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Review

The diverse landscape of AB5-type toxins

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ABSTRACT

AB_5-type toxins are a group of secreted protein toxins that are central virulence factors for bacterial pathogens such as *Shigella dysenteriae, Vibrio cholerae, Bordetella pertussis,* and certain lineages of pathogenic *Escherichia coli* and *Salmonella enterica.* AB_5 toxins are composed of an active (A) subunit that manipulates host cell biology in complex with a pentameric binding/delivery (B) subunit that mediates the toxin's entry into host cells and its subsequent intracellular trafficking. Broadly speaking, all known AB_5-type toxins adopt similar structural architectures and employ similar mechanisms of binding, entering and trafficking within host cells. Despite this, there is a remarkable amount of diversity amongst AB_5-type toxins; this includes different toxin families with unrelated activities, as well as variation within families that can have profound functional consequences. In this review, we discuss the diversity that exists amongst characterized AB_5-type toxins, with an emphasis on the genetic and functional variability within AB_5 toxin families, how this may have evolved, and its impact on human disease.

1. Introduction

Many bacterial pathogens secrete protein toxins as a major mechanism of virulence that directly or indirectly contributes to their capacity to cause disease. A substantial proportion of bacterial toxins have evolved to cross the host cell plasma membrane, allowing them to manipulate cellular processes within the host cell. These toxins are known as AB-type toxins, since their biological activities and cell binding/entry mechanisms are generally carried out by distinct subunits or domains; A subunits (or "active" subunits) modify specific host cell targets thereby altering host cell biology, and B subunits ("binding" or "delivery" subunits) mediate host cell binding, entry and trafficking. AB toxins can be further classified based on the stoichiometry of the A and B subunits within the toxin complex. AB_5-type toxins, composed of an active subunit that is carried by a pentameric B subunit delivery platform, represent a surprisingly widespread and diverse class of toxins. AB_5 toxins have a considerable impact on human health, since they are principal virulence factors for several important bacterial pathogens. In addition, due to the efficient manner in which they enter human cells and the targeted ways in which they manipulate cell biology, AB_5 toxins have a great deal of potential in biotechnology or as therapeutic agents.

AB_5 toxins can be divided into families on the basis of sequence similarity as well as the enzymatic and biological activities of their A subunits (see Table 1). The breadth of the AB_5 toxin families varies from those composed of a single toxin encoded by select strains within a single species (*e.g.* the subtilase toxin family), to those composed of numerous diverse toxins that are encoded by taxonomically distant bacterial lineages (the pertussis toxin family). Regardless of the family, all known AB_5 toxins share several common structural and functional features. Perhaps the most prominent conserved feature is the overall holotoxin architecture, which consists of the A subunit sitting atop a donut-shaped ring of five B subunits. The A and B subunits form a stable complex using non-covalent interactions. A salient and conserved feature of the AB_5 structure is the insertion of a C-terminal α-helix from the A subunit into the central pore of the B subunit pentamer [1,2]. The A-B complex is further stabilized by additional interactions where the A subunit sits on the apical surface of the B subunit, although the nature and extent of these interactions is highly variable amongst different toxins. The A subunits of diverse AB_5 toxins are composed of an enzymatically active A1 domain, and an A2 domain that anchors the active component to the delivery platform [2–6]. The A1 and A2 domains are connected by a linker that is proteolytically cleaved, however the domains remained covalently tethered by a disulfide bond until the latter stages of the toxin's trafficking within target host cells. The B subunits of AB_5 type toxins all adopt similar oligosaccharide/oligonucleotide binding (OB) folds and assemble in an analogous way to yield the canonical pentameric ring with a central pore that is lined with an α-helix from each monomer [1–5]. Broadly speaking, the strategies employed by different AB_5 toxins to enter and traffic within host cells are also similar. The pentameric nature of the delivery platform yields a toxin complex with multiple glycan binding sites on each toxin, and the B subunits bind glycan receptors on the cell surface in a manner that is thought to involve multivalent in-

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Table 1
Summary of AB_5 toxin families: intrafamily diversity and unique evolutionary features.

Toxin family	Major toxins, types, subtypes	A subunit(s): Activity	A subunit(s): Structure and diversity	B subunit(s): Family	B subunit(s) Structure and diversity
Shiga Toxin	Shiga toxin (*S. dysenteriae*) Shiga toxin type 1 subtypes Stx1a/c/d, Shiga toxin type 2 subtypes Stx2a-f (*E. coli*)	RIP[&]	Single A subunit with RIP activity. Activity differences amongst different family members	Shiga family	Homopentamers. B subunit diversity amongst different types/subtypes confer different functional properties.
Cholera Toxin	Cholera toxin (*V. cholerae*) Heat-labile toxin: LT-I, LT-IIa/b/c (*E. coli*)	ADP-RT[%]	Single A subunit with ADP-RT activity. Activity differences amongst different family members	Cholera family	Homopentamers. Substantial B subunit diversity amongst heat-labile toxins in terms of both sequence and functional properties.
Pertussis toxin	Pertussis toxin (*B. pertussis*), ArtAB type 1/2 (*Salmonella*), ECPlt (*E. coli*) & other similar toxins. Substantial diversity amongst ArtAB/ECPlt-like toxins	ADP-RT	Single A subunit with ADP-RT activity. Likely some activity differences in different family members, but not well characterized	Pertussis family	Heteropentamers (pertussis toxin) or Homopentamers (ArtAB/ECPlt-like toxins). Very diverse glycan binding properties amongst different family members.
Typhoid toxin	PltB typhoid toxin and PltC typhoid toxin (*Salmonella*). Substantial typhoid toxin diversity, but not formally divided into types/subtypes	ADP-RT and DNase	Two A subunits tethered by a disulfide bond. Unknown if there are activity differences in different family members	Pertussis family	Homopentamers. Some strains produce two toxins with the same A subunit, but different B subunits. Variable B subunit sequences/ functional properties in different *Salmonella*
CfxAB/ EcxAB	CfxAB (*Citrobacter*), EcxAB (*E. coli*)	MFMP[#]	Single A subunit with putative MFMP activity. Evolved via A subunit replacement of cholera family toxin ancestor?	Cholera family	Homopentamers
Subtilase toxin	Subtilase toxin (*E. coli*)	SP[$]	Single A subunit with SP activity. Evolved via A subunit replacement of pertussis family toxin ancestor?	Pertussis family	Homopentamers

[%] ADP-ribosyltransferase.
[&] ribosome inactivating protein (RNA N-glycosidase).
[#] putative metzincin family metalloprotease.
[$] serine protease.

teractions (avidity). Toxin binding triggers toxin uptake and retrograde transport via the trans-Golgi network to the endoplasmic reticulum (ER) [7–10]. Once within the lumen of the ER, the A1-A2 disulfide bond is reduced and the A1 subunit is unfolded in a process that involves protein disulfide isomerase (PDI). For most toxins, the unfolded A-subunit is subsequently translocated to the cytoplasm by hijacking the host cell ER associated degradation (ERAD) pathway. This pathway proteolytically degrades misfolded ER proteins, but the A1 subunit is able to escape proteolysis and enters the cytosol, where it refolds to adopt its active state [7,11–13].

Despite these common features, different AB_5 toxins can share little or no significant sequence similarity, and can exhibit very different biological activities. For example, two of the most prominent AB_5 toxins, Shiga toxin and pertussis toxin, are encoded by different phyla of bacteria, and display no significant sequence similarity in their A or B subunits. Although the B subunits of both toxins contain the conserved OB-fold, their delivery platforms differ substantially in their size, composition, structure and glycan binding properties. The A subunits of these two toxins share no overt evolutionary, structural or functional connection, and they target unrelated aspects of host cell biology. The dichotomy between the similarities and differences amongst AB_5 toxins points to an ancient origin and a complex evolutionary history. Evidence for substantial evolutionary diversification is not restricted to comparisons between different toxin families, but can also be observed with a given family. Indeed, toxins are generally carried by mobile genetic elements, and the nature of the toxins encoded by closely-related organisms can vary, sometimes strain-by-strain. These differences can have important implications, since variants of the same toxin can have functional differences that result in altered virulence or disease properties for the organisms that produce them.

There is a great deal we do not know about AB_5 toxin evolution, however decades of intense study and the expansion of genomic databases have shed some light on this subject. A major factor that distinguishes different AB_5 toxin families from one another is the enzymatic activity of the A subunit. There are known instances wherein two toxin families have homologous B subunits, but unrelated A subunits. This suggests that new AB_5 toxin families can emerge as a result of “A subunit replacement”, where an enzyme encoded within the same genome as an AB_5 toxin evolves the capacity to form a complex with the B subunit, and ultimately supplants the original A subunit (Fig 1a). Although the A subunit often drives the differences between different AB_5 toxin families, the evolutionary diversification within a given family often stems mostly from changes to the B subunit(s). Some of the major mechanisms that are thought to have driven the functional diversification of AB_5 toxin families are summarized in Fig. 1b. The most common way by which novel toxin variants emerge is through sequence changes (either by accumulating mutations or by genetic recombination with similar toxins) that result in new toxin phenotypes. Recently, it has become increasingly clear toxin diversification can also result from acquiring additional B subunits, either via genome duplication (and subsequent diversification by mutation), or by horizontal acquisition of an evolutionarily distant B subunit. This new B subunit can potentially supplant the original, leading to a toxin with a similar A subunit but a distinct B subunit. Alternatively, the new B subunit and the original B subunit can coexist, either assembling into a heteromeric delivery platform, or assembling into separate delivery platforms that can both interact with the same A subunit.

In this review, we discuss the evolutionary diversification of AB_5-type toxins, with an emphasis on genetic and functional differences within AB_5 toxin families. For simplicity, we discuss the subtilase and CfxAB/EcxAB toxin families within the sections concerning pertussis and cholera families, respectively, since they share clear evolutionary connections. For those seeking additional information concerning AB_5 toxins outside the scope of this review, we direct you to other AB_5 toxin reviews [2,6], as well as reviews focused on individual toxins or toxin families: Shiga toxin [14,15], cholera family toxins [16,17], pertussis toxin [18,19], subtilase toxin [20] and typhoid toxin [21].

2. The Shiga toxin family

2.1. Overview

Shiga toxins are a potent family of toxins produced by *Shigella dysenteriae* type 1 and an assortment of pathogenic *Escherichia coli* strains that

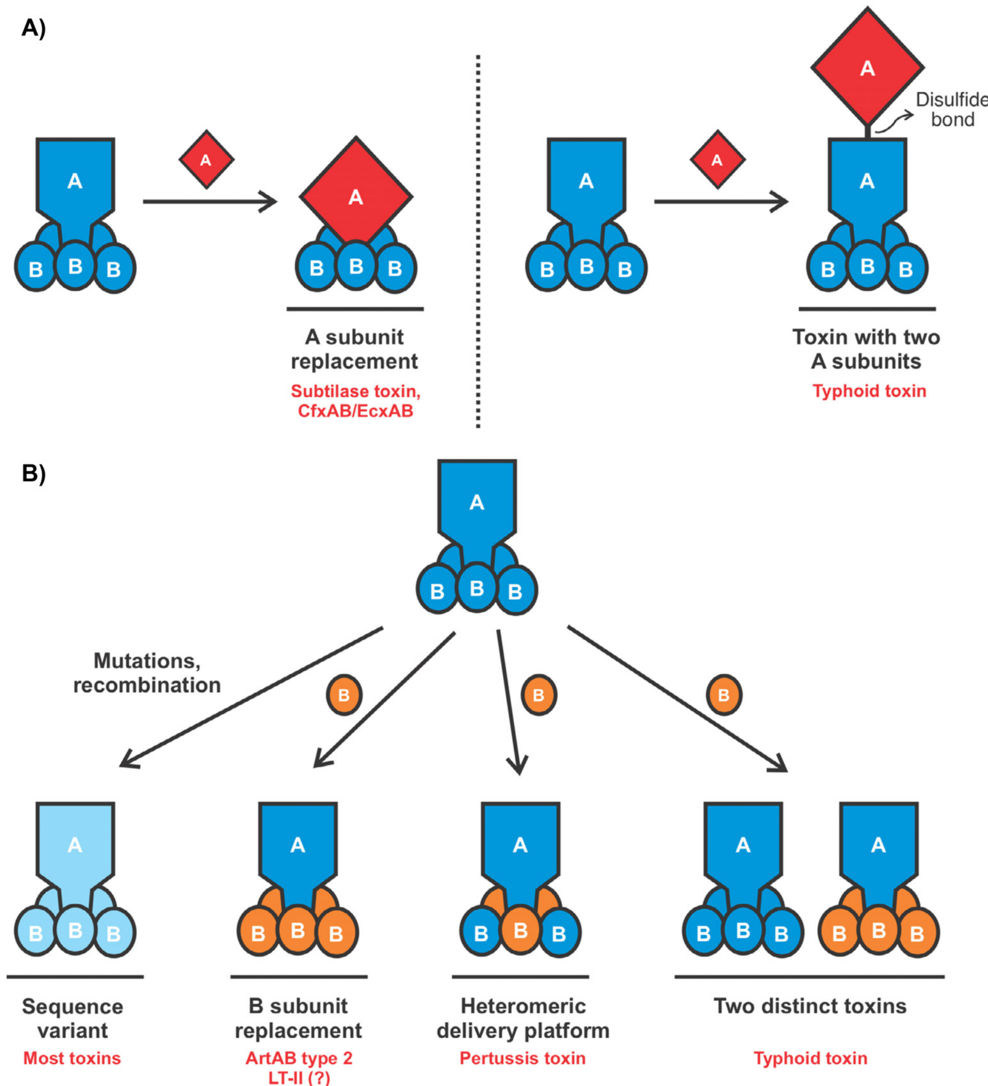

Fig. 1. Major mechanisms of evolutionary change for AB_5 toxins. (A) Mechanisms that incorporate a novel A subunit, thereby creating a new AB_5 toxin family. Left, a novel enzyme (red) evolves the capacity to interact with the B subunit of an existing AB_5 toxin (blue) and ultimately replaces the original A subunit. Right, a novel enzyme (red) evolves to form a disulfide bond with the A subunit of an existing AB_5 toxin (blue), resulting in a novel toxin with two active subunits. (B) Mechanisms of evolutionary diversification within AB_5 families. In most AB_5 families there is sequence diversity amongst the known members generated by the accumulation of mutations; in some cases homologous recombination amongst related toxin variants might further contribute to this sequence diversity. In other cases, diversity amongst different toxins appears to stem from larger-scale changes to the B subunit. This can include genetic duplication of the B subunit and/or horizontal acquisition of a novel B subunit, resulting in a new B subunit that can engage with the toxin's A subunit. Newly acquired B subunits can either (i) replace the original B subunit, (ii) interact with the original B subunit to form a heteromeric delivery platform, or (iii) co-exist with the original B subunit, with both B subunits interacting separately with the A subunit, resulting in two different versions of the toxin. Examples of toxins thought to have been subject to the various toxin evolutionary mechanisms are given in red text.

are collectively referred to as Shiga toxin-producing *E. coli* (STEC) [14]. For both of these lineages, Shiga toxin constitutes a central virulence factor and is thought to be directly responsible for the most severe disease outcomes commonly associated with infection. Shiga toxins can also be found in the genomes of assorted (and relatively rare) strains of other enteric bacteria, although the roles of these toxins in disease are generally not well understood [22]. Shiga toxins adopt a typical AB_5 structure featuring the A subunit perched atop a ring-shaped delivery platform comprised of five identical copies of the B subunit [4,23]. Shiga toxins are ribosome inactivating protein (RIP) toxins, a biological activity that has not been described for any other AB_5 toxin. Similar to other RIP toxins (most prominently ricin), the A subunit of Shiga toxin is an RNA N-glycosidase that depurinates a specific rRNA nucleotide near the 3′ end of the 28S rRNA [24–26]. This residue is located in the sarcin-ricin loop, a critical region of the 28S rRNA due to its interaction with elongation factors, and Shiga toxin activity effectively inactivates the ribosome, terminating translation. In addition to efficiently inhibiting protein synthesis, Shiga toxins also exert other biological effects on target host cells. The ribosome damage elicited by the A subunit instigates a proinflammatory host cell response mediated by MAP kinase signaling that has been dubbed the ribotoxic stress response [27–30]. Furthermore, the binding of the B subunit to surface receptors can also alter the physiological state of the host cell [14]. Ultimately, the cumulative effects of Shiga toxin can lead to cell death, but can also (or alternatively) trigger substantial signaling changes that are thought to contribute to disease, including, notably, the production and release of various cytokines [14,15,31]. The B subunit of Shiga toxin is generally specific for globotriaosylceramide (Gb3), a glycolipid that can be found on the cell surface. Gb3 levels vary significantly by tissue and cell type; cell types with relatively high Gb3 levels can be found within the kidneys, the central nervous system, and lymphoid tissues, for example [32]. Upon binding Gb3, Shiga toxin is endocytosed, undergoes retrograde transport to the ER, and then the A subunit undergoes ERAD-mediated translocation to the cytosol where it re-folds and can access its rRNA target [12,33]. Shiga toxin is not the only virulence factor that dictates the extent or nature of the virulence of the various STEC strains, but its activities are thought to be directly responsible for much of the symptomology associated with severe STEC infections. In STEC infections, Shiga toxin's effects can be seen both in the intestines at the local site of infection, as well as at distal systemic sites where tissues express high levels of Gb3 [34,35]. Locally, Shiga toxin-induced damage to the intestinal tissue contributes to the development of hemorrhagic colitis, which is commonly associated with STEC infections [36–38]. In more severe cases, Shiga toxin-induced kidney damage can lead to the development of hemolytic uremic syndrome (HUS), a life-threatening disease that accounts for the majority of acute cases of kidney failure in infants and young children [39–41]. More rarely, Shiga toxin can also induce severe neurological damage, which can be associated with sudden mor-

tality [42]. *S. dysenteriae* type 1 was the source of numerous severe pandemics in the 19th and 20th centuries, however its global burden has diminished in modern times, and currently represents < 5% of all shigellosis cases [43,44]. STEC remains an important pathogen worldwide; in the US alone, STEC causes over 250,000 infections per year, a significant percentage of which lead to serious complications [45].

2.2. Functional differences amongst Shiga toxins

Within the *E. coli* species there is substantial genetic variation amongst the Shiga toxins that are produced, and sequence variants can have functional differences that substantially affect the virulence of the encoding strain [46]. In *E. coli*, the Shiga toxin (stx) genes are encoded by lambdoid prophages, and the diversity and spread of Shiga toxin within *E. coli* has presumably been driven by its association with these mobile genetic elements [47,48]. The Shiga toxins produced by STEC are divided into two types, Stx1 and Stx2, which are antigenically distinct and are ~60% identical at the amino acid level (both the A and B subunits). Within each type, there are multiple sequence variants that are classified as subtypes. Three subtypes have been described for Stx1: Stx1a (the founding member) as well as the rarely encountered subtypes Stx1c and Stx1d [46]. Based on its amino acid sequence, the Shiga toxin produced by *S. dysenteriae* belongs within the Stx1a subtype, but for historical reasons it is generally referred to as a distinct Shiga toxin type. Stx2 is more heterogenous and consists of numerous subtypes including Stx2a-Stx2f, as well as a handful of recently identified subtypes that have rarely or never been isolated from clinical samples [46,49–52]. Interestingly, it is not uncommon for a STEC strain to encode multiple different Stx types/subtypes [53–57]. Some subtypes are much more commonly associated with human infection than others; for example, the 1a, 2a, 2c are relatively common, whereas the remaining subtypes range from uncommon to vanishingly rare [56–58]. Within this framework, the proportion of infections caused by STEC encoding the various subtypes varies by location and over time.

The characteristics of the various toxin subtypes and their impact on virulence has been evaluated by assessing their properties *in vitro,* in cell culture or animal models of infection, as well as through clinical/epidemiological studies. It is evident from studies that correlate disease outcome with Stx subtype in patients infected with STEC that strains encoding the most common Stx2 subtypes (2a, 2c) are more frequently associated with severe disease outcomes such as HUS than are strains encoding Stx1 toxins [54,56,59]. This observation has been supported by animal models of infection and of intoxication, suggesting that this correlation is due to the toxin itself and not (solely) genetic linkages to other virulence factors [60,61]. Indeed, purified Stx2a exhibits substantially greater toxicity than Stx1 in different animal models (such as mice, gnotobiotic pigs, and primates) and using various routes of toxin administration (oral, intravenous, intraperitoneally) [61–64]. Interestingly, patient studies indicate that strains encoding only Stx2a are more likely to elicit severe disease than strains encoding both Stx1a and Stx2a [54,56,65–69]. This suggests that Stx1 production dampens the effects of the more potent Stx2 toxin, a hypothesis that is supported by studies using animal models [70,71]. The Stx2 subtypes 2c and 2d, which exhibit only a few amino acid changes relative to Stx2a, are also associated with a propensity to cause severe disease and increased potency in animal models of infection/intoxication [55,72,73]. Stx2d is noteworthy because it is known to be activated substantially by proteolytic removal of the two C-terminal residues of the A subunit by elastase, an enzyme found in intestinal mucus (Fig 2A) [73–75]. Although less is known about the activities of the rarer Shiga toxin subtypes, it has generally been observed that they are associated with milder disease outcomes [53,58,76,77]. Stx1 and Stx2 have different potencies toward cultured cell lines, with Stx1 showing increased toxicity toward some cell types, including the Vero cell line used in numerous Shiga toxin studies [75,78,79]. However, Stx2 has been observed to have a greater potency for human renal microvascular endothelial cells (HRMEC) than Stx1, highlighting that the relative potencies of these toxins are cell-line specific [46,79].

2.3. Mechanisms of Shiga toxin functional diversification

Differences in the biochemical and functional properties between the various Shiga toxin types/subtypes have been observed, although it is not completely clear how these factors culminate in the observed differences *in vivo*. In general, the functional differences between toxin types appear to be due to sequence differences in the B subunit, or sequence differences in the C-terminal residues of the A subunit that protrude through the B-subunit pore and reside on the binding face of the holotoxin. This has been shown through mutational analyses and through using Stx1/Stx2 hybrid (or chimeric) toxins featuring the A subunit from one toxin type in complex with a B subunit of the other, where the functional differences track with the B subunit and not the A subunit [62,80,81]. There is some evidence that the A subunits from different Shiga toxin types can differ functionally, however this is not as well established [82,83]. Numerous studies have revealed differences in the interactions of different Shiga toxins with glycan receptors. One noteworthy subtype in this regard is Stx2e, which shows a binding preference for globotetraosylceramide (Gb4) over Gb3 [84] (Fig 2A). STEC producing Stx2e is found mainly in pigs, where Gb4 is highly expressed on epithelial cells of the intestinal ileum and kidney [85,86]. Stx2e-encoding STEC are rarely isolated from the human population, but represent the dominant STEC in pigs, where they cause of severe edema and neurological impairment [87]. Interestingly, Stx1a exhibits greater affinity for Gb3 than does the more toxic Stx2a [61]. Importantly, however, Shiga toxin's interaction with receptors is complex and is impacted by factors such as the fatty acid content of Gb3, its local density, and the composition of the local membrane; differences in such factors have been shown to differentially impact Stx1 and Stx2 [88–98]. Shiga toxin's B subunit has been shown to have as many as three glycan binding sites, leading to 15 total sites per toxin, and multivalent interactions also have important impacts on toxin binding [91,94,95,99,100]. Structural studies show that there are differences in the nature of the ligand binding pockets between Stx1 and Stx2 [23]. How differences in receptor interactions between Stx1 and Stx2 translate into the observed virulence differences is not clear. One possibility is that Stx1's greater affinity for Gb3 (as well as Gb4) leads to the toxin being sequestered by cells that express lower levels of Gb3, and thus less toxin reaches cells/tissues rich in Gb3 that are associated with severe disease, such as renal endothelial cells [101]. This is supported by animal models that show that Stx1 and Stx2 toxins exhibit differences in tissue tropism, including a more specific targeting of the kidneys by Stx2 and a reduced serum half-life for Stx1 [78]. One factor that might contribute to this phenomenon is the kinetics of Gb3 binding, as it has been observed that Stx1 exhibits a faster on rate than Stx2, but Stx2 exhibits a slower off rate, which could improve the efficiency of its uptake into Gb3-rich cells [95].

3. The cholera toxin family

3.1. Cholera toxin

Cholera toxin (Ctx) is the predominant virulence factor for the pathogen *Vibrio cholerae*, the etiological agent of cholera, a disease that afflicts ~3 million people annually, resulting in ~100,000 deaths [102]. It adopts the canonical AB_5 structure featuring the A subunit, CtxA, in complex with a homopentamer of the delivery subunit, CtxB [103]. *V. cholerae* is a non-invasive, enteric pathogen that predominantly infects the lumen of the small intestines [104]. Cholera toxin is produced and secreted at high levels from this location, and subsequently enters and intoxicates the neighbouring intestinal epithelial cells [16]. CtxA is an ADP-ribosyltransferase that covalently modifies the α subunit of the G_s protein signaling protein, inhibiting its GTPase activity and thus locking it in its active state [105–107]. This leads to a substantial activation of

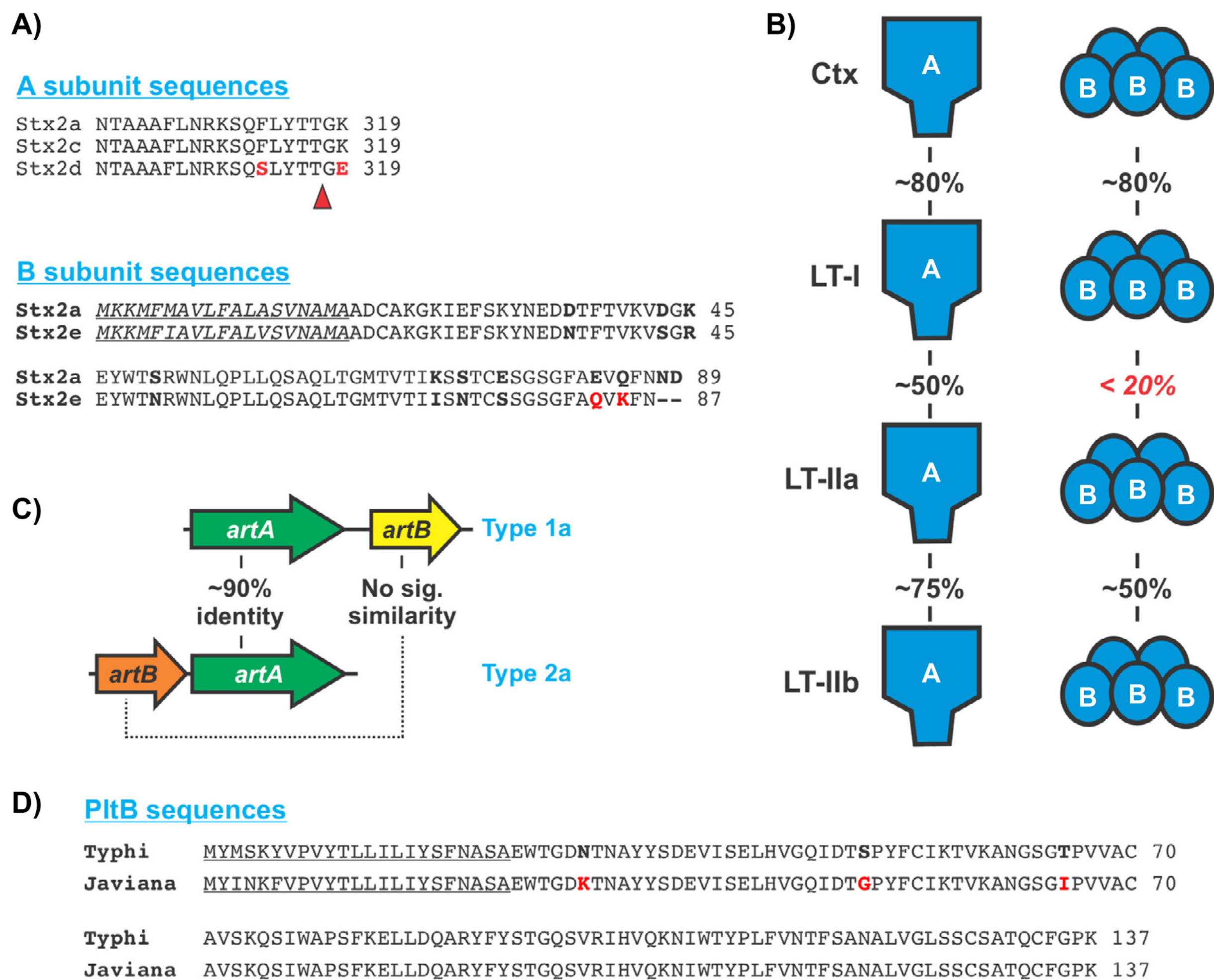

Fig. 2. Examples of sequence diversity within AB_5 toxin families. (A) Sequence differences amongst type 2 Shiga toxin subtypes that confer unique properties. Top: Amino acid sequence alignment showing the C-terminal 19 amino acids of the A subunits of the 2a, 2c, and 2d subtypes. The A subunit of the 2d subtype is activated by proteolytic removal of the final two amino acids by elastase (cleavage site shown with red triangle), an enzyme found in the intestinal lumen. This processing activates Stx2d, leading to increased toxin potency. Unlike Stx2d, other subtypes such as 2a and 2c are not activated by elastase; this difference is thought to stem from two amino acid differences at the C-terminus of the A subunit (red). Bottom: Amino acid sequence alignment comparing the B subunits of the 2a and 2e subtypes. Unlike all other known subtypes, Stx2e preferentially binds Gb4 rather than Gb3. Sequence differences that might contribute to this are in bold, and two residues that have been shown to be particularly important for this are highlighted in red. **(B)** Illustration showing the extent of sequence similarity amongst some major cholera-family toxin types. Percent amino acid sequence identity values amongst the indicated pairs of A or B subunits are shown. **(C)** Genome diagram and DNA sequence comparisons between type 1 and type 2 ArtAB toxins. These toxins have very similar A subunits, but their B subunits are very distantly related and share no significant DNA sequence similarity. Coupled with their reversed gene order, this suggests that one subtype evolved from the other by horizontal acquisition of a new B subunit, which supplanted the original (see B subunit replacement in Fig. 1B). **(D)** Amino acid sequence comparison of the PltB sequences of the typhoid toxins encoded by *S.* Typhi and *S.* Javiana. These proteins have only three amino acid differences (bold/red), but exhibit very different potencies and glycan binding properties. In panels (A) and (D), underlined residues represent sequences removed during Sec-mediated secretion to the periplasm.

the downstream effector adenylate cyclase, resulting in increased concentrations of the second messenger cAMP. Most relevant for the pathogenesis of *V. cholera*, elevated cAMP levels culminate in the activation of ion channels that efflux chloride ions, triggering an accompanying flow of water out of the cell [16]. Ultimately, the actions of cholera toxin cause considerable fluid loss in the form of the “rice-water diarrhea” that is characteristic of cholera, which can lead to the rapid onset of dehydration in patients [102]. The excreted fluid contains large numbers of *V. cholerae*, and it is thought that cholera toxin benefits this bacterium by facilitating transmission [108]. Recently, it was shown that cholera toxin also contributes to *V. cholerae* growth within the gastrointestinal (GI) tract by creating an iron-depleted environment that confers a selective growth advantage due to *V. cholerae*’s capacity to acquire haem and fatty acids from the host under these conditions [109]. Cholera toxin gains access to target cells through the binding of CtxB to glycans present on the cell surface. CtxB exhibits very high affinity for the ganglioside monosialotetrahexosylganglioside (GM1), which is well established to be an important cholera toxin receptor [1,110–112]. More recently, numerous studies have identified fucosylated glycoproteins as functional cholera toxin receptors that bind with a lower affinity at a distinct binding site on the CtxB subunit [113–116]. Accumulating evidence suggests that, in addition to GM1, these glycoprotein receptors also appear to be relevant for cholera toxin pathogenesis. Interestingly, CtxB is able to recognize histo-blood group antigens via this secondary binding site with a preference for H determinant associated with the O blood type, providing a potential explanation for the increased risk for severe disease observed for those with this blood type [1,117–119].

Amongst *V. cholerae* strains that carry cholera toxin, the toxin’s amino acid sequence is well conserved. The sequence diversity that has

been observed is limited to the B subunit, with sequence variants differing by only a few amino acids. The *ctx* genes are encoded on a filamentous prophage known as CTXφ [120] and Ctx-producing *V. cholerae* strains from the O1 serogroup can be classified into biotypes based on the nature of this prophage [121]. The two major biotypes are dubbed "classical" and "El Tor", although more recently strains that represent hybrids of these two biotypes have become more prevalent [122–124]. The CtxA sequence is identical between the classical and El Tor biotypes, but there are two amino acid residues that differ in their CtxB sequences. The functional implications of this sequence variation are not fully understood, but it has been proposed that these amino acid differences affect receptor binding at the secondary binding site, impacting their association with blood group antigens [1]. In addition to the common El Tor and Classical genotypes, several other *ctxB* genotypes have been found in *V. cholerae*, many of which are rare [123,124]. These stem from different combinations of amino acid changes to a handful of residues, the impacts of which are not well understood [123,124]. One recent study provided evidence that in the *ctxB7* allele of the Haitian variant of O1, a single amino acid change within the CtxB signal sequence might improve the efficiency of its secretion/processing, contributing to the increased toxin production that has been reported for these strains [125–127].

3.2. Heat labile toxins

Toxins that share a clear evolutionary, structural and functional relationship with cholera toxin are also found within the *E. coli* lineage, and are referred to as heat-labile toxins [128]. *E. coli* is a genetically and ecologically diverse species, and different lineages or strains of this species have been found to encode genetically divergent heat-labile toxins. Heat labile toxins have been grouped into 4 types: type I (LT-I), type IIa (LT-IIa), type IIb (LT-IIb) and type IIc (LT-IIc); there is also significant sequence diversity within these types [129,130]. LT-I toxins are commonly encoded on plasmids carried by *E. coli* of the ETEC (enterotoxigenic *E. coli*) pathotype, a major cause of traveller's diarrhea [128]. LT-I is genetically similar to cholera toxin, exhibiting ~80% amino acid sequence identity compared to cholera toxin (both the A and B subunits), and the two toxins adopt very similar structures (Fig 2B) [3,101]. In the context of ETEC infections, LT-I elicits similar symptomology as cholera toxin (although the symptoms are generally less severe) and it does so using an analogous mechanism involving GM1-mediated toxin uptake into intestinal epithelial cells via its B subunit, and ADP-ribosylation of $G\alpha_s$ via its A subunit [128,131]. The primary ligand binding pocket of LT-I is very similar to that of CtxB, and GM1 is the dominant ligand recognized by both toxins [3,111,132,133]. However, LT-I has been observed to have less stringent specificity for GM1 and also recognizes certain related gangliosides, albeit with a lower affinity [133–135]. Like cholera toxin, LT-I also binds glycoproteins and blood group antigens at a secondary site [136–139]. Contrary to cholera toxin's preference for blood group H antigens, LT-I appears to prefer binding to blood group A or B antigens [137,139–142]. Despite LT-I's similarity to cholera toxin, the two toxins are not functionally equivalent [143–145]. The B subunit-driven differences in glycan binding described above are likely a major driving force for this, but activity differences in the A subunits of cholera toxin and LT-I have also been noted [144]. Furthermore, a recent study found that cholera toxin is more efficiently unfolded in the ER by PDI than is LT-1 due to differences in the holotoxin structure, providing another potential explanation for its increased potency [146].

3.3. Functional differences amongst heat-labile toxins

Type II heat labile toxins adopt a similar overall structure compared to LT-I and cholera toxin, and are also able to ADP-ribosylate $G\alpha_s$ [147–149]. However, *E. coli* strains encoding these toxins are rarely associated with human infection, and it is not clear if they represent *bona fide* virulence factors within a human host. Strains encoding type II heat-labile toxins, which are encoded within prophages, have been isolated from various animal hosts [130,150–153]. The ecology and the relevant environmental reservoirs of *E. coli* strains that encode the various subtypes of LT-II toxins is not clear. When compared to cholera toxin and LT-I, the A subunits of LT-IIa, LT-IIb and LT-IIc are all ~50–60% identical. The B subunits of these toxins, by contrast, share little or no significant sequence similarity with cholera toxin and LT-I (Fig 2B). Despite this, the B subunits of LT-II toxins exhibit a surprising extent of structural similarity to the cholera toxin and LT-I B subunits [147,148]. There are, however, substantial differences in the nature of the A-B subunit interactions between LT-I and LT-II toxins, and coupled with their highly divergent B subunit sequences, this suggests that type I and type II heat labile toxins are distantly related [147]. Alternatively, the two types of heat labile toxins might be the product of divergent B subunits evolving to form a toxin with a similar A subunit. For example, the LT-II toxins could have emerged as a result of a horizontally acquired LT-II-like B subunit supplanting the original B subunit of an LT-I toxin (see Fig. 1B, "B subunit replacement") [154]. This would be analogous to what has been proposed for the two types of ArtAB toxins (described below). In most models of intoxication, type II heat labile toxins are less potent than LT-I, with the notable exception of the Y1 mouse adrenal cell line [151,155]. The B subunits of type-II heat labile toxins also recognize gangliosides, but with different specificity compared to LT-I and cholera toxin. For example, cholera toxin strongly binds GM1 but does not recognize GD1a, whereas LT-IIb exhibits the reverse binding specificity, showing high affinity for GD1a but no capacity to bind GM1 [133,156]. In addition to being different from LT-I, the different subtypes of type II heat labile toxins also differ from one another. The A subunits and B subunits of type II LT-IIa, IIb and IIc share ~70% and ~50% amino acid sequence identity, respectively [130]. LT-IIa, IIb and IIc heat labile toxins exhibit distinct glycan-binding preferences, which presumably confer these toxin types with different functional characteristics in the hosts they have evolved to target [148,151,157–160].

3.4. CfxAB/EcxAB toxins

Certain rare *E. coli* and *Citrobacter* strains encode a B subunit with >70% amino acid sequence similarity to LT-I and cholera toxin, but do not encode a homolog of the cholera toxin A subunit [161]. In place of the *ctxA* gene, these strains encode a putative metzincin family metalloprotease immediately upstream of the cholera toxin-like B subunit [161–163]. It has been demonstrated that these two proteins assemble into a canonical AB_5 toxin architecture, and this novel AB_5-type toxin is able to induce physiological changes to Chinese hamster ovary cells [162,163]. Beyond its structure, very little is known about this toxin, which has been named CfxAB/EcxAB (depending on the origin species). However, this toxin is a noteworthy example of AB_5 toxin evolution, wherein an enzyme with no prior connection to AB-type toxins appears to have evolved to associate with the B subunit of an existing AB_5-type toxin, resulting in a unique AB_5 toxin with a novel enzymatic activity (see Fig. 1A, "A subunit replacement").

4. The pertussis toxin family

4.1. Pertussis toxin

Pertussis toxin is a major virulence factor for *Bordetella pertussis*, a human-adapted bacterial pathogen that is the causative agent of whooping cough (also known as pertussis), a disease that affects an estimated ~24 million people each year, resulting in over 160,000 deaths [164]. *B. pertussis* differs from most other bacteria that produce well-characterized AB_5 toxins with respect to both phylogeny and site of infection: most AB_5 toxin producers are Gammaproteobacteria that infect the GI tract, whereas *B. pertussis* is a Betaproteobacterium that infects the respiratory tract. Whooping cough disproportionately affects young children

(although vaccine regiments have shifted pertussis' population dynamics in recent years), and the salient feature of the disease is developing intermittent coughing fits that can be severe and debilitating, often persisting for weeks, or longer [165–167]. In infants, *B. pertussis* infections can produce more severe disease, including pulmonary hypertension and pneumoniae, which can be fatal [168]. The pathogenesis of *B. pertussis* requires the coordinated effects of numerous virulence factors, however there is strong evidence that pertussis toxin is a central factor with respect to both immune evasion and eliciting disease symptomology [18,19,169–172]. Pertussis toxin's delivery platform is promiscuous, and is able to bind diverse glycans and thus target numerous assorted cell types. Its active subunit - an ADP-ribosyltransferase that modifies the activity of the α subunit of G proteins from the $G_{i/o}$ family - affects cell signaling in a manner that can trigger a wide range of physiological changes [173–175]. Accordingly, pertussis toxin is thought to elicit wide-ranging effects on its host during infection, however the relevance of the assorted phenotypes that have been observed in cell culture or animal models for human infection is not always clear. Through its capacity to modulate host cell signaling, pertussis toxin is an important aspect of how *B. pertussis* evades the immune system. It has various immunomodulatory effects such as altering cytokine signaling, impacting immune cell migration, suppressing antibody responses, and more [176–180]. Furthermore, there is strong evidence that pertussis toxin is partially or entirely responsible for many of the severe and/or systemic symptoms associated with pertussis, such as histamine sensitisation, leukocytosis, and pulmonary hypertension [18,171,181,182]. It is not fully understood what causes the characteristic cough of whooping cough, but there is evidence that pertussis toxin contributes to this as well, including recent work that has shed light on this subject using a mouse coughing model [171,183–185].

4.2. Other pertussis family toxins: ArtAB and EcPlt

Genes with significant sequence similarity to pertussis toxin subunits can be found in the genomes of numerous other bacterial species, including certain strains of *E. coli, S. enterica* and assorted *Yersinia* species [154,186,187]. They are generally encoded within mobile genetic elements such as prophages, and thus have an irregular phylogenetic distribution that includes only specific clades or strains of a given species. Pertussis family AB_5-type toxins that share many structural and functional similarities with pertussis toxin have been identified and characterized from *E. coli* (dubbed EcPlt) and *Salmonella* (dubbed ArtAB) [186,188,189]. EcPlt and ArtAB exhibit functional differences compared to pertussis toxin, but both toxins have been shown to be capable of ADP-ribosylating G protein subunits [186,189,190]. These toxins have not yet been thoroughly studied, however, and little is known about their functions or their roles in disease. Despite their different names, EcPlt and ArtAB appear to be similar enough that they would be better thought of as different types or subtypes of the same toxin. Indeed, within both the *E. coli* and the *Salmonella* lineages there is substantial sequence diversity amongst the ArtAB/EcPlt toxins they encode [154,186]. The assorted ArtAB type toxins in *Salmonella* differ primarily in their B subunit sequences, with some of the most variable amino acids lying within the glycan binding pockets, suggesting that different sequence variants likely have different glycan binding properties [154]. *Salmonella* ArtAB toxins have been divided into two types; type 1 and type 2. Interestingly, the A subunits of type 1 and type 2 ArtAB share substantial nucleotide and amino acid sequence similarity (>90% identity in some cases), but their B subunits are highly divergent, exhibiting <30% amino acid sequence identity and no significant nucleotide sequence similarity (Fig 2C) [154]. Based on this finding, it has been proposed that type 2 ArtAB toxins are the result of a horizontally-acquired B subunit supplanting the original *artB* gene, yielding a new toxin with the same A subunit but a new B subunit (see Fig 1B, 2C) [154]. In support of this hypothesis, type 2 ArtAB toxins have a reversed gene order (*artBA* instead of *artAB*), and remnants of a transposase can be found upstream of the type 2 *artB* gene. All pertussis toxin family B subunits studied to date bind sialic acid terminated glycans, however there is remarkable diversity amongst different members of this class of B subunit with respect to their genetic sequences and their glycan-binding specificities [191,192]. Diverse pertussis family B subunits have been identified as the delivery components of pertussis toxin, ArtAB/EcPlt-like toxins, subtilase toxin and typhoid toxin (described below), as well as appearing as "orphan" B subunits without a genetically-linked A subunit [191,193,194]. The binding specificities of these different family members vary with respect to both the nature of the sialic acid and the underlying sugar chemistry that they recognize [191,192]. In general, pertussis family B subunits exhibit the capacity to recognize numerous assorted sialoglycans *in vitro*, and can generally bind both glycoproteins and glycolipids. It is likely that the spatial arrangement of compatible sialoglycans is a major factor that dictates receptor specificity *in vivo*, with receptors that permit multiple binding sites on the pentameric delivery platform to bind simultaneously being strongly preferred.

4.3. The unique heteropentameric delivery platform of pertussis toxin

Pertussis toxin is exceptional within the pertussis family of toxins, and indeed amongst all AB_5 toxins, due to the more elaborate nature of its delivery platform. Indeed, all other known AB_5 toxins have a homopentameric delivery platform, whereas the pertussis toxin B pentamer is composed of four different polypeptides. The pertussis toxin locus consists of five genes that encode toxin subunits (one active subunit and four delivery subunit genes) as well as a nine-gene operon that encodes a type IV secretion system that appears to be dedicated exclusively to pertussis toxin secretion [173,195]. The 14-gene pertussis toxin locus therefore stands in stark contrast to the two-gene loci that encode many AB_5 toxins. It is likely that pertussis toxin is the culmination of a substantial evolutionary process that began with a genetically simpler toxin similar to the ArtAB/EcPlt toxins. Pertussis toxin's unique heteromeric delivery platform consists of one copy of the proteins S2, S3 and S5 and two copies of S4 [5]. The S2 and S3 subunits share approximately 70% amino acid sequence similarity, and are the closest relatives of the B subunits from other pertussis-family toxins. The S4 and S5 subunits exhibit little-to-no significant sequence similarity to one another or to S2/S3. However, despite their divergent sequences, S2-S5 all share structural similarities and S4 and S5 have structural features also observed in S2/S3 that are absent from other AB_5 toxin B subunits. This suggests that all 4 pertussis toxin B subunits likely evolved from the same parental protein [5]. In the pertussis toxin structure, the S2 and S3 subunits each associate with S4 to form two dimers (S2-S4 and S3-S4), which are linked together by the S5 subunit. This arrangement creates a unique delivery platform architecture that lacks the five-fold symmetry present in other AB_5 toxins. S2 and S3 share a conserved binding pocket with other members of the pertussis family of toxins, including conserved sequence features such as a serine residue that is ubiquitous (and essential for binding at this site) amongst divergent pertussis family B subunits [188,196–198]. In addition to this binding site, S2 and S3 contain a discrete ~100 amino acid N-terminal Aerolysin/Pertussis Toxin (APT) domain that structurally resembles domains found in eukaryotic lectins [5,196]. APT domains are not found in any other known AB_5 toxins, including other pertussis-family B subunits. The S2 and S3 APT domains, which differ from one another, contain multiple putative glycan binding sites that have different glycan binding specificities [175,199]. Neither S4 nor S5 have established glycan binding sites, and they are thought to serve a predominantly structural role [5,196]. The hypothetical evolution of the unusual pertussis toxin B pentamer from an ArtB-like ancestor would therefore have involved several steps including (i) acquiring an APT domain, (ii) multiple genome duplication events, (iii) sequence changes in S2/S3 to confer each with unique glycan binding properties, (iv) substantial sequence changes in the S4/S5 subunits to alter their structure and primary function. From a broad viewpoint, the major outcome of this evolutionary process is that per-

tussis toxin has multiple glycan binding pockets that all differ from one another, and which have different receptor specificities; this contrasts with all other AB_5 toxins, which have five identical copies of each glycan binding site.

4.4. Subtilase toxin

Amongst the many diverse pertussis toxin-like B subunits that have been identified, the vast majority form toxins with ADP-ribosyltransferase active subunits. A remarkable exception to this is subtilase toxin (SubAB), an AB_5 toxin that is encoded by certain STEC serovars that have been associated with outbreaks of HUS [193]. The delivery subunit of subtilase toxin (SubB) is noteworthy due to its strong specificity for *N*-glycolylneuraminic acid (Neu5Gc)-terminated sialoglycans over those terminating in *N*-acetylneuraminic acid (Neu5Ac) sialic acids, which is unique amongst pertussis family B subunits characterized to date [191,197]. SubB is, however, structurally-similar to other pertussis family B subunits and utilizes a similar sialoglycan binding pocket [197]. The A subunit of subtilase toxin (SubA), by contrast, is a serine protease that is structurally and functionally unrelated to the ADP-ribosyltransferase A subunits of the pertussis toxin family [20,193,200,201]. SubA targets and proteolytically cleaves Binding immunoglobulin Protein (BiP), an ER chaperone that is essential for maintaining ER integrity though its roles in protein folding, as well as cell signaling via the unfolded protein response [193,202]. Subtilase toxin can therefore be cytotoxic, and it has also been reported to have immunomodulatory effects [193,201,203–205]. Due to its unique enzymatic activity, subtilase toxin is categorized as a unique AB_5 toxin family [2]. It is not impossible that subtilase toxin evolved before pertussis family toxins, and served as the evolutionary precursor to this family. However, the diversity and broad distribution of pertussis family toxins suggests that it likely evolved first, and that subtilase toxin might have emerged as a result of a SubA-like serine protease acquiring the capacity to interact with the delivery subunit of a pertussis family toxin, supplanting its ADP-ribosyltransferase subunit, resulting in a novel AB_5 toxin family with a discrete enzymatic activity (see Fig. 1A, “A subunit replacement”).

5. The typhoid toxin family

5.1. Overview

Typhoid toxin is a prominent virulence factor for the human-adapted pathogen *Salmonella enterica* serovar Typhi, the bacterium that causes typhoid fever. According to Global Burden of Disease estimates, there are ~6–14 million cases of typhoid fever each year, resulting in ~50,000–200,000 deaths [206]. The precise role that typhoid toxin plays in *S*. Typhi pathogenesis and the development of typhoid fever is not yet clear. Human volunteer infection studies suggest that typhoid toxin does not have a substantial impact in the early stages of *S*. Typhi infection [207]. However, *S*. Typhi causes a prolonged systemic infection that elicits a diverse range of complications, and animal intoxication studies suggest that typhoid toxin elicits certain symptoms that are associated with severe typhoid fever [194,208–210]. Animal infection studies, which are complicated by the fact that *S*. Typhi is a human-adapted pathogen that does not infect laboratory animals, have provided evidence that typhoid toxin plays a role at the systemic stage of infection [211–213]. A small but significant proportion of *S*. Typhi infections result in a long-term persistent infection, and these “carriers” are thought to be important for *S*. Typhi transmission [214–216]. It has been proposed that typhoid toxin is important for *S*. Typhi to reach and/or persist at sites of long-term carriage (typically the gallbladder) [217]. Typhoid toxin is a very unusual AB_5 toxin that is unique in several ways. For example, all other AB_5 toxins are thought to be produced and secreted by extracellular bacteria, whereas typhoid toxin is only produced by *S*. Typhi that reside within host cells [194,218]. Salmonellae are facultative intracellular bacteria that actively invade host cells, sculpting and living within a vacuolar niche dubbed a *Salmonella* Containing Vacuole (SCV). *S*. Typhi expresses typhoid toxin genes in response to cues present within the SCV, and the toxin is subsequently secreted from the bacterium using a unique secretion mechanism that appears to require environmental conditions present within the SCV [9,218–220]. The B subunit of secreted toxin then engages with a host cell receptor within the SCV, which was recently identified to be the cation-independent mannose-6-phosphate receptor (M6PR), triggering a toxin trafficking pathway that culminates in typhoid toxin being exocytosed from the cell into the extracellular space [221–223]. From this location, typhoid toxin adopts the canonical AB_5 toxin biological program, and binds specific glycosylated receptors on the surface of target host cells, which leads to toxin uptake, trafficking, and ultimately cellular intoxication [9,209,223]. This is a remarkably complex biological program for a toxin, and it creates a scenario where intracellular bacteria can potentially intoxicate nearby cells without damaging the host cell in which they reside. Although it is not clear how this plays out *in vivo*, having the ability to manipulate the biology of surrounding cells (such as immune cells) from a safe haven could confer *S*. Typhi with obvious advantages in the context of establishing a long-term infection.

5.2. Typhoid toxin’s unique A_2B_5 architecture

In addition to its unique biological program, the composition of typhoid toxin is also distinct amongst AB_5 toxins. In fact, typhoid toxin is not an AB_5 toxin, it is an A_2B_5 toxin. However, it has all of the structural and functional hallmarks common to $A_{(1)}B_5$ toxins, and is thus grouped in this toxin family. Typhoid toxin can be thought of as having a pertussis toxin-like AB_5 toxin core that is structurally similar to ArtAB [188,209,224]. The A subunit of this AB_5 core, PltA, forms a disulfide bond with a second active subunit, CdtB, creating the final A_2B_5 holotoxin (see Fig. 1A, “toxin with two A subunits”) [209]. CdtB does not directly interact with the delivery platform and its incorporation into the toxin strictly relies on its covalent association with PltA. It is not known whether *S*. Typhi secrete toxin complexes that lack CdtB, but *cdtB* is expressed from a distinct promoter and its expression is regulated in a subtly different manner compared to the other typhoid toxin genes, suggesting this could be the case [218]. The two active subunits of typhoid toxin have seemingly unrelated activities. Like other members of the pertussis toxin family, PltA is an ADP-ribosyltransferase. It is catalytically active, however, the biologically relevant target(s) of PltA have not yet been identified, and no definitive PltA-dependent intoxication phenotypes have been identified [209]. *S*. Typhi CdtB exhibits clear homology to the enzyme of the same name which serves as the active subunit of the AB_2-type toxin cytolethal distending toxin (CDT) [209,225,226]. CdtB is a DNase that introduces single-stranded and/or double-stranded breaks into host cell genomic DNA, leading the G_2M cell cycle arrest and/or cell death depending on dose and cell type [209,227,228]. Strains or purified toxins featuring a mutation to an essential catalytic residue of CdtB do not exhibit any detectable intoxication phenotypes in cell culture and animal infection/intoxication models [209,210,229]. This indicates that CdtB is responsible for all typhoid toxin activities that have been identified to date. Both CDT and ArtAB toxin can be found in assorted (rare) *Salmonella* genomes, and it has been proposed that typhoid toxin evolved within the *Salmonella* genus through the amalgamation of these two toxins [188]. The critical step in this evolutionary process would have been the accumulation of mutations that generated appropriately positioned cysteine residues for PltA-CdtB disulfide bond formation. Typhoid toxin’s unusual A_2B_5 architecture represents a simple yet elegant mechanism to expand the functionality of a toxin, enabling a single delivery platform to simultaneously deliver two different activities to target cells.

5.3. The two versions of typhoid toxin: diversification by delivery

A third unique aspect of typhoid toxin biology is that *S*. Typhi produces two different versions of typhoid toxin in which the same active subunits (PltA and CdtB) form toxins with either PltB or PltC homopentamers (see Fig. 1B, "two distinct toxins") [194]. PltB and PltC are both pertussis toxin family B subunits, but they are distantly related and share <30% amino acid sequence identity. *pltB* is encoded at the typhoid toxin locus alongside *pltA* and *cdtB* and presumably represents the original form of the toxin that evolved. *pltC*, which is genetically similar to the *artB* gene from *S*. Typhimurium serovar DT104, is encoded at a distant *S*. Typhi genomic locus downstream of a degraded *artA* pseudogene [154,194]. The genetic evidence therefore strongly suggests that PltC is derived from an *artB* gene that was co-opted to serve as a alternate typhoid toxin delivery subunit. Several lines of evidence indicate that *S*. Typhi produces two distinct toxins, and not heteropentameric PltB/PltC toxin complexes, including (i) PltB and PltC both efficiently assemble typhoid toxins in the absence of the other, (ii) the structures of both the PltB and the PltC typhoid toxin have been solved, and based on these structures it is predicted that electrostatic repulsion between PltC and PltB would deter the formation of heteromeric pentamers, and (iii) interaction studies using both heterologous over-expression systems and immunoprecipitation from typhoid toxin-expressing *S*. Typhi indicate that PltB and PltC strongly interact with PltA/CdtB, but not with each other [194,209,224]. *S*. Typhi produces both versions of typhoid toxin in cell culture infection models, as well as when grown *in vitro* in medium designed to promote typhoid toxin expression [194]. Interestingly, in the conditions tested to date, *S*. Typhi appears to produce higher levels of the PltC version of typhoid toxin, which is consistent with *in vitro* interaction studies and structural data that indicate that PltC is able to out-compete PltB for binding to PltA [194,224]. Importantly, however, *pltB* and *pltC* are expressed from different promoters that are regulated by different two-component regulatory systems, and their expression is also known to be affected differently by certainly metabolic cues [194]. This indicates that *S*. Typhi is able to adjust the relative expression levels of the two typhoid toxin B subunits in response to its environment, thereby adjusting the proportion of PltB and PltC typhoid toxins produced.

PltB and PltC typhoid toxins exhibit different properties in both cell culture and animal model systems [194]. Notably, the PltB toxin elicits greater morbidity and mortality in a mouse intoxication model, but administration of the PltC toxin results in a greater loss in circulating white blood cells, suggesting that the different toxins preferentially target different cell/tissue types *in vivo*. Furthermore, unlike the PltB toxin, the PltC typhoid toxin remains associated with the SCV in cell culture models of infection and is not exocytosed from the cell [194]. In light of recent findings, this suggests that PltC may be unable to productively interact with M6PR to trigger the formation of exocytic vesicles [221]. The significance of this difference in trafficking is not yet clear, but it is possible that the PltC toxin is exocytosed by other cell types. Alternatively, PltC toxin could be maintained within the SCV, which might be beneficial to *S*. Typhi. For example, this pool of toxin could protect *S*. Typhi from immune detection in the event that the host cell in which they reside lyses. *In vitro*, PltB binds diverse sialic acid terminated glycans, generally preferring those in which the terminal sialic acid is linked to a Gal-GlcNac disaccharide via an α2–3 or α2–6 linkage [191,198,209]. It exhibits exquisite specificity for glycans terminated in the sialic acid Neu5Ac over analogous Neu5Gc-terminated sialoglycans [198,209], which is noteworthy given that *S*. Typhi is a human-adapted pathogen and unlike most other mammals, humans are unable to produce Neu5Gc. PltB has also been reported to preferentially recognize chemically modified sialic acids, and to exhibit enhanced affinity for multiantennal sialoglycans that enable multiple PltB binding sites within the homopentamer to engage the same glycan [210,230]. Less is known about the binding specificity of PltC, however it also recognizes diverse sialic acid-terminated glycans. Its glycan-binding preferences have been shown to be different from those of PltB, most notably in that PltC is able to efficiently bind both Neu5Ac and Neu5Gc-terminated sialoglycans [224]. The nature of the biologically-relevant receptors for typhoid toxin and how this varies between the two version of the toxin will be an interesting avenue of future research.

5.4. Typhoid toxin diversity

The *S. enterica* species is composed of thousands serovars that have diverse ecologies and virulence properties. Outside of the Typhi serovar, typhoid toxin is also encoded by other lineages of the *Salmonella* genus with a sporadic phylogenetic distribution [154,194,231,232]. The Javiana serovar has served as an important model system to study typhoid toxin biology in nontyphoidal *Salmonella* [212,231,233–236]. Like the *S*. Typhi version of typhoid toxin, the *S*. Javiana typhoid toxin elicits DNA damage and cell cycle arrest in cell culture models of infection [212,233,234]. Furthermore, murine infection studies comparing wild-type *S*. Javiana to a typhoid toxin mutant indicate that strains encoding typhoid toxin accumulate at greater numbers at certain systemic sites [212]. Like *S*. Typhi, the Javiana serovar also encodes *pltC,* and cell culture infection experiments indicate that both the PltB and the PltC toxin can elicit CdtB-mediated DNA damage arrest during infection [212,231]. Although the sequence of the typhoid toxin genes is very similar in *S*. Typhi and *S*. Javiana, mouse intoxication experiments demonstrated that the Javiana version of the (PltB) typhoid toxin is markedly less potent than its Typhi counterpart [235]. The differences were found to stem from three amino acid differences in the PltB sequences, which reduce Javiana PltB's affinity for glycans carrying α2–3-linked terminal sialic acid compared to *S*. Typhi PltB (Fig 2D). More broadly, analysis of typhoid toxin genetic sequences suggests that there is substantial diversity amongst different typhoid toxins produced by various salmonellae, which likely reflects the evolutionary adaptation of these toxins to target the diverse hosts *Salmonella* infections or colonizes ([154] and unpublished results). Interestingly, phylogenetically diverse *S. enterica* strains encode both the core typhoid toxin locus and PltC, which is surprising given that both loci are genetically mobile and they are usually not genetically linked. This suggests that encoding two versions of typhoid toxin is a widespread feature of typhoid toxin biology that serves an evolutionarily beneficial function [154,231].

6. Concluding thoughts

Table 1 summarizes the major evolutionary diversification that has occurred amongst the AB_5 toxins that have been characterized to date. These toxins and toxin families presumably represent only a fraction of those that exist in nature. Indeed, despite the fact that *S*. Typhi is a major human pathogen and that salmonellae are amongst the most highly studied bacteria, typhoid toxin was not discovered until 2008. Many uncharacterized genes with sequence similarity to AB_5 subunits can be found by searching DNA sequence databases, and such searches only unveil putative toxins that share detectable sequence similarity with known toxins. The large and heterogeneous collection of AB_5 toxins that exists today is a testament to both the efficacy of the AB_5 paradigm, and to the power of bacterial evolution. Although mutation and homologous recombination are clearly major factors in generating AB_5 toxin diversity, it is becoming increasingly clear this is not the only way that diversity is generated. The AB_5 scaffold is surprisingly amenable to incorporating novel subunits into existing toxins. In the case of B subunits, it is noteworthy that homologous proteins that share an ancient ancestor appear to represent an environmental reservoir for evolutionary diversification. For example, type 2 ArtAB toxins and the PltC typhoid toxin both appear to have evolved as a result of an A subunit forming a toxin with a B subunit that is distantly-related to its original B subunit. This suggests that, although the functional properties of A and B subunits appear to evolve quickly, the A/B interface is relatively well conserved over evolutionary time. Indeed, it has been noted that there are conserved aspects of

the A–B interaction in divergent AB_5 toxins, such as the nature of the B pentamer pore, which is generally lined with hydrophobic amino acids for the apical portion, and with charged and/or polar residues for the basal half [147]. Multiple distinct enzymes appear to have evolved the capacity to engage with AB_5 toxin B subunits, yielding novel AB_5 toxins families. That this has apparently occurred on numerous independent instances suggests that the A/B interface of AB_5 toxins is quite flexible. Collectively, this conserved-but-flexible A-B interface creates ideal conditions for the emergence of novel AB_5 toxins with unique activities and cell targeting properties.

As highlighted above, the evolutionary diversification of AB_5 toxins has important consequences for human health by impacting the virulence properties of prominent bacterial pathogens. Conversely, AB_5 toxins have been useful tools to study eukaryotic cell biology, they have diagnostic utility, and they have a great deal of potential as components of novel measures to combat human disease [2,14–17,237–239]. This includes approaches that target AB_5 toxins in order to prevent or treat the diseases caused by the bacteria that produce them, a strategy that has been explored with significant promise for many of the toxins described above. The most noteworthy example of this is pertussis toxin, which is a principal component of the widely used acellular Pertussis vaccines (such as TDaP/DTaP) administered to prevent whooping cough [16,240–243]. Additionally, AB_5 toxins and their constituent subunits have been widely investigated as therapeutic agents to treat other (unrelated) diseases. For example, cholera toxin family B subunits are highly immunogenic and have a great deal of promise as mucosal adjuvants [244]. Another example of this is the application of Shiga toxins or their B subunits in anti-cancer agents, a promising strategy because Gb3 is present at significantly higher levels in many cancer cell types relative to normal tissue [238]. In light of the many potential applications of AB_5 toxins, there is a great deal that can be learned from analyzing the wide range of biological properties found within the natural reservoir and AB_5 toxins. Furthermore, nature is the greatest engineer, and efforts to design customized AB_5 toxins will benefit from understanding the mechanisms by which AB_5 toxins have evolved naturally.

CRediT authorship contribution statement

All authors contributed to the writing of the original manuscript, the preparation of the figures/table, and manuscript editing. C.C.F. oversaw all aspects of this project.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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