Structure–function analyses of a pertussis-like toxin from pathogenic Escherichia coli reveal a distinct mechanism of inhibition of trimeric G proteins

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Abstract

Pertussis-like toxins are secreted by several bacterial pathogens during infection. They belong to the AB₅ virulence factors, which bind to glycans on host cell membranes for internalization. Host cell recognition and internalization are mediated by toxin B subunits sharing a unique pentameric ring-like assembly. While the role of pertussis toxin in whooping cough is well established, pertussis-like toxins produced by other bacteria are less studied and their mechanisms of action are unclear. Here, we report that some extra-intestinal Escherichia coli pathogens (i.e. those that reside in the gut but can spread to other bodily locations) encode a pertussis-like toxin that inhibits mammalian cell growth in vitro.

We found that this protein, EcPlt is related to toxins produced by both nontyphoidal and typhoidal Salmonella serovars. Pertussis-like toxins are secreted as disulfide-bonded heterohexamers in which the catalytic ADP-ribosyltransferase subunit is activated when exposed to the reducing environment in mammalian cells. We found here that the reduced EcPlt exhibits large structural rearrangements associated with its activation. We noted that inhibitory residues tethered within the NAD⁺-binding site by an intramolecular disulfide in the oxidized state dissociate upon the reduction and enable loop restructuring to form the nucleotide-binding site. Surprisingly, while pertussis toxin targets a cysteine residue within the α-subunit of

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inhibitory trimeric G proteins, we observed that activated EcPlt toxin modifies a proximal lysine/asparagine residue instead. In conclusion, our results reveal the molecular mechanism underpinning activation of pertussis-like toxins, and we also identified differences in host target specificity.

Pathogenic Escherichia coli strains preferentially inhabit different sites within their hosts, with enteric or extra-intestinal niches presenting distinctly different challenges. Infection by enteric or diarrheagenic E. coli can result in gastroenteritis but seldom spreads beyond the intestinal tract except in immunocompromised individuals(1,2). In contrast, extra-intestinal E. coli (ExPEC) strains such as uropathogenic E. coli (UPEC) or neonatal meningitis E. coli (NMEC) can reside passively within the gut until conditions permit their expansion into the urinary tract, blood or nervous system where they may potentially cause life-threatening disease(3). To do so, ExPEC strains express a range of virulence factors, often encoded on mobile genetic elements, including AB5 toxins. Such phage-encoded toxins are secreted by several major bacterial pathogens including: enterotoxigenic E. coli (ETEC), and enterohemorrhagic E. coli (EHEC)(4), Vibrio cholerae(5), Shigella(6), Salmonella(7) and Bordetella pertussis(8,9). Modern sequencing techniques frequently identify novel AB5 toxins related to those previously studied, but their conservation at the level of function remains to be determined.

AB5 virulence factors bind to glycans present on the surface of eukaryotic host cell membranes resulting in their internalization. Once inside the host cell the enzymatic A subunits are released allowing them to disrupt host biochemistry and physiology. Host cell recognition and internalization is mediated by the toxin B subunits that share a unique pentameric ring-like assembly. This juxtaposes with the A subunit cargos that are class-specific and structurally divergent, their activation inside mammalian cells occurs through distinct intracellular detection and release mechanisms(10). Five AB5 toxin families currently exist: the enzymatic components of the Subtilase cytotoxin(11) and EcxAB toxin(12) are proteases while those of the Shiga group(6) are ribosome inhibitors; cholera and heat labile enterotoxins(4,5) carry related ADP-ribosyltransferases, as do the related Pertussis(8,9) and typhoid toxin (7) proteins.

Toxin ADP-ribosyltransferases (ARTs) hydrolyze the nicotinamide group from NAD+ and transfer the ADP-ribose moiety onto specific host proteins. Pertussis toxin specifically targets inhibitory trimeric G-proteins by modifying a conserved cysteine located four residues from the C-termini of the Gα subunit(13). This modification renders the Gαi/o subunits unable to associate with their cognate G-protein coupled receptors (GPCRs) thus modulating the host’s immune response. A series of related pertussis-like (Plt) toxins exist within the genomes of pathogenic bacteria including strains of E. coli, Salmonella and Yersinia. The bacterial strains harboring such virulence factors are diverse and their evolutionary relationship complicated due to the spread of these genes on mobile genetic elements. Proteins within the Plt family include
the atypical archetype member whose glycan binding B-subunits have expanded and diverged within the *B. pertussis* genome to form four separate genes yielding a pseudopentameric glycan-binding platform. In contrast, other pertussis-like toxins display a homopentameric glycan-binding stoichiometry. All Plt proteins nonetheless carry a conserved catalytic A subunit.

In the secreted state the enzymatic ART domain of pertussis-like toxins lies atop the 5 glycan-binding subunits allowing its C-terminus to thread through a U-shaped NAD$^+$-binding cleft before plunging into the pore of the B subunit pentamer(7,9). When these C-terminal residues are truncated from Pertussis toxin its ART domain is constitutively active *in vitro* but is unable to associate with its pseudopentamer or enter cells(14). Enzymatic activation *in vivo* requires proteolytic separation of the inhibitory C-terminus and reduction of a connecting disulfide. While the molecular mechanisms underlying an alternate activation mechanism of cholera toxin are understood(15) the changes that occur following activation of a Pertussis-like protein have not previously been characterized.

While the association of Pertussis toxin with whooping cough is well established, orthologous pertussis-like toxins present within other pathogenic bacterial infections are less studied and their mechanism(s) of action are unclear(7). Here we identify a Pertussis-like AB$_5$ protein (EcPltAB) from clinical *E. coli* isolates that is related to the Typhoid and ArtAB toxins observed in typhoidal and nontyphoidal *Salmonella* serovars. We provide structures that confirm that Pertussis-like toxins are secreted as inactive forms in which an intramolecular disulfide holds an occluding C-terminal tail within the NAD$^+$ binding site. This conserved disulfide also serves as a redox-switch that senses host cell entry, with reduction of the bond allowing displacement of the occluding C-terminal residues, facilitating NAD$^+$ binding and maturation of catalytic activity. At a global level, the existence of EcPltAB type proteins expands the family of known bacterial Pertussis-like toxins capable of modulating the human immune system. Furthermore, we show that while AB$_5$s proteins are segregated into evolutionarily related enzyme families these may not always modify the same residues within host proteins.

**Results**

**Identification of EcPltAB**

The frequency with which pathogenic bacterial strains are isolated from clinical settings doesn’t necessarily correlate with their representation in genomic databases, yet remains a useful proxy measure. We thus determined the prevalence of genes encoding AB$_5$-type toxins among complete and draft *E. coli* genome sequences available on the NCBI database. *E. coli* genomes were queried using previously identified A and B subunit sequences. As expected, most genes discovered corresponded to previously known *E. coli* toxins; by far the most prevalent were the Shiga toxins produced by Shiga toxigenic *E. coli* (STEC), proteins whose action can cause hemolytic-uremic syndrome following infection, identified in 19% of genomes (Fig. S1). The next most prevalent were toxins from the cholera family LT-I and LT-II heat-labile
Activation mechanism of Pertussis-like toxins

enterotoxins secreted by ETEC (Fig. 1), identified in approximately 8% of the genomes. The properties of these enteric *E. coli* toxins are well established(4,5,11). However, the third most common set of AB₅ genes identified were a series of related Pertussis-like proteins found in ExPEC genomes, particularly from phylogroup B2 (Fig. 1). The B subunit of these toxins shared 69% sequence identity to ArtB and the upstream A subunit 70% identity to ArtA, two components of a Pertussis-like toxin produced by *Salmonella* Typhimurium DT104(16). In contrast to these *Salmonella* and *Escherichia* orthologs, the archetype Pertussis toxin has an expanded set of four glycan-binding B subunits (named S2-S5) associated with a single catalytic A or S1 subunit. The *Salmonella* ArtAB protein is an active ADP-ribosyltransferase(17), while a related Typhoid toxin additionally serves as cargo-carrier for an accessory cytolethal distending toxin (7). Our analysis indicates that the *E. coli* Plt toxins are more evolutionarily diverse than currently identified *Salmonella* variants, with at least three semi-separate lineages sharing between 70-80% sequence identity. We refer to the product of these Pertussis-like genes as *E. coli*-Pertussis-like toxins (*Ec*Plt).

**Cytotoxicity of EcPltAB**

We characterized the *EcPlt* toxin family by cloning a number of representative members and expressing them using standard recombinant techniques, focusing primarily on the *EcPlt* genes (E9YZW8 and E9YZW9) cloned from UPEC isolate PA26B. The oxidized *EcPltAB* holotoxin was purified after co-expressing both subunits using a periplasmic expression system(18). The 96kDa soluble protein complex eluted on gel-filtration with a molecular weight consistent with that of an AB₅ heterohexameric stoichiometry. To ascertain whether this holotoxin was functional it was added to human embryonic kidney (HEK293T) or African green monkey kidney epithelial cells (Vero). Mammalian cell lines grown to 40-50% confluence were exposed to different concentrations of *EcPltAB* (Fig. 2a, Fig. S3). The characteristic response profile of Pertussis toxin involves a slow cellular-entry mechanism followed by an accumulative interruption of signaling resulting in clustered growth (19). Over an 18-hour period control treated cells remained unaffected and continued to proliferate, while those exposed to *EcPltAB* displayed cell clustering and an absence of growth (see Fig. S3a-c) compared with buffer alone controls (Fig. S3d).

Next we performed a glycan array analysis to determine the specificity of *EcPltAB* (see Table S2). Its B subunit displayed avidity for a broad range of branched eukaryotic glycans, especially those with N-acetylneuraminic acid (Neu5Ac) termini; implying *EcPlt* targets a similar range of cell-types as Typhoid toxin (7).

We next sought to establish which eukaryotic proteins were targeted by *EcPltA*. The *EcPltA* toxin shares 50-70% identity with *Salmonella* ArtA proteins and 31% identity with the Pertussis toxin’s catalytic S1 subunit; two known ADP-ribosyltransferases that modify the α-subunits of inhibitory trimeric GTP-binding proteins (17,20). We set up an assay to identify equivalent *EcPltA* substrates by using biotin-conjugated NAD⁺ as substrate for the toxins. In such assays ADP-ribosylation also adds a biotin
PT is responsible for many of the pathogenic features of *B. pertussis*. However, other *Bordetella* species also contain *ptx*-like genes yet fail to produce the toxin because of inactive promoters(21). Using our assay, we sought to identify whether ADP-ribosyltransferase activity was detectable in the supernatant of UPEC strain PA26B. UPEC PA26B was cultured in the presence of prophage inducing agents. After 12 hours growth, bacterial cells were removed by filtration and the supernatant tested for ADP-ribosyltransferase activity using *HsGaα3ΔN* as a substrate. In control reactions employing purified *EcPltAB*, a robust ADP-ribosyltransferase response was observed using 20nM of pure toxin (Fig 2c lane 1). Prior to commencing the reaction, the supernatant of PA26B cells were seen to contain a natively biotinylated 20-kDa protein, but this was readily distinguishable from our host *HsGaα3ΔN* target (Fig 2c lane 2). When *HsGaα3ΔN* was added to the filtered supernatant of PA26B we observed ADP-ribosylation of the substrate (Fig. 2c lane 3) suggesting, but not conclusively proving, that *EcPltAB* is actively expressed.

The B subunit of the Typhoid toxin impacts cell-signaling pathways independent of its associated catalytic A subunits(22). To determine whether the growth-arrest phenotype induced by *EcPltAB* resulted from its enzymatic activity, inactivating mutations were incorporated into the A subunit. A highly conserved Q/ExE motif is present in many ADP-ribosyltransferases and is essential for activity(23) (residues 116QNE118 in *EcPltA*). Mutation of residue E118 to an aspartic acid residue resulted in a requirement for 1000-fold higher concentrations of toxin in order to
induce the same level of growth arrest as wild-type EcPltAB toxin (Fig. 2d). Furthermore, a Q116D, E118D double mutant that retains a wild-type B subunit, showed no effect on growth and was indistinguishable from control reactions even at 50 µg/mL of toxin (Fig. 2d, Fig. S3e), as discussed later this mutant displayed no discernible enzymatic activity (Fig. 7c). EcPltAB thus appears to be expressed by ExPEC and its ADP-ribosyltransferase activity is cytotoxic to mammalian cells.

The presence of ATP, phosphate and detergent assist activation of the PT S1 subunit by promoting dissociation from its pseudopentamer and increasing its exposure to the local microenvironment(24). ATP binds within the Pertussis toxin central pseudopentameric pore, destabilizing the quaternary structure(25). We sought to determine whether the activation requirements for the EcPltAB toxin were similar. Our assay recapitulated the finding that reducing agents activate PT and that this process is synergistically enhanced by the presence of ATP (Fig. 3a lanes 1-5). Like PT, the EcPltAB holotoxin is inactive when oxidized and higher concentrations of reducing agent result in increased catalytic activity in vitro (lanes 6-8). However, ATP did not synergistically enhance this process (in Fig. 3a lane 4 is 95% more intense than lane 2 whilst the intensities of lane 7 and 9 are within 1%). The ATP-binding site of the PT pseudopentamer appears absent within the homopentameric EcPltAB. We examined whether the nature of the reducing agent affected EcPltAB activation by repeating the ADP-ribosylation assays in the presence of intracellular (2mM) or serum (2µM) concentrations of glutathione using different redox ratios. Reduced glutathione alone was ~20% more effective at inducing toxin activation in vitro than an equivalent concentration of TCEP (Fig. 3c), yet even small quantities of oxidized glutathione (GSSG) drastically slowed activation. Thus, while some characteristics are shared, the sequence divergence that exists between Pertussis toxin and Pertussis-like toxins results in differences in their respective activation mechanisms.

The protein substrates

PT targets the α-subunits of human inhibitory trimeric G-proteins by transferring the ADP-ribose moiety of NAD⁺ onto a conserved cysteine four residues from their C-termini. PT-sensitive substrates primarily belong to the heterotrimeric Gi/Go protein subfamily, which in humans includes the 40.5-kDa proteins Ga₁₁, Ga₂₂, Ga₁₃ and Ga₆₆, as well as the equivalent subunit from transducin. The Salmonella ArtA Pertussis-like toxin modifies this same set of proteins(17), indicating some degree of target conservation. As the HsGa₃ was shown to be an EcPltA substrate, we sought to test for activity against the remaining trimeric Gi proteins enriched from brain lysate using equal amounts of a commercial Gβ- immunoprecipitated source of trimeric inhibitor G proteins. Mammalian inhibitory Ga₁ proteins possess similar molecular weights but have divergent pIs (Ga₁₂/Ga₆ (5.34), Ga₁₃ (5.50) and Ga₁₁ (5.7)), allowing for their separation as three distinct bands on an isoelectric focusing gel. PT-catalyzed ADP-ribosylation of brain lysate (left panel Fig. 3c) showed three distinguishable IEF bands, roughly reproducing its substrate
preference of Ga\textsubscript{i2}/Ga\textsubscript{o} > Ga\textsubscript{i3} > Ga\textsubscript{i1}(17). In contrast, when the same experiment was performed using EcPltA, at least three subunits were modified, with a slight relative preference for Ga\textsubscript{i3} over Ga\textsubscript{i2}/Ga\textsubscript{o} and Ga\textsubscript{i1} (right panel Fig. 3c). EcPlt thus represents a functional AB\textsubscript{3} toxin that retains some, but not all, characteristics of the archetypical PT.

**The protein substrate ADP-ribosylation site**

Pertussis toxin specifically modifies a conserved cysteine four amino acids from the C-terminus of Ga\textsubscript{i/o} subunits(26) (C351) and in doing so renders them unable to interact with their cognate receptors (Fig. 4a). In our ADP-ribosyltransferase assay, and as reported by others(27), C351S mutations within Ga\textsubscript{i/o} proteins leave them resistant to PT modification, but surprisingly not modification by EcPltA (see Fig. 4b). To determine whether EcPltA targeted another cysteine residue we mutated each cysteine residue and repeated the experiment; all such HsGa\textsubscript{i3} mutants were simultaneously treated with PT as a control. As shown in Figure 4b, all other cysteine knockout mutants act equally well as substrates for either toxin. When Cholera toxin’s primary site of modification within stimulatory Ga\textsubscript{s} proteins is removed, lower affinity sites instead become targeted(28). In case EcPltA targeted more than one cysteine, we pre-treated HsGa\textsubscript{i3} with iodoacetamide for 24 hours and re-purified the protein prior to the assay (Fig. 4c). Alkylating all available cysteines renders HsGa\textsubscript{i3} resistant to PT modification as expected, but does not prevent the protein from being a substrate for EcPltA, indicating an alternate chemical linkage. The ADP-ribosylation products produced by EcPltA and PT were not sensitive to 1M hydroxylamine treatment suggesting the modification is on residues other than Glu and Asp. However, lysine methylation of HsGa\textsubscript{i3} rendered it entirely resistant to EcPltA modification (Fig. 4c). PT could still act on methylated HsGa\textsubscript{i3} substrate but at a slightly reduced rate compared with controls (Fig. 4c).

To identify the residue(s) within HsGa\textsubscript{i3} modified by EcPltA, the reaction product was subjected to MS-MS analysis. ADP-ribosylation results in a +541 Da mass shift to precursor peptides, fragmentation of the pyrophosphate bond also liberates a characteristic 348.1 Da adenine monophosphate during product ion scans (Fig. S5). We obtained full peptide coverage of HsGa\textsubscript{i3} and could not detect any evidence of cysteine modification, instead ADP-ribosylated modification sites were identified in HsGa\textsubscript{i3} C-terminal sequences: 331NVQFVFDAVTDVII-K(ADP-ribosylated)-NNLK\textsuperscript{349} and 346N-N-(ADP-ribosylated)-LKECGLY\textsuperscript{354}. Thus, EcPltA ADP-ribosylates two residues (K345 and N347) at the C-terminus of HsGa\textsubscript{i3}, sites distinct from PT modification of C351. We next constructed three mutants: HsGa\textsubscript{i3}(K345A), HsGa\textsubscript{i3}(N346A) and HsGa\textsubscript{i3}(N347A) and tested their suitability as substrates for both toxins. HsGa\textsubscript{i3}(K345A) was seen to be resistant to ADP-ribosylation by EcPltA yet was readily modified by the constitutively active PT S1 (Fig. 4d); HsGa\textsubscript{i3}(N346A) was readily ADP-ribosylated by either toxin and HsGa\textsubscript{i3}(N347A) by neither toxin (Fig. 4e). It is noteworthy that mutation of either K345 or N347 to alanine also reduced enzymatic activity against the other site, suggesting a concerted mechanism of recognition. Together,
these experiments suggest that different Pertussis-like toxins can target distinct but nearby residues within the C-termini of Ga\textsubscript{i} proteins.

**Ga\textsubscript{i}/o recruitment to GPCRs is disrupted by EcPltAB**

We next examined what effect EcPltAB had on GPCR signaling pathways in cells. The Relaxin family peptide 4 receptor (RXFP4) is a GPCR that recruits Ga\textsubscript{i}/o subunits upon binding its ligand: insulin-like peptide 5 (INSL5); the induced nucleotide exchange inhibits forskolin-stimulated cAMP production and promotes Erk phosphorylation(29), a response that can be abolished through PT pretreatment. Ga\textsubscript{i}/o proteins with a C351I mutation (mGa\textsubscript{oA}, mGa\textsubscript{OB}, mGa\textsubscript{i1}, mGa\textsubscript{i2} or mGa\textsubscript{i3}) are PT-insensitive and can be used to rescue the PT-abolished INSL5-stimulated ERK1/2 response in order to determine which Ga\textsubscript{i}/o subunits are recruited to RXFP4. We firstly confirmed that PT could completely abrogate the INSL5-stimulated ERK1/2 response within Chinese hamster ovary (CHO)-RXFP4 cells transfected with the pcDNA3 vector control (Fig. 5a); an identical result was obtained with EcPltAB pretreatment (Fig. 5a). Next, we demonstrated that ERK1/2 responses were partially restored in PT-treated cells transfected with mGa\textsubscript{oA}, mGa\textsubscript{OB}, mGa\textsubscript{i2} and to a smaller extent with mGa\textsubscript{i3} but negligibly with Ga\textsubscript{i1} (Fig. 5b-f). GPCR RXFP4 can thus recruit all isoforms of Ga\textsubscript{i}/o although it does so in a preferential manner, predominantly recruiting Ga\textsubscript{oA} = Ga\textsubscript{oB} = Ga\textsubscript{i2} > Ga\textsubscript{i3} ≥ Ga\textsubscript{i1}. None of the PT-insensitive Ga\textsubscript{i}/o mutants tested were able to restore INSL5-stimulated ERK1/2 responses in cells treated with EcPltAB (Fig. 5b-f), underlining that within cells the residue(s) on Ga\textsubscript{i} targeted by EcPltAB differ from the canonical C351 ADP-ribosylated by PT and that once modified, GPCR signaling is disrupted.

As Ga\textsubscript{i}/o subunits carrying the PT-resistant C351I mutant remain sensitive to EcPltAB treatment we next sought to identify alternate EcPlt-resistant mutations. To do so we monitored the cAMP concentration in cells responding to the adenylyl cyclase activator Forskolin (Fig. S6a). This stimulatory response can be counteracted by inhibitory trimeric G-proteins coupled to INSL5-induced RXFP4 signaling which acts to restore cAMP-levels to a near basal state (Fig. S6a). The RXFP4-transduced inhibitory signal can be abrogated by either toxin (Fig. S6a), reconstituting C351I Ga\textsubscript{i}/o subunits allows partial recovery of the inhibitory signal in the presence of PT (Fig. S6b-f) but not EcPltAB. In an attempt to achieve an equivalent response for the entereobacterial toxin we mutated its two identified ADP-ribosylation sites within HsGa\textsubscript{i3} to alanine (N347A and K345A) and repeated the experiment (Fig. S6g-h). Neither HsGa\textsubscript{i3}(N347A) nor HsGa\textsubscript{i3}(K345A) facilitated the recovery of PT treated cells. In contrast, EcPltAB treatment of cells transfected with HsGa\textsubscript{i3}(N347A) displayed a 26 ± 5% difference in the cAMP response in the presence of INSL5/forskolin or forskolin alone (Fig. S6g-h); consistent with the N347A mutant providing a degree of protection for EcPltAB but not for PT. In the same experiment HsGa\textsubscript{i3}(K345A) did not obviously allow recovery of INSL5-mediated signaling (Fig. 7h).
Activation mechanism of Pertussis-like toxins

Structure of the secreted EcPltAB holotoxin

To understand the secreted ExPEC toxins in more detail we determined the 2.4 Å crystal structure of the EcPltAB holotoxin (see methods and Table 1 for details). This heterohexameric complex resembles a blunted pyramid in which the EcPltA subunit sits atop a pentameric B subunit base, thereby adopting an overall quaternary structure of the AB₅ family (Fig. 6a). In the secreted oxidised state the enzymatic A subunit contains a single intramolecular disulfide bond between the two conserved cysteines (C41 and C192, Fig. 6b); each of the five EcPltB subunits contains a further two intramolecular disulfides. EcPltA shares 52% sequence identity to the Typhoid toxin’s A subunit(30) and 31% identity to the S1 subunit of Pertussis toxin(9), its two closest known structural homologues (root mean squared deviation of 1.1 Å over 207 of 224 Cα; and 2.0 Å over 186 Cα respectively PDBeFOLD). In all three holotoxins the respective orientation of the A subunit atop the B pentamer is similar, with the Cα backbone structure of the EcPlt holotoxin and the lower AB₅ section of the Typhoid toxin overlaying over most of their length. The conserved intramolecular disulfide of Pertussis group toxins holds their inhibitory C-terminal residues inside their nucleotide binding sites (Fig. 6b), preventing catalytic activity prior to entry into the reducing environment of the host cytoplasm.

Structure of the cytoplasmic NAD⁺ bound EcPltA

To determine what structural changes occur following release of the C-terminal redox-switch we determined a 1.8 Å resolution structure of the constitutively active catalytic EcPltA subunit (residues 5-181) purified under reducing conditions (Table 1). As ART enzymes display off-target NAD⁺-glycohydrolase activity in the absence of substrate, we utilized the catalytically inactive mutant (Q116D, E118D) to slow this reaction and permit stable co-complex formation with the NAD⁺ substrate(30). These crystals diffracted in space group P1 with four complexes of NAD⁺:EcPltAcat within the asymmetric unit allowing independent models of the ART domain to be compared. The enzymatic core of the ART fold consists of ~150 amino acids and has a U-shape with a concave cleft creating the NAD⁺ binding site (Fig. 6c). The two β-sheets of EcPltA are on either side of this nucleotide binding cleft: on one side is the β-sheet with strands s1, s3, s6 and s7 sandwiched between helix 3 and helix 1; whilst on the other side is the fold’s smaller s2, s5 and s4 β-sheet along with its h2, h4 and h5 helices. Unbiased omit maps showed NAD⁺ to be bound inside the U-shaped ART domain with two distinct binding modes observed, one extended and the other semi-extended (Fig. 7)(31). The extended conformation (Fig. S7a) dominates in the A, B and C protomers and the semi-extended conformation in D (Fig. S7b). Both bound forms of NAD⁺ adopt highly folded nicotinamide mononucleotide (NMN) sections, whereas the adenine base transits between alternate aromatic stacking interactions. The common factor within the two distinct nucleotide conformations is the folded NMN group, forcing the nucleotide to adopt this form is proposed to be an integral part of catalysis(32). The residues stabilizing the compact NMN are contributed from both sides of the binding cleft. Strand s1 provides a conserved...
phosphate-binding nucleophilic amino acid (Arg-9) also found in PT while the amide group of the nicotinamide moiety forms hydrogen-bonds to the peptide backbone (Fig. 7a). On the other side of the cleft residues within a semi-conserved “STS”-motif within the s2 strand position the scissile bond for catalysis by clasping the nicotinic ring of NAD$^+$ (actual residues $^{52}$ATT$^{54}$, Fig. S7c). The inactivating E118D mutation of the catalytically essential glutamate shifts the negatively charged carboxyl away from the nucleotide (Fig. 7a/b); if the E118 rotamer observed in the wild-type holotoxin were to be maintained following reduction its carboxyl group would be positioned within hydrogen-bonding distance of the N-ribose’s 2’-hydroxyl (Fig. 7e). This interaction is highly conserved within ART enzymes and is thought to promote nicotinamide scission by stabilizing the formation of an oxocarbenium ion intermediate(33). The movement of the carboxyl group caused by the E118D mutation helps explain why this otherwise relatively conservative substitution is inactivating in the ART enzymes (Fig. 7c).

In the extended NAD$^+$ conformation the adenine base interleaves between the guanidinium group of R13 and the indole ring of H26. A similar interleaved interaction is seen in the NAD$^+$ bound Cholera toxin structure(15). In contrast, the semi-extended NAD$^+$ conformation has the adenine base stacked atop Y67 (Fig. 7b). The Y67A mutation was seen to abrogate catalytic activity in the ADP-ribosyltransferase assays using $HsG_{i3}$ as substrate (Fig. 7c), but ~25% wild type activity is retained by Y67F mutants suggesting it is the aromatic base-stacking role that is its more vital feature (Fig. 7c). Moreover an S70W mutation, which would disrupt the adenosine-binding site of the semi-extended but not the extended conformation, also displayed minimal ADP-ribosyltransferase activity in vitro (Fig. 7b and c) suggesting this conformation may play a role during catalysis.

Several EcPltA residues located at the intersubunit interface of the holotoxin become solvent-exposed following subunit dissociation; this includes I111 on h5, I58 and S56 on h3 (grey labels in Fig. 7b). To determine if any such residues are also involved in protein-interactions with the transfer-target they were mutated to large bulky residues to disrupt potential recognition interfaces. All mutations on the exterior face of h3 had no effect (Fig. 7c), while equivalent mutations on h5 (I111Y) were inactivating. This h5 helix is N-terminal to the catalytic E/QxE-motif, a region of the ART-domain that in Cholera and C3-like toxins corresponds to a conserved flexible loop between strands s4-s5 known to be necessary for substrate recognition(34).

Redox-induced structural changes

We compared the inactive oxidized and active NAD$^+$-bound states of EcPltA (Fig. 7e; Supplementary video). This shows that the activation mechanism of pertussis-like toxins is fundamentally different from the loop-refolding mechanism of cholera toxin (15). The conserved intramolecular disulfide within the EcPltA oxidized state (C41-C192) is located inside the NAD$^+$-binding cleft directly above the catalytic glutamate (E118), C192 being the C-terminal residue of the short inhibitory A2 helix. In the holotoxin the binding pocket for the nicotinamide ring is filled by F193 from hA2 (Fig. 7d);
subsequent A2 backbone residues then rise to thread through the cleft with R199 and R200 occupying the same site as the adenosine ring in the extended-conformation (*pink loop in Fig. 7d). The A2 loop subsequently doubles back on itself before entering the pentamer pore; during this descent it blocks the adenosine-binding site near helix 3 for the semi-extended conformation (Fig. 7b). The nucleotide-binding site of EcPltA is comprised from dynamic regions of the protein that move following release of the inhibitory C-terminal residues as well as regions that remain stationary. A static-floor capable of accommodating either NAD\(^+\) or the A2 peptide includes: the Adenine base-interleaving residues R13 and H26 (Fig. 7a); the phosphate binding nucleophile Arg-9; and the nicotinamide “STS”-clasp equivalent residues. In contrast, structural movements are required to form the side-walls of the NAD\(^+\)-binding site. This is necessary as the occluding C-terminal helix is more bulky than the substrate nucleotide.

After the redox-sensing disulfide is reduced C41 and the remainder of the active-site loop undergo a ~2.5Å rigid-body inward movement towards the adenosine ribose and phosphate (Fig. 7e). This loop-movement carries the h2 helix with it until it abuts the 3’-hydroxyl of the N-ribose, in doing so the catalytic residue His-35 (35) is shifted towards the scissile N-glycosidic bond (Fig. 7a and e) where it may interact with E118. Following NAD\(^+\)-binding, helix 3 pivots about its N-terminus resulting in a maximal displacement from the oxidized form of ~8.2Å (Fig. 7e). This movement allows the side chains of Y67 and I63 to move in and neighbor the nicotinamide ring. These NAD\(^+\)-binding associated movements are likely to be conserved features of activation within Typhoid toxin and Pertussis toxin.

**Discussion**

We provide the structural details of the *E. coli* Pertussis-like toxin in its intracellular reduced form, detailing different substrate binding modes and the redox-induced structural changes that occur during activation. In the oxidized state, the disulfide-linked C-terminus of the A subunit inactivates the protein, by occluding the NAD\(^+\) binding site (Fig. 6), a mechanism clearly conserved within Pertussis toxin as suggested by modeling studies(36). Loss of the inhibitory A2 domain from within the nucleotide-binding cleft allows significant lobe-closure and reorganization of residues about NAD\(^+\) particularly within helices h2, h3 and the active-site loop. This contrasts with the activation mechanism of the Cholera toxin whose A2 domain is largely external to the catalytic cleft and requires no lobe movements upon activation, but involves significant loop restructuring(30). Our high-resolution co-crystal structure details NAD\(^+\) binding in two distinct conformations: the dominant extended conformation is similar to that observed in nucleotide-soaked CT crystals(30); a second lower-occupancy semi-extended conformation also exists and may play a role during catalysis as C-terminal h3 mutations predicted to disrupt the base-stacked adenine abrogate activity.

The residues within h2 are relatively well conserved, suggesting the redox-induced movement of this structural element and the
Restructuring of the preceding active-site loop are a conserved features of Pertussis-like proteins. In contrast, helices h3 and h5 show more significant structural divergence between the Pertussis and the Enterobacterial holotoxins, with these regions more likely involved in binding to the transfer target. GPCR-binding is disrupted by pertussis toxin through modification of the Ga residue C351, the toxin is highly specific and unable to modify mutants lacking this residue (Fig 6). The extent to which Pertussis-like toxins are conserved could have correlated with conservation of target chemistry, yet we show EcPlt does not modify cysteine residues within Ga proteins, but instead a lysine and asparagine several residues away. Moreover, our cAMP experiments confirmed that EcPltAB enters cells and inhibits the action of Ga proteins via a residue other than C351 (Fig 4). This is not unprecedented, human Poly(ADP-ribose) polymerase 1 initiates polymer formation on C-terminal chromatin lysines(37) but ADP-ribosylation at such sites has not previously been described within bacterial toxins nor in human Ga subunits. Why would this site be targeted? GPCR:Ga-protein coupling occurs when the predominantly helical C-terminus of the Ga subunit inserts into a pocket built from the cytoplasmic facing ends of the receptor TM helices. The co-complex structure of opsin in complex with the C-terminus of transducin (38) shows that C351 penetrates deep into the binding-pocket, highlighting why Pertussis toxin’s modification would be incompatible with receptor coupling. As the C-terminus of receptor-bound Ga-subunits are predominantly helical N347 lies a turn beneath C351 interacting with the same transmembrane helix (TM3). K345 is on the opposing side of the G-protein C-terminus interfacing with TM6. Thus both N347 and K345 reside nearer to the cytoplasmic “mouth” of the receptor’s binding pocket than C351 when coupled to their cognate GPCRs.

EcPltA-catalysed ADP-ribosylation may thus block receptor recruitment of inhibitory heterotrimeric G-proteins using a related but distinct mechanism to that used by Pertussis toxin. Our attempts to create an EcPlt-resistant Ga3 subunit were partially successful (Fig. S6), and could be interpreted as indicating that the N347 modification may dominate over the K345 site in vivo. In vitro both K345 and N347 were required for EcPltA to modify HsGa3, so both residues may be involved in toxin:G-protein binding. Peptides consisting of the 10-20 C-terminal residues of HsGa3 can act as minimal pertussis toxin substrate-inhibitors (39) suggesting the entire protein is not necessarily required for recognition. Our work might imply that EcPlt has shifted its respective recognition motif within Ga proteins several residues downstream from that of PT with N347 playing distinct roles in both EcPlt and PT toxins recognition interfaces; we note that N347 appears to be more essential for the action of EcPlt as its mutation did not provide sufficient protection to reconstitute signalling in the presence of PT. Further work will be required to validate our reconstitution experiments; mutants that disrupt the toxin-G protein interface may also influence GPCR binding and/or GTPase activity.

Finally, Pertussis toxin has become a valuable tool for dissecting GPCR pathways, and the identification of an expanded toolset of Pertussis-like proteins with varied inhibitory
mechanisms may permit additional experiments to be performed. Given the proven utility of PT in this role, it may be beneficial to further characterize the behavior of other Pertussis-like toxins in reconstituted systems in order to fully understand how this toolset functions.

EXPERIMENTAL PROCEDURES

Materials.
All chemicals were purchased from Sigma-Aldrich (St. Louis, MI, USA) unless otherwise stated. Mouse INSL5 was synthesised and purified at The Florey Institute of Neuroscience and Mental Health (Melbourne, Australia) by Dr. Akhter Hossain(40). FBS, DMEM Ham’s/F12, Lipofectamine 2000 and PT were all from Life Technologies (Carlsbad, CA, USA). AlphaScreen SureFire® ERK1/2 phosphorylation kits, acceptor beads and donor beads were all from PerkinElmer (Wellesley, MA, USA).

Bioinformatic analysis and bacterial strains
E. coli strains containing AB5-type toxin-like genes were identified from an E. coli dataset (EcoDS) comprising 1700 genome sequenced strains deposited on the NCBI database up to January 2014 (PMID: 27904885) using a querying dataset based on characterized AB5 toxins (Art(16), Shiga(41), Subtilase(42) and Cholera(43) toxins). Sequence comparisons were performed using the FASTA36 package(44) and ORFs showing an amino acid sequence identity >75% with the querying dataset were extracted from the public dataset. A neighbor-joining tree was generated using the concatenated AB toxin subunits with ClustalW(45). The phylogenetic group was determined using an in silico triplex scheme(46,47) and MLST analysis was performed according to a seven-gene scheme previously described(48). UPEC strain PA26B was isolated from a patient presenting with a urinary tract infection at the Princess Alexandra Hospital (Brisbane, Australia) (PMID: 26858103).

Toxin expression
Toxin expression was induced by stressing PA26B cells and a control K12 laboratory strain, overnight cultures grown at 37°C were diluted 1:40 into 40mL of LB broth without antibiotics, grown for 4 hours at 37°C before addition of Mitomycin C or 10mM H2O2. After 24 hours of growth all proteins secreted into the media were harvested and clarified by passing through a 0.2 µm filter. 10nM of bovine catalase was added to the supernatant of cells stressed by H2O2.

Cytotoxicity assay
Kidney epithelial cells from the African green monkey (Vero cells) or Human embryonic kidney (HEK293T) were expanded to 40-50% confluency in 10cm² dishes covered with 2mL of Dulbecco’s modified eagle medium supplemented with 10% (v/v) fetal calf serum. Cytotoxicity assays were performed by adding recombinant EcPltAB holotoxins at 50 µg/mL-500 pg/mL. Cells were left for 18hours then stained with crystal violet and imaged. The surface area covered by the cells was determined by the number of blue pixels within each image normalised against buffer controls.

Cloning
A co-expression vector for the EcPltAB toxin was created as in Ng et al. 2013. Briefly both the full-length EcPltA and EcPltB genes were chemically synthesised (GenScript, New Jersey, USA), with the addition of restriction sites NdeI and Xmal and XbaI and XhoI flanking the EcPltA and EcPltB open reading frames, respectively. The EcPltA gene containing its native signal sequence was cloned directly into pBAD18. EcpltB was sub-cloned into pET-23 (Novagen) via XbaI and XhoI sites, resulting in a protein with a C-terminal poly-His tag and N-terminal PelB signal sequence, this construct was used for the expression of unliganded EcPltB pentamer. To produce the co-expression vector for EcPltAB, the ribosome binding site and EcPltB was excised from pET-23 using the XbaI and HindIII restriction sites prior to being cloned downstream of EcPltA within the pBAD vector.

The catalytic domain of the Pertussis toxins S1 subunit, EcPltA and human HsGaα3 proteins were cloned into a pET28 expression vector fused to a hexahistidine tag through a Human Rhinovirus 3C cleavage site. The constitutively active EcPltA construct encompassed residues 5-181. The HsGaα3 constructs encompassed residues 034-354 or 005-354. Mutants used in enzymatic assays were created by oligonucleotide-directed mutagenesis followed by DpnI digestion of the parental vector, with all resulting constructs confirmed by sequencing.

**Protein expression and purification**

For protein expression EcPltAB was transformed into *E. coli* BL21(DE3) cells then grown in LB at 37°C containing 100 µg/mL ampicillin to OD<sub>600</sub> = 0.6 followed by a 4 hr induction at 37°C with 0.2% L-arabinose. Cell culture pellet was collected and resuspended in HEPES-buffered saline (HBS) (20 mM HEPES pH 7.0, 150 mM NaCl) and stored at -20°C. The cell pellet was thawed, lysed and centrifuged with the supernatant collected and loaded onto 5 mL of nickel-sepharose beads (GE Healthcare), equilibrated in 50 mM HEPES pH 7.0, 250 mM NaCl, 60 mM imidazole. Unbound material was removed by washing with the same buffer. Protein was eluted with 1 M imidazole buffer (20 mM HEPES pH 7.0, 250 mM NaCl, 1 M imidazole), with 5 mL fractions collected. Fractions containing EcPltAB were pooled, buffer exchanged into HBS, and loaded on a Superdex S200 16/60 gel filtration column (GE Healthcare) where it eluted with a molecular weight consistent with that of a heterohexamer (~100 kDa).

Protein expression of EcPltA constructs were performed in BL21 (DE3) *E. coli* cells which were grown at 37°C in LB medium supplemented with 30 µg/mL Kanamycin. After reaching an OD<sub>600nm</sub> of 0.6-0.8 the temperature was dropped to 18°C and the cells induced for 18 hours with 0.2mM of Isopropyl β-D-1-thiogalactopyranoside. Cells were harvested and resuspended in HBS with 20mM Imidazole and 1mM TCEP then lysed by adding 1 mg/mL lysozyme and DNase then sonicated. Cleared lysate was applied to a Ni-NTA affinity column and washed extensively before eluting in 20mM HEPES pH 7.0, 250mM NaCl, 400 mM Imidazole, 1 mM TCEP. The protein’s hexahistidine tag was cleaved by incubating the eluted protein with 0.1 mg/mL GST-fused HRV 3C overnight at 4°C. The HRV 3C protease was
removed by passing over 0.1mL GSH-agarose resin with the flow through subsequently purified by gel filtration on a Superdex S75 16/60 (GE Healthcare Life Sciences). The constructs eluted as a single peak corresponding to monomeric EcPltA. The pertussis toxin’s catalytic S1 domain was purified using a similar manner.

Expression of the different HsGa\textsubscript{i3} constructs was identical to that of EcPltA except they required 0.01% (v/v) Triton x100 during HRV 3C cleavage and the protein was buffer exchanged into 20mM HEPES, 50mM NaCl, 1mM TCEP to allow HiTrap Q purification prior to gel-filtration. When HsGa\textsubscript{i3} mutants were being purified for enzymatic assays chromatographic systems and columns were purged with two intervening dummy runs: one of 0.5 M NaOH followed by 1 M HEPES pH 7.0. When required HsGa\textsubscript{i3}ΔN was methylated using a reductive alkylation kit (Hampton Research #HR2-434) or carbamidomethylated with iodoacetamide (Pierce #90034) according to manufacturer’s instructions prior to the ion exchange column during purification.

**Enzymatic assay**

Activity assays were performed using 6-Biotin-17-NAD\textsuperscript{+} (BPS Biosciences #80610) as a substrate for the modification of different G-protein substrates. The resulting biotin adducts were visualized using Streptactin conjugated horseradish peroxidase (Bio-RAD #161-0381). Briefly each reaction mix contained ~1µM of protein substrate which consisted of recombinant HsGa\textsubscript{i3}; purified trimeric Bovine Brain G-proteins (EMD Millipore 371739) or whole Vero-cell lysate. Standard assay conditions consisted of ~1 µM G-protein in 20 mM HEPES pH 7.0, 140 mM NaCl, 5 mM TCEP and 10 µM Biotin-NAD\textsuperscript{+} which was incubated for 5min at 37°C with 20nM of toxin. Holotoxin assays were performed with up to 100mM TCEP to induce activation and consisted of PT purified from *Bordetella* lysate (Sigma Aldrich P7208) or recombinant EcPltAB. Reactions were stopped by adding SDS-loading buffer and heating at 100 °C for 5 min before analysis by SDS-PAGE. Analysis by 2-D gel electrophoresis was done by the Proteomics Facility, Monash University; samples were separated in the 1st dimension over broad IPG pH strip (pH 3-10) before separation in the second dimension by SDS-PAGE (gradient of 9-16% acrylamide). 1D and 2D gels were processed for analysis by Western blot, proteins were transferred to a membrane prior to being visualized using Streptactin-HRP and Pierce chemiluminescence substrate. Assays performed with recombinantly produced G-proteins were subsequently stained by coomassie to ensure equal loading. All assays were repeated 3 times to assure reproducibility. Densitometry was performed using an Epson V700 scanner and data quantification via ImageQuant.

**Crystallization data collection and structure determination**

The EcPltAB holotoxin crystals were obtained by setting up 1:1 ratio hanging drop experiments with 16 mg/ml protein over a reservoir of 0.2 M MgCl\textsubscript{2}, 0.1 M Tris-HCl pH 8.5, 15% (w/v) polyethylene glycol 3350 at 293 K; crystals took 2-3 weeks to grow and were then harvested by flash freezing in liquid nitrogen after
soaking in a cryoprotectant solution containing an additional 25% (v/v) glycerol prior to data collection. *EcPltAB* crystals diffracted to 2.35 Å in space group *P6_3* with one heterohexamer in the asymmetric unit, initial maps were generated using the lower AB_3 section of the Typhoid toxin\(^7\) as a molecular replacement model\(^{49}\) in the program Phaser\(^\text{50}\). The resulting electron density allowed rounds of manual building and refinement using the programs COOT\(^\text{51}\) and PHENIX\(^\text{49}\). Due to the moderate-resolution NCS constraints were maintained throughout refinement, as releasing restraints did not result in significant improvements in \(R_\text{free}\). The final model encompasses residues 1-114 across all 5 protomer B subunits and residues 4-226 in the A subunit.

The reduced EcPltA(Q116D, E118D) mutant at 6-8 mg/mL was complexed with 20 mM NAD\(^+\) for thirty minutes before setting up in hanging drop crystallisation experiments using a 1:1 ratio at 277 K. Clusters of plate-like crystals grew within 2-3 days over reservoirs containing 4-7% (v/v) polyethylene glycol, 0.1 M Tris pH 8.5, and 150 mM Li\(_2\)SO\(_4\). Crystals clusters were broken into single pieces prior to freezng in reservoir complemented with 25% (v/v) glycerol and flash frozen in liquid nitrogen, subsequently diffracting in a monoclinic space group to 1.8 Å with four molecules in the asymmetric unit. A molecular replacement solution was obtained using a trimmed version of the holotoxin’s A1 subunit as a model with four molecules found within the asymmetric unit. In protomers A-D clear electron density was observed for residues 4-174, there were few differences in the \(C_\alpha\) backbone structure amongst protomers except for a shift in protomer D within the loop C-terminal to h3. The final model contains 4 NAD\(^+\) molecules and all but the C-terminal residue of the construct.

Data completeness for the *EcPltA* in spacegroup P1 was a little lower than ideal. When PT or EcEPlt dissociates from their B subunits they mimic unfolded proteins in order to translocate towards the cytosol. In such structures h5 is highly flexible so crystals often grew as oddly shaped clusters that needed to be fragmented. In the four molecules in the asymmetric unit the h5 loop adopts different forms, moreover such conformations varied when data was collected at different points in the crystal. This structural complexity caused issues for merging of datasets, and crystals showed some radiation damage after 150-200°, the dataset was collected from multiple points on a single crystal.

**Glycan array analysis**

Purified *EcPltAB* protein at 1 or 100 µg/ml was applied to a PA_v51 glycan microarray at the Consortium of Functional Glycomics Protein-glycan Interaction core at Emory University, binding was detected using Alexa488-conjugated anti-hexahistidine antibody. The data are reported as average relative fluorescence units of four of six replicates (after removal of the highest and lowest values) for each glycan represented on the array.

**Mass spectrometry analysis**

*HsGαi3* at 2 mg/mL was treated for 1 hour with a 100:1 molar ratio of *EcPltA* in 20mM HEPES, 150mM NaCl, 2.5mM NAD\(^+\), 0.5mM
Activation mechanism of Pertussis-like toxins

CHO cells stably expressing RXFP4 (CHO-RXFP4) were a gift from A/Prof. Ross Bathgate (Florey Institute of Neuroscience and Mental Health, Melbourne). Cells were grown and maintained in 175 cm² flasks in DMEM/F12 medium supplemented with 5% (v/v) foetal bovine serum (FBS) at 37 °C in humidified air containing 5% CO₂. cDNA constructs for G protein α-subunits (GαoA, GαoB, Gαi1, Gαi2 or Gαi3) containing the C351I mutation were from the cDNA Resource Center (Bloomsberg, PA, USA; http://www.cdna.org). HsGαi3 containing K345A and N347A mutations were generated via site-directed mutagenesis using wild-type HsGαi3 pcDNA3 vector as template. HsGαi/o constructs carrying the mutations were transiently transfected into CHO-RXFP4 cells grown on 6-well plates using Lipofectamine 2000 as per the manufacturer’s protocol.

ERK1/2 phosphorylation.

18 hours after transfection, CHO-RXFP4 cells were trypsinised and seeded onto 96-well plates at a density of ~50,000 cells per well and incubated for 6 – 8 hours in complete media to allow cell adhesion. Cells were then incubated with PT, EcPltAB or control (serum-free DMEM/F12) for 18 hours before stimulation with mouse INSL5 (0.1 to 100 nM) for 5 minutes and then lysed. Phosphorylation of ERK1/2 (T202/Y204) was detected using the AlphaScreen SureFire® kit. In brief, 4 µl of cell lysates was added to a white 384-well microplate (ProxiPlate; PerkinElmer) containing 5 µl acceptor bead mix (40 parts reaction buffer/10 parts activation buffer/1 part protein A acceptor beads) and incubated for 2 hours at 23 °C. Then, 2
µl donor bead mix (20 parts dilution buffer/1 part streptavidin-coated donor beads) was added and incubated for 2 hours at 23°C. All additions and incubations were carried out under low light conditions to avoid photobleaching. Fluorescence readings were made using the Envision multilabel plate reader (PerkinElmer; excitation = 680 nm; emission = 520 - 620 nm). ERK1/2 phosphorylation is expressed as a percentage response to 10% (v/v) FBS (positive control).

**Inhibition of cAMP accumulation**

18 hours after transfection, CHO-RXFP4 cells were trypsinised and seeded onto 96-well plates at a density of ~50,000 cells per well and incubated for 6 – 8 hours in complete media to allow cell adhesion. Cells were then incubated with PT, EcPltAB or control (serum-free DMEM/F12 medium) for 18 hours. On the day of experiment, we changed the medium to HBSS supplemented with 500 µM IBMX, 5 mM HEPES and 0.1% (w/v) BSA and equilibrated for 1 h at 37°C. Cells were treated with INSL5 (100 nM) for 15 minutes, followed by 30 minutes forskolin stimulation and then lysed with 100% ice-cold ethanol. Once ethanol has evaporated, cAMP was resuspended in 100 µL of lysis buffer containing 0.3% (v/v) Tween-20, 5 mM HEPES and 0.1% (w/v) BSA. The amount of cAMP generated by forskolin and its inhibition by INSL5 was measured using a homogenous time-resolved fluorescence resonance energy transfer (TR-FRET) LANCE cAMP kit (Perkin-Elmer) as per the manufacturer’s protocol.

**Acknowledgements**

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**Conflict of interest:** The authors declare that they have no conflicts of interest with the contents of this article.
Author contributions: DRL wrote the manuscript, purified proteins and obtained the EcPlt structures, MTT and MJ cloned constructs and performed the ADP-ribosylation assays, OK performed mass-spectrometry experiments, DGM and MAS identified EcPltAB and performed the bioinformatics analysis. SY, MK and RS devised and performed the GPCR activity assays, AWP, JCP, JR, MAS and TB provided experimental design oversight and interpretation.
References

Typhimurium DT104 ArtA-dependent modification of pertussis toxin-sensitive G proteins in the presence of [32P]NAD. Microbiology 155, 3710-3718


### Table 1 Data collection and refinement statistics

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¹ R_{pim} = Σ_{hkl} | |I_{hkl} - <I_{hkl}>| / Σ_{hkl} <I_{hkl}>

² R_{free} = (Σ | |F_o| - |F_c| | ) / (Σ | |F_o| ) - for all data except as indicated in footnote 3.

³ 5% of data was used for the R_{free} calculation.
**Figure 1 - E. coli AB₅ toxins**

Cladogram of AB₅ toxin sequences identified in genome sequenced *E. coli* strains available on the NCBI database. Archetype sequences from the querying dataset are indicated with an asterisk. *E. coli* strains harbouring more than one AB₅ group are indicated by a red star. The inner circle represents the *E. coli* phylogenetic groups (A, B1, B2 and D). Additional colour-coded circles indicate the place, year and source of each strain, as well as the associated infection (where known). Since STEC strains are overrepresented in the NCBI database, only archetypical Shiga toxin variants are presented. The EcPltA toxin focused on in this study is highlighted with a blue star. A schematic of the A and B subunit domain’s displayed with the positions of the intramolecular disulfides (yellow) and the inhibitory C-terminus (pink) is at the bottom of the figure. Insert: an expanded view of the pertussis toxin branch. A complete overview of all Shiga toxin variants and their associated strains is presented in Fig. S1.
Figure 2 - Cytotoxicity of recombinant *EcPltAB*

18 hours after treatment with buffer or different concentrations of *EcPltAB* Vero cells were stained and imaged.  **A)** Representative images for left: untreated controls and right: 50ng/mL *EcPltAB* are shown.  **B)** ADP-ribosyltransferase assays in which Pertussis toxin or EcPltAB were used to modify different protein substrates including: trimeric G protein extracts, recombinant human G\(\alpha_i3\) or Vero cell lysates. Reactions at 37°C were performed in the presence of biotin-NAD\(^+\) then quenched after 5 minutes, separated by SDS-PAGE, transferred to a Western blot membrane and imaged with either left: luminescence following streptactin-HRP exposure or right: coomassie.  **C)** Western blots showing the incubation of ExPEC culture medium with NAD\(^+\)-biotin with and without the presence of HsG\(\alpha_i3\)ΔN.  **D)** Vero cells were imaged in a similar manner to those in panel (a) were monitored for the degree of cell growth relative to buffer controls following exposure to different concentrations of EcPltAB or A subunit mutants. Surface area is calculated from the percentage of each image stained by crystal violet. Data represents the mean ±SD from three replicates.
Figure 3 - Activity assays for EcPltAB
ADP-ribosyltransferase assays similar to those used in Figure 2 using biotinylated NAD$^+$ as a substrate to modify recombinant HsGai3ΔN A) Pertussis (PT) and EcPltAB holotoxins were monitored for activity for 5 min at 37°C in the presence of 10mM (+) or 100mM (+++) TCEP and with or without 10mM ATP. B) EcPltAB activation in the presence of different ratios of reduced (GSH) or oxidized (GSSG) glutathione within the ADP-ribosylation assay. C) 2D isoelectric focusing separation of different Ga$_i$ isoforms isolated from bovine brain extract following treatment with EcPltAB or PT indicate multiple Ga$_i$ isoforms act as substrates.
Activation mechanism of Pertussis-like toxins

Figure 4 - Proposed model of the Pertussis-like toxins transfer target.

**a)** Structure of Opsin in complex with the C-terminus of transducin (Scheerer et al. pdb 3DQD), their K345L mutant was reverted to lysine for this figure. Gαi3 is shown in teal color, the position of the C-terminus, and ADP-ribosylated residues discussed in text are shown. The plasma membrane and GPCR receptor (green) are at the top of the image. **b)** ADP-ribosyltransferase assays using EcPtA or Pertussis toxin in which potential acceptor cysteines within Gαi3ΔN were mutated to serine, or **c)** following iodoacetamide and lysine-methylation, **d)** mutation of K345 or **e)** N346 and N347 to alanine.
FIGURE 5 – EcPltAB disrupts G-protein coupled signaling.
RXFP4 recruits G_10 α-subunits upon ligand activation. PT pretreatment abolishes INSL5-stimulated ERK1/2 phosphorylation and this is rescued to differing degrees by transfection of individual PT-insensitive G_10 mutants. In a) Chinese Hamster Ovary-RXFP4 cells were transiently transfected with pcDNA3 vector control, or (b-f) G_10 constructs carrying the C351I mutation (mGα1A, mGα1B, mGα1I, mGα2 or mGα3).
Blockade of the response to PT but not EcPltAB pretreatment was rescued by transfection of the mutant G proteins. Cells were stimulated with mouse INSL5 alone (0.1 to 100 nM), or after pretreatment with PT (100 ng/ml) or EcPltAB (100 ng/ml). Data points represent mean ± S.E.M. of three independent experiments and are expressed as the % response of the positive control (10% v/v FBS).
FIGURE 6 - Activation mechanisms of EcPlt and Cholera toxin

a) Cartoon representation of the EcPltAB holotoxin in which α-helices are colored marine, β-strands wheat and loop regions green; the C-terminal A2 domain is highlighted pink showing its interaction with the B-subunits of the glycan-binding pentamer (gray).  
b) Cartoon representation highlighting the holotoxin’s EcPltA subunit. The conserved redox-sensing disulfide is labeled and rough position of the B-pentamer shown as a partially transparent structure. Predicted potential proteolytic sites are shown with partially transparent scissors and the C-terminus marked (Ct).  
c) The reduced active NAD⁺ co-complex with all secondary structural elements labeled. The enzymatically cleaved N-glycosidic bond cleaved during the course of the reaction is highlighted.
FIGURE 7 - The NAD⁺ binding site of EcPltA

(a) Structure of EcPltA with NAD⁺ bound in the extended conformation, residues investigated for their role in binding and catalysis are labeled, as are the nicotinamide “STS”-clasp equivalent residues 52ATT54. 

(b) Structure of the semi-extended NAD⁺ conformation, approximate position of occluding residue mutants are shown in grey.

(c) ADP-ribosyltransferase assay response relative to wild-type protein for various point-mutants within the NAD⁺ binding site. Error bars are the SD of three independent replicates.

(d) Depiction of where the residues important for NAD⁺ binding are located in the oxidized holotoxin and their interactions with the occluding A2 helix (pink).

(e) An overlay highlighting the structural changes occurring during activation/NAD⁺ binding. The semi-extended NAD⁺ bound structure (blue helices and orange loop) is compared with the oxidised holotoxin (cyan helices and yellow loop).
Structure and function analyses of a pertussis-like toxin from pathogenic Escherichia coli reveal a distinct mechanism of inhibition of trimeric G proteins

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