important colonization factor <i>in vivo</i> ; overcomes bacterial clearance by mucociliary escalator; enhances expression of ICAM-1 on target cells [46-48]	Model for two-partner secretion in Gram-negative bacteria including: HMW proteins in <i>Haemophilus</i> ; ShlA cytolysin in <i>Serratia marcescens</i> ; CdtA from <i>E. coli</i>
Pertactin	Autotransporter (T5SS)	Sole gene in operon	BvgAS regulated	Contributes to attachment to CHO	Unique cargo domain, transport domain

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Tracheal colonization factor (TCF)	Autotransporter (T5SS)	Sole gene in operon	BvgAS regulated	and Hela cells <i>in vitro</i> , <i>in vivo</i> role uncertain [44,49] Required for colonization of mice [44,50]	homologous to other autotransporters
Vag8	Autotransporter (T5SS)	Sole gene in operon	BvgAS regulated	Null mutants colonize mice as efficiently as wildtype strains [44, 51]	C-terminal domain is homologous to pertactin and other autotransporters
BcfA	Autotransporter-like	Adjacent to putative dehydrogenase; relationship with BcfA is unknown	BvgAS regulated	Required, along with BipA, for colonization of murine respiratory tract [52]	Homologous to BipA
BipA	Autotransporter-like	Sole gene in operon	BvgAS regulated	Bvg "intermediate phase" gene; combinatorial role with BcfA in tracheal colonization [52,53]	Similar structure to intimins and invasins in enteric pathogens, homologous to BcfA
Unknown	T6SS	Structural genes identified by homology are present in <i>B. bronchiseptica</i> and <i>B. paratuberculosis</i>	Unknown	Unknown	Homologous to known T6SS structural components [54]
Dermonecrotic toxin (DNT)	Unknown, may be released following cell lysis	Sole gene in operon	BvgAS regulated	Activates small Rho family GTPases; causes skin lesions following injection; role in <i>B. bronchiseptica</i> - induced turbinate lesions [17]	Member of dermonecrosis- inducing bacterial toxin family; <i>E. coli</i> CNF-1 and CNF-2, <i>Y. pseudotuberculosis</i> CNF-γ
Tracheal cytotoxin	Cell wall derived disaccharide tetrapeptide; released during growth	Release is due to insertion in <i>ampG</i>	Unknown	Induces cytopathology including loss of ciliary action and extrusion of ciliated cells from respiratory epithelium; increases production of iNOS in host cells; activates Nod1 intracellularly [17, 55]	TCT is released during growth by <i>Neisseria</i> and <i>Vibrio</i> species