

Mechanism of association of adenylate cyclase toxin with the surface of *Bordetella pertussis*: a role for toxin–filamentous haemagglutinin interaction

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Summary

Adenylate cyclase (AC) toxin from *Bordetella pertussis* is unusual in that, unlike most other members of the repeats-in-toxin family that are released into the extracellular milieu, it remains associated with the bacterial surface. In this study, we investigated the nature of the association of this toxin with the surface of *B. pertussis*. AC toxin was extracted from crude outer membrane preparations of *B. pertussis* with 8 M urea, but only partially with alkaline sodium carbonate and not at all with octylglucoside, suggesting that denaturation of the toxin is necessary for its removal from the membrane. *B. pertussis* mutants lacking filamentous haemagglutinin (FHA) released significantly more AC toxin into the medium, and AC toxin association with the bacterial surface was partially restored by expression of FHA from a plasmid, suggesting a role for FHA in surface retention of AC toxin. AC toxin distribution was unaffected by the absence of pertactin, or full-length lipopolysaccharide, or a defect in secretion of pertussis toxin. Using overlay and immunoprecipitation, we found that a direct physical association can occur between AC toxin and FHA. Combined, these findings suggest that FHA may play a role in AC toxin retention on the surface of *B. pertussis* and raise the possibility of an involvement of adherence mediated by FHA in delivery of AC toxin from the bacterium to the target cell.

Introduction

Bordetella pertussis, the causative agent of whooping cough, has re-emerged as an important pathogen among young children and adults throughout the world (Hewlett, 1999). *B. pertussis* produces a number of proteins essential to its virulence, including adenylate cyclase (AC) toxin,

filamentous haemagglutinin (FHA), pertactin (PRN), pertussis toxin (PTX) and fimbriae (FIM) (Locht, 1999). AC toxin is a unique member of the repeats-in-toxin (RTX) family of bacterial toxins (Welch, 1991; Coote, 1992; Ludwig and Goebel, 1999), which includes *Escherichia coli* haemolysin and leukotoxins from *Pasteurella haemolytica* and *Actinobacillus actinomycetemcomitans*. These proteins are characterized by the presence of glycine/aspartate-rich nonameric repeats.

Adenylate cyclase toxin is unique because, unlike other RTX family members, it contains two functionally separable domains, one of which possesses AC enzymatic activity and the second, which haemolyses red blood cells (Glaser *et al.*, 1988; Hanski, 1989; Ehrmann *et al.*, 1991; Ladant and Ullmann, 1999). After translocation into a target cell, the AC domain is activated by host cell calmodulin and catalyses the uncontrolled production of intracellular cAMP from ATP, a process termed intoxication (Hanski, 1989; Ehrmann *et al.*, 1991; Gray *et al.*, 1998). Supraphysiological levels of cAMP suppress leukocyte functions such as chemotaxis, phagocytosis and oxidative burst, potentiating *B. pertussis* survival (Confer and Eaton, 1982; Hanski and Farfel, 1985; Friedman *et al.*, 1987; Pearson *et al.*, 1987).

Like other RTX toxins, of which the most extensively studied is *E. coli* haemolysin, AC toxin is secreted by a type I secretion apparatus that in *B. pertussis* is encoded by *cyaB*, *cyaD* and *cyaE*, which are arranged in an operon with the AC toxin structural gene, *cyaA* (Glaser *et al.*, 1988). Whereas *E. coli* haemolysin is released into the extracellular medium (Ludwig and Goebel, 1999), *B. pertussis* AC toxin is not, with the majority of the toxin remaining associated with the bacterial surface (Hewlett *et al.*, 1976). *A. actinomycetemcomitans* leukotoxin also remains associated with intact organisms (Tsai *et al.*, 1984), an interaction that appears to be mediated by binding to nucleic acids on the surface of the bacterium (Ohta *et al.*, 1993).

Although secretion of *E. coli* haemolysin and *B. pertussis* AC toxin is presumed to occur by the same mechanism, it is unclear why haemolysin is primarily present in the medium of *E. coli* whereas AC toxin remains associated with the bacterium, with only a small proportion of the toxin released into the supernate. One possibility is

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that outer membrane proteins of *B. pertussis*, such as FHA and PRN, which are involved in adherence to host cells (Urisu *et al.*, 1985; 1986; Relman *et al.*, 1989; Leininger *et al.*, 1991), influence the association of AC toxin with the bacterial surface. Recently, Kachlany and co-workers reported that in *A. actinomycetemcomitans*, expression of the *tad* operon that encodes long fibrils responsible for rough colony morphology and adherence to surfaces, is important for leukotoxin localization to the bacterial outer membrane (Kachlany *et al.*, 2000). Supporting a role for FHA in AC toxin association with the surface of *B. pertussis* are the observations that FHA-deficient mutants release more AC toxin into the medium (Weiss *et al.*, 1983) and FHA co-purifies with AC toxin during some purification protocols (E. L. Hewlett, unpublished). In addition, early studies indicated that PRN contained contaminating AC activity (Novotny *et al.*, 1985). Combined, these findings suggest that AC toxin may associate with FHA and/or PRN, however, this hypothesis has never been directly tested.

The purpose of this study was to investigate the nature of the association of AC toxin with the surface of *B. pertussis* as a first step towards understanding how AC toxin is delivered from *B. pertussis* to target cells. We evaluated the ability of buffers and detergents to extract AC toxin from *B. pertussis* membrane preparations to determine whether AC toxin is a peripheral or integral membrane protein and to learn if AC toxin associates with the membrane by ionic interactions. To examine the role of outer membrane virulence determinants in the association of AC toxin with the surface of *B. pertussis*, we examined AC toxin distribution in a variety of *B. pertussis* mutants lacking expression of FHA, PRN, or full-length LPS, or defective for PTX secretion. Because results from these initial studies suggested an association between AC toxin and FHA, we further investigated this possibility by overlay and immunoprecipitation experiments. Results from these experiments suggest that a previously unrecognized physical association exists between AC toxin and FHA; this interaction may potentiate delivery of AC toxin during infection.

Results and discussion

Denaturation is required to remove AC toxin from B. pertussis outer membranes

Previous work has shown that AC toxin is extracytoplasmic, but remains primarily associated with, and exposed on the surface of *B. pertussis* (Hewlett *et al.*, 1976). Whereas only 18% of AC enzymatic activity is measured in the supernate of exponentially growing *B. pertussis* organisms, the remaining 82% of AC enzymatic activity is associated with *B. pertussis*; up to 93% of this activity is

abolished after exposure of intact *B. pertussis* organisms to trypsin (Hewlett *et al.*, 1976). These findings demonstrate that AC toxin is not only extracytoplasmic, but that the majority of the protein, at least the portion of it responsible for AC activity, is probably on the outer face of the outer membrane of *B. pertussis*.

Adenylate cyclase toxin contains a 200-amino-acid hydrophobic domain, but lacks amino acid sequence motifs consistent with β -sheet formation (Hanski, 1989), a characteristic of known bacterial transmembrane outer membrane proteins (Tamm *et al.*, 2001). Thus, it is likely that AC toxin associates with the outer membrane of *B. pertussis* in a fashion that does not include insertion, but rather by a mechanism that may involve ionic or hydrophobic interactions. To understand the nature of the interaction by which AC toxin remains associated with *B. pertussis*, we investigated the ability of various buffers and detergents to extract AC toxin from crude outer membrane preparations.

Total membranes (inner and outer) were prepared from *B. pertussis* and treated with Triton X-100. The insoluble fraction represents crude outer membranes (Schneider and Parker, 1982; Johnson and Burns, 1994) and contains the majority of AC toxin as determined by Western blot analysis (data not shown). Consistent with the inability of Triton X-100 to solubilize AC toxin, octylglucoside (a non-ionic detergent in the same family as Triton X-100) did not extract AC toxin from crude outer membranes (Fig. 1). In addition, neither the ionic detergent, deoxycholate, nor the zwitterionic detergent, CHAPS, extracted AC toxin (data not shown). In contrast, 8 M urea-TEE (Tricine/EDTA/EGTA) almost completely extracted AC toxin into the soluble fraction (Fig. 1), whereas TEE alone had no effect. When crude outer membranes were exposed to sodium carbonate (100 mM, pH 11.5), which disrupts ionic interactions resulting in the removal of peripheral membrane proteins (Fujiki *et al.*, 1982), a minute amount of full-length AC toxin was released into the soluble fraction (Fig. 1). Thus, it is unlikely that AC toxin associates with the outer membrane of *B. pertussis* through an ionic interaction. The majority of the cell-associated toxin, however, is strongly bound, perhaps in a fashion involving the hydrophobic domain that can only be disrupted by denaturation of the toxin, as occurs upon treatment with urea.

Association of AC toxin with the B. pertussis surface is dependent on the presence of FHA

One possible mechanism by which AC toxin remains associated with the bacterial surface is through an interaction with other outer membrane proteins. A number of past observations provide a basis for this hypothesis. It has been reported that more AC toxin is released into the supernate of FHA-deficient *B. pertussis* organisms than

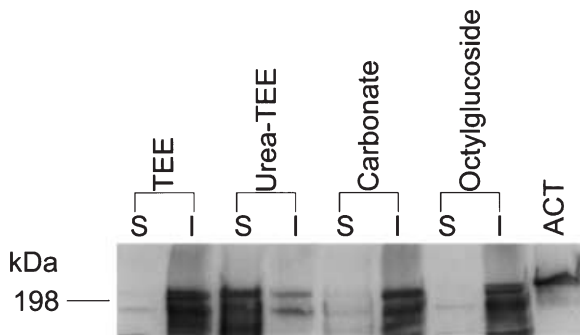


Fig. 1. Urea extracts adenylate cyclase (AC) toxin from *Bordetella pertussis* outer membranes. Crude outer membranes were prepared from *B. pertussis* (see *Experimental procedures*) and 80 µg were incubated in 1 ml of either TEE (10 mM Tricine, 0.5 mM EDTA, 0.5 mM EGTA-pH 8.0), 8 M urea/TEE, 100 mM sodium carbonate (pH 11.5) or 10 mM octylglucoside/150 NaCl (pH 7.4) overnight at 4°C. The soluble fraction (S) was obtained after centrifugation of the membranes at 107 000 *g* for 1 h at 4°C. The insoluble fraction (I) was suspended in 1 ml of PBS, pH 7.5. Equal volumes of the soluble and insoluble fractions were subjected to SDS-PAGE and AC toxin was visualized by immunoblot analysis using a rabbit polyclonal antibody against AC toxin, followed by secondary HRP-conjugated anti-rabbit IgG and chemiluminescent detection.

WT (Weiss *et al.*, 1983). FHA co-purifies with AC toxin and AC toxin may contaminate PRN preparations. Because previous studies analysing AC toxin distribution utilized FHA mutants with transposon insertions in the *fhaA* accessory gene (Weiss *et al.*, 1983; Lochter *et al.*, 1993), we asked whether a similar amount of AC toxin is released into the supernate of a *B. pertussis* mutant containing an in-frame, non-polar deletion of the FHA structural gene, *fhaB* (Lochter *et al.*, 1992).

Consistent with previous reports, greater than 90% of the total AC enzymatic activity was associated with the surface of wild-type *B. pertussis* (BP338) compared with less than 17% on the surface of an FHA transposon mutant (BP353) ($p < 0.0001$; Fig. 2 and Table 1) (Weiss *et al.*, 1983). Similarly, much more of the total AC enzymatic activity (90%) was associated with the surface of a different wild type strain (BPSM) than its isogenic *fhaB* deletion mutant, BPGR4 (14.1%; $p < 0.0001$; Fig. 2 and Table 1). On the other hand, the distribution of FHA was not altered in an AC toxin-deficient *B. pertussis* mutant (BP348) as determined by Western blot analysis (data not shown). These data suggest that the presence of FHA is a major determinant in AC toxin association with the surface of *B. pertussis* but that the absence of AC toxin does not effect FHA localization.

We added soluble, purified AC toxin to BPSM or BPGR4 to determine if the presence or absence of FHA affects the ability of exogenously added, purified AC toxin to adhere to the bacterial surface. In three independent experiments, BPSM bound 3.3 ± 0.33 -fold (mean \pm SEM) more exogenous AC toxin than BPGR4, providing further

evidence that AC toxin may associate with FHA on the surface of *B. pertussis*. Some exogenously added AC toxin did, however, bind to BPGR4, suggesting that FHA is not absolutely required for this type of association. The interaction of AC toxin with BPGR4 is probably due, at least in part, to the fact that this strain contains some AC toxin on its surface to which exogenous AC toxin may bind. This notion is supported by the finding that AC toxin binds less well to an AC toxin-negative *B. pertussis* mutant (F.R. Zaretsky, M.C. Gray, T. Kim and E.L. Hewlett, manuscript in preparation) and that AC toxin forms dimers in solution (S. J. Lee, M.C. Gray and E.L. Hewlett, manuscript in preparation) and binds to itself on blots (see below).

Defect in surface retention of AC toxin in BPGR4 can be complemented by expression of FHA

One way to establish a role for FHA in AC toxin retention on the surface of *B. pertussis* would be to demonstrate that expression of FHA in the FHA-deficient mutant restores the cell-surface association of AC toxin. We used BPGR4 harbouring a plasmid (pBG1) that contains a 10 kb *EcoR1* fragment of *fhaB* minus the last 990 bp of the gene. Expression of FHA from pBG1 doubled the retention of AC toxin on the surface of BPGR4 (28% versus 14%; $p = 0.037$) but did not restore AC toxin association to wild-type levels (Fig. 2 and Table 1). One possible explanation for this finding is that less FHA is expressed from pBG1 in BPGR4 than in the wild-type background; this possibility was supported by Western blot analysis (data not shown). Another possibility, although not mutually exclusive from the first, is that the truncated form of FhaB that is expressed from pBG1 (lacking the final 330 amino acids) has reduced ability to interact with AC toxin. To explore this possibility further, we

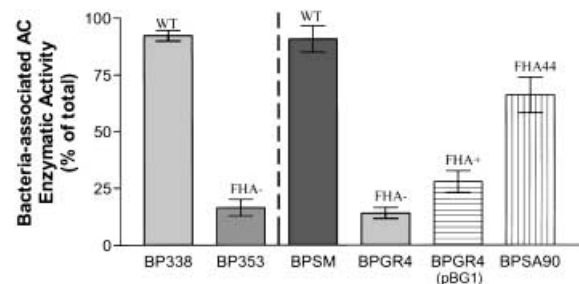


Fig. 2. Association of AC enzymatic activity with *B. pertussis* organisms. *B. pertussis* strains were grown to mid-exponential phase, centrifuged at 6000 r.p.m. for 10 min, and the supernate and pellet (whole bacteria) were assayed in duplicate for AC enzymatic activity (see *Experimental procedures*). Results are expressed as mean percent \pm SEM of total (supernate + pellet) AC enzymatic activity present on the bacterial pellet and are from at least three independent bacterial cultures.

Table 1. Adenylate cyclase (AC) toxin distribution in *Bordetella pertussis* mutants and clinical isolates.

Strains	^a Bacteria-associated AC enzymatic activity (% of total; mean ± SEM)	Relevant characteristics	Reference
BP338	92.3 ± 2.37	WT naladixic acid-resistant Tohama I derivative	Weisis <i>et al.</i> (1983)
BP353	16.4 ± 3.72	BP338 with Tn5 in <i>thaA</i> ; naladixic acid- and kanamycin-resistant; result, no FHA production	Weisis <i>et al.</i> (1983)
BPSM	90.8 ± 5.93	WT streptomycin- and naladixic acid-resistant Tohama I derivative	Menozzi <i>et al.</i> (1994)
BPGR4	14.1 ± 2.436	BPSM with 10 kb deletion of <i>thaB</i> ; streptomycin- and naladixic acid-resistant; result, no FHA production	Locht <i>et al.</i> (1992)
BPGR4 (pBG1)	27.9 ± 4.77	BPGR4 expressing FHA from plasmid pBG1 that contains a 10 kb <i>EcoRI</i> fragment of <i>thaB</i> except the last 990 bp; streptomycin-, naladixic acid- and kanamycin-resistant	Locht <i>et al.</i> (1992)
BPSA90	66.0 ± 7.77	BPSM with deletion of C-terminal half of FHA; result-expresses 82 kDa N-terminal FHA fragment	Provided by C. Loch (unpublished)
BPLC5	74.5 ± 3.753	BPSM with 626 bp internal deletion in <i>sphB1</i> ; streptomycin- and naladixic acid-resistant; result, FHA not cleaved	Coutte <i>et al.</i> (2001)
BP536	93.5 ± 1.25	WT streptomycin- and naladixic-resistant Tohama I derivative	Relman <i>et al.</i> (1990)
Wlb	91.6 ± 3.55	BP536 with deletion of <i>wlb</i> locus; streptomycin- and naladixic acid-resistant; result, produces only lipid A and core oligosaccharide	Allen and Maskell (1996)
BBC9	92.9 ± 3.05	kanamycin-resistance cassette in PRN structural gene; result, no PRN production	Leininger <i>et al.</i> (1991)
BPM3171	95.3 ± 1.87	BP338 with Tn5 in <i>ptlC</i> ; naladixic acid- and kanamycin-resistant; result, produces all of the PTX subunits but is defective for PTX secretion	Weiss <i>et al.</i> (1993)
18323	85.5 ± 3.5	WT Mouse-virulent strain used in vaccine testing	ATCC #9797
4069	96.8 ± 0.62	WT clinical isolate from Adult Acellular Pertussis Vaccine Efficacy Trial (APERT)	
4205	92.3 ± 1.43	WT clinical isolate from APERT trial	

a. The total amount of AC toxin activity produced by each strain was similar.

used a construct (BPSA90) in which the 3'-half of *thaB* is deleted from the chromosome of BPSM; this strain produces an 82 kDa N-terminal FHA truncate (FHA44) (C. Loch, personal communication). More AC activity was associated with the surface of BPSA90 compared with BPGR4 (Fig. 2 and Table 1), making it less likely that the C-terminal half of FHA is required for AC toxin interaction. However, significantly less AC toxin interacted with the surface of BPSA90 than with BPSM (Fig. 2 and Tables 1, $p = 0.044$). This finding may be related to the amount of cell-associated FHA in *B. pertussis* expressing FHA44, which is reportedly less than wild type (Renauld-Mongenie *et al.*, 1996). These data support a role for FHA in AC toxin association with the bacterial surface and suggest that AC toxin may interact with the N-terminal 82 kDa portion of FHA.

Filamentous haemagglutinin is produced as a 367 kDa precursor protein (Domenighini *et al.*, 1990; Renauld-Mongenie *et al.*, 1996) that has been recently shown to be proteolytically processed by the SphB1 subtilisin-like protease into its 220 kDa mature form (Coutte *et al.*, 2001), which comprises the N-terminal 60% of the FHA precursor (Domenighini *et al.*, 1990; Renauld-Mongenie *et al.*, 1996). A portion of the mature 220 kDa form of FHA is released into the extracellular medium, however, a significant proportion remains associated, or re-associates, with the surface of *B. pertussis*. The C-terminal portion

of FHA has been proposed to act as a chaperone to prevent premature folding of the protein before its export to the cell surface, where it is subsequently processed (Domenighini *et al.*, 1990; Renauld-Mongenie *et al.*, 1996).

Coutte and colleagues have recently reported that a *B. pertussis* mutant with deletion in *sphB1* (BPLC5) is deficient for FHA processing and, as a result, the precursor 367 kDa protein is never cleaved and remains associated with the bacterium (Coutte *et al.*, 2001). We were interested in determining whether this mutant would have an altered distribution of AC enzymatic activity. The percentage of AC toxin activity released into the medium by BPLC5 was not significantly different from that released by the wild-type strain, BPSM ($p = 0.088$; Table 1).

AC toxin distribution is not dependent on other secreted B. pertussis virulence determinants

In light of the dramatic effect of FHA deficiency on AC toxin retention on the bacterial surface, we evaluated secreted virulence determinants for their effect on the association of AC toxin with *B. pertussis*. As shown in Table 1, FHA was the only virulence determinant examined whose absence or alteration was associated with release of AC enzymatic activity into the supernate. Lack of PRN or full-length LPS, or impaired secretion of PTX,

had no effect on AC toxin distribution. To be certain that the wild-type strains used in these studies (BP338, BPSM, BP536) are representative, we also examined AC toxin distribution in two recent clinical isolates of *B. pertussis* and the mouse challenge strain, 18323. Similar to wild-type laboratory strains, greater than 80% of AC enzymatic activity was bacteria-associated in both clinical isolates analysed, and in 18323 (Table 1).

During the course of this study, a number of mutants that had been constructed using a spontaneous streptomycin-resistant BP338 derivative (SM001) (Barry, 1985) were found to contain high percentages of AC enzymatic activity in the supernate (data not shown). In fact, these mutants were reported to lack FHA production by Western blot analysis (Barry, 1985), thus explaining their reduced ability to retain AC toxin on their surface.

Overlay and immunoprecipitation demonstrate an interaction between AC toxin and FHA

Because our initial studies indicated that four to five times more AC toxin remains associated with the bacterial surface when FHA is produced, we asked whether purified AC toxin and FHA could interact directly. For the initial approach to this question, we used an overlay protocol, which is commonly used to determine if a protein can interact with proteins on a solid matrix (Sojar *et al.*, 2002). Purified preparations of PRN, FHA, PTX, bovine serum albumin (BSA) and Δ N549 (AC toxin mutant lacking the N-terminal 549 amino acids) were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and then overlaid with purified wild-type AC toxin. Binding of AC toxin to proteins on the nitrocellulose membrane was visualized using a monoclonal antibody (mAb), 3D1, directed against amino acids 373–399 of AC toxin.

As shown in Fig. 3, AC toxin bound to Δ N549, supporting the finding that AC toxin can associate with itself in solution (S. J. Lee, M.C. Gray and E.L. Hewlett, manuscript in preparation) and that the first 549 amino acids of AC toxin are not necessary for this interaction. AC toxin did not interact with PRN or PTX on the nitrocellulose membrane, consistent with the earlier observation that these virulence determinants had no effect on AC toxin distribution in *B. pertussis*. Most importantly, AC toxin bound to FHA on the nitrocellulose membrane, suggesting that a direct physical interaction occurs between AC toxin and FHA (Fig. 3). On control blots, containing full-length AC toxin as a positive control, mAb 3D1 did not bind to Δ N549 or FHA in the absence of the AC toxin overlay (data not shown).

To determine if AC toxin and FHA interact in solution, purified AC toxin and FHA were incubated together, then immunoprecipitated with 9D4 (a mAb against the repeat region of AC toxin) bound to protein G beads. Proteins

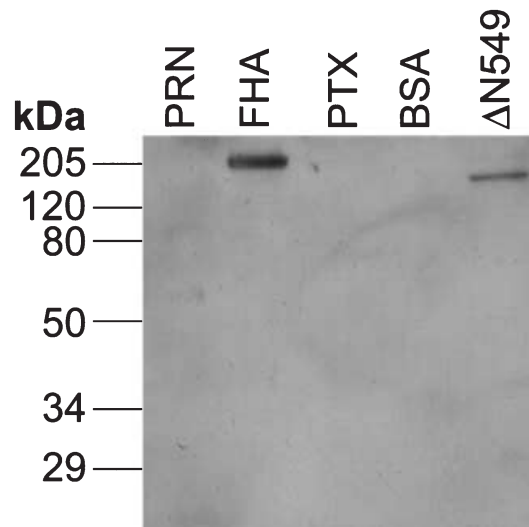


Fig. 3. AC toxin interacts with filamentous haemagglutinin (FHA) on nitrocellulose blots. Purified preparations of pertactin (PRN, 1 μ g), FHA (1 μ g), bovine serum albumin (BSA; 1 μ g), pertussis toxin (PTX; 2 μ g) or Δ N549 (10 ng) were subjected to SDS-PAGE and proteins were transferred to nitrocellulose. Blots were incubated with 5 μ g ml⁻¹ of wild-type AC toxin. Binding of AC toxin to proteins on blots was visualized by using mAb 3D1 followed by secondary HRP-conjugated anti-mouse IgG and chemiluminescent detection.

were eluted from the mAb-coated beads using Laemmli buffer, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with either X3C, a mAb against FHA, or 9D4. As shown in Fig. 4, FHA was immunoprecipitated as a complex with AC toxin, as FHA was eluted from 9D4-coated beads only when AC toxin was present. No cross-reactivity of X3C with AC toxin or 9D4 with FHA was observed. These data extend the overlay results and establish that a direct physical association occurs between purified AC toxin and FHA in solution.

Conclusions and future directions

Although the general concept regarding RTX toxins has been that they are secreted into the extracellular milieu, it has been known for over 25 years that AC toxin remains primarily associated with the surface of *B. pertussis*. The mechanism for this interaction and its relevance to target cell intoxication has gone uninvestigated. With the recognition that another RTX toxin, leukotoxin from *A. actinomycetemcomitans*, is cell-associated, came an analysis of the mechanism by which that toxin interacts with the bacterial surface. Leukotoxin has been shown to bind to the bacterial surface through an interaction with nucleic acids (Ohta *et al.*, 1993) and, more recently, a role for fibrils encoded by the *tad* locus in retention of leukotoxin on the surface of *A. actinomycetemcomitans* has been demonstrated (Kachlany *et al.*, 2000). Until now, leukotoxin was

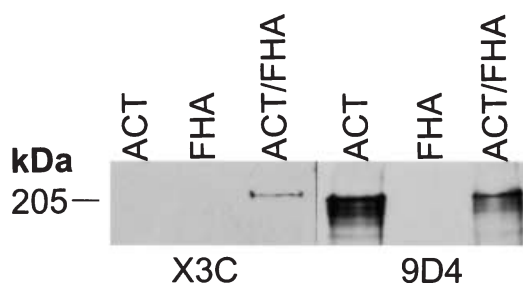


Fig. 4. FHA co-precipitates with AC toxin on beads coated with anti-AC toxin antibody. AC toxin alone (ACT; 4.4 μ g), FHA alone (FHA; 4.4 μ g), or AC toxin and FHA (ACT/FHA; 4.4 μ g each) were incubated for 2 h at 4°C. Proteins were immunoprecipitated with beads coated with 9D4, a monoclonal antibody against AC toxin. Samples eluted from beads were subjected to SDS-PAGE and transferred to nitrocellulose. The presence of FHA in ACT–FHA complexes was detected using the monoclonal anti-FHA antibody, X3C; FHA was not immunoprecipitated by 9D4-coated beads in the absence of ACT. AC toxin was detected by probing blots with 9D4; as expected, AC toxin bound to beads. Antibody binding to nitrocellulose was detected by chemiluminescence.

the only cell-associated RTX toxin in which the mechanism of toxin interaction with the bacterial surface has been extensively studied.

In the present study, we have shown that AC toxin can interact with FHA and that the presence of FHA is important for the association of AC toxin with the *B. pertussis* surface. However, a small percentage of AC toxin remains associated with *B. pertussis*, even in the absence of FHA, thus the interaction of AC toxin with the surface of *B. pertussis* is likely more complicated. Current concepts concerning the structures, subcellular locations, and functions of the *B. pertussis* type I secretion apparatus CyaBDE are mostly based on what is known regarding the HlyBD/TolC proteins involved in *E. coli* haemolysin secretion (Ludwig and Goebel, 1999; Koronakis *et al.*, 2000). In *E. coli*, secretion of haemolysin is believed to be initiated by binding of the C-terminal signal sequence to HlyB and/or HlyD, subsequent binding of the periplasmic domain of HlyD to TolC, formation of a HlyD/TolC trimeric pore, then release of haemolysin to the extracellular milieu without a periplasmic intermediate (Thanabalu *et al.*, 1998).

CyaB and D are highly homologous to HlyB and D respectively. CyaE is proposed to perform a similar function to that of TolC, but it is the most diverged of the TolC-like protein family. Of particular interest is that the N-terminal (1–200) and C-terminal (202–419) residues of CyaE share significant sequence similarities with each other (Gross, 1995). Although TolC does not possess this repetitive structure, its N-terminal residues (23–178) are similar to the repetitive regions of CyaE (Gross, 1995). AC toxin is released into the medium of *E. coli* producing

HlyBD/TolC (Sebo and Ladant, 1993). Is it possible that the amino acid sequence differences between CyaE and TolC play a role in whether the toxin is released into the medium or remains associated with the bacterium? This is certainly one possibility that must be considered.

Like other RTX toxins that are activated by acyl-modification (Hardie *et al.*, 1991), AC toxin is palmitoylated at lysine 983 by the product of *cyaC* (Barry *et al.*, 1991; Hackett *et al.*, 1994). Another possibility is that the palmitoyl group plays a role in surface retention of AC toxin through an interaction with LPS. This modification alone is unlikely to represent the basis for surface retention, however, as many RTX toxins appear to be acylated (Lally *et al.*, 1999) and most are released into the medium. Other bacteria that produce RTX toxins do not produce FHA, however, which may, in *B. pertussis*, interact with the palmitoyl group of AC toxin.

Finally, a role for the hydrophobic and repeat regions of AC toxin in surface retention cannot be ruled out. When expressed in *E. coli*, secretion of AC toxin into the medium by HlyBD/TolC is increased fourfold by deletion of at least the hydrophobic domain, with or without the repeat region, from the toxin (Sebo and Ladant, 1993). Although it is likely that this occurs because the presence of these domains facilitates aggregation and inclusion body formation in *E. coli*, a role for the hydrophobic domain and repeat region in the interaction of AC toxin with the *B. pertussis* surface warrants further study.

Bordetella pertussis can deliver AC toxin to host cells by contact (Hewlett *et al.*, 1988; Mouallem *et al.*, 1990). Preliminary studies in our laboratory suggest that live, intact bacteria intoxicate target cells more efficiently than soluble toxin isolated from the medium of FHA mutants, suggesting that the association of AC toxin with the bacterial surface induces a conformation that is much more effective for insertion of the catalytic domain into the target cell membrane. The interaction of AC toxin with FHA may help to increase the local concentration of AC toxin on the *B. pertussis* outer membrane and adherence to host cells mediated by FHA may be coupled to AC toxin delivery. These concepts, derived from demonstration of the direct interaction of AC toxin with FHA, are serving as the basis for current studies of AC toxin delivery from *B. pertussis* to target cells.

Experimental procedures

Growth of *B. pertussis* strains

All *B. pertussis* strains were cultured on Bordet–Genou agar (Gibco) containing 20% defibrinated sheep blood (Cocalico) or in Stainer–Scholte broth (Gibco) (Hewlett *et al.*, 1989) with the appropriate antibiotics at the following concentrations: nalidixic acid, 30 μ g ml⁻¹; kanamycin, 25 μ g ml⁻¹; and streptomycin, 100 μ g ml⁻¹. Antibiotics and all other chemicals,

unless otherwise stated, were obtained from Sigma. Bacteria on plates were grown at 37°C for 3–5 days then subcultured to 12 mls of broth at 35.5°C for 24 h. Bacteria were then subcultured into 50–200 mls of broth and grown for an additional 24 h before experimentation.

The *B. pertussis* strains used in this study are listed in Table 1. BPSM, BPGR4, BPSA90, BPLC5 and BPGR4(pBG1) were provided by Dr Camille Locht (Institute Pasteur, Lille, France), BP536 was received from Dr Scott Stibitz (Center for Biological Evaluation and Review, Food and Drug Administration, Bethesda, MD, USA), BBC9 was received from Dr Alison Weiss (University of Cincinnati School of Medicine, OH, USA), and BPM3171 was received from Dr Drusilla Burns (Center for Biological Evaluation and Review, Food and Drug Administration, Bethesda, MD, USA). The LPS mutant, Wlb, was a gift of Dr Duncan Maskell (Center for Veterinary Science, University of Cambridge, UK). The clinical isolates were received from Drs Joel Ward and Swei-Ju Chang (University of California Los Angeles Center for Vaccine Research, Torrance, CA, USA). *B. pertussis* strain 18323 was purchased from ATCC.

Immunoblotting

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (Laemmli, 1970) using 10% PA gels (Bio-Rad). Proteins were transferred to nitrocellulose membranes (Osmonics) at 100 V for 1 h in 20 mM Tris, 150 mM glycine, 20% methanol, blocked overnight at 4°C with 5% skimmed milk in TBS (50 mM Tris pH 7.5, 200 mM NaCl), and probed for 1–2 h at room temperature (RT) with either a rabbit polyclonal antibody (1:1000 dilution) or mouse monoclonal antibodies (mAbs), 3D1 (1:15 000 dilution) or 9D4 (1:1000 dilution), against AC toxin (Lee *et al.*, 1999). AC toxin is proteolytically sensitive, thus several high molecular weight bands are often visualized by immunoblot analysis. FHA was detected on membranes using mAb X3C (provided by Dr Drusilla Burns). All antibodies were diluted into TBS plus 5% skimmed milk. Membranes were washed three times (5–15 min each) in TBS plus 0.1% Tween 20 (TBS-T) and were incubated for 30 min to 1 h at RT in anti-rabbit or anti-mouse immunoglobulin G secondary antibody conjugated to horseradish peroxidase (HRP; 1:1000 dilution) (Jackson ImmunoResearch Laboratories). After three more washes with TBS-T, ECL Western blotting detection reagents (Amersham-Pharmacia Biotech) were added to the membranes and the bands were visualized by chemiluminescence.

Isolation and treatment of *B. pertussis* crude outer membranes

Crude outer membranes of *B. pertussis* strain BP338 were isolated by the protocol initially described by Schneider and co-workers (Schneider and Parker, 1982) and subsequently modified by Johnson and Burns (Johnson and Burns, 1994). Briefly, exponentially growing *B. pertussis* were harvested using centrifugation at 6000 r.p.m. for 10 min, washed once in phosphate-buffered saline (pH 7.5; PBS), and the bacterial pellet was resuspended in PBS and frozen at –70°C. The

pellet was thawed in cold water and the bacteria were sonicated (Model W-375; Heat Systems Ultrasonics) on power setting '3' and 50% duty cycle for 5 min on ice three times with 1 min between cycles. Unbroken cells were removed by centrifugation at 2000 *g* for 10 min and the supernate was subjected to ultracentrifugation at 107 000 *g* for 1 h to pellet the total (inner and outer) membrane fraction.

To solubilize the inner membrane, total membranes were suspended in 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) buffer, pH 7.4, containing 2% Triton X-100 and 7.5 mM MgCl₂, and were incubated on a vertically rotating wheel overnight at 4°C. The membranes were centrifuged again at 107 000 *g* for 1 h to pellet the insoluble fraction (crude outer membranes) and the presence of AC toxin in the outer membrane fraction was confirmed by immunoblot analysis using rabbit polyclonal anti-AC toxin antibody.

Crude outer membranes (80 µg) were incubated in either TEE (Tricine 10 mM, EDTA 0.5 mM, EGTA 0.5 mM-pH 8.0), 8 M urea-TEE, 10 mM octylglucoside/150 mM NaCl (pH 7.3), or 100 mM sodium carbonate (pH 11.5) overnight at 4°C and the membranes were centrifuged 107 000 *g* for 1 h. The supernate, representing the soluble fraction was removed and the pellet, representing the insoluble fraction, was resuspended in PBS to an equal volume as the soluble fraction. Equal volumes of the soluble and insoluble material were subjected to SDS-PAGE, the proteins were transferred to nitrocellulose, and the presence of AC toxin was determined by immuno-blotting with anti-AC toxin polyclonal antibody. Interestingly, whereas urea extracted AC toxin from crude outer membrane preparations, it did not extract other outer membrane proteins, such as FHA. Octylglucoside and carbonate, however, did release FHA into the soluble fraction.

Because the inner and outer membranes of *B. pertussis* cannot be separated by isopycnic centrifugation (Ezzel *et al.*, 1981), Triton X-100 is commonly used to differentially solubilize the inner membrane proteins. The procedure used here to isolate crude *B. pertussis* outer membranes has been used, with some modifications, to demonstrate the outer membrane localization of the S1 subunit of PTX (Farizo *et al.*, 2002) and proteins responsible for PTX secretion (PtIF and PtIG) (Johnson and Burns, 1994) in *B. pertussis*.

Distribution of AC toxin or FHA in mutant *B. pertussis*

Strains were cultured as described above. When the OD₆₅₀ reached between 0.7 and 1.0, cultures were centrifuged at 6000 r.p.m. for 10 min and the supernate was removed and frozen at –70°C for later determination of AC enzymatic activity. The resultant pellet was washed once with Hanks Balanced Salt Solution (HBSS; Gibco), centrifuged as above, resuspended in HBSS to the original volume, and frozen at –70°C. AC enzymatic activity in supernate and pellet fractions was determined as described previously (Hewlett *et al.*, 1989). The percentage of AC toxin associated with the bacterial pellet was determined by dividing the AC enzymatic activity in the bacterial pellet by the total AC enzymatic activity (supernate plus bacterial pellet) multiplied by 100. Results are representative of at least two independent experiments. Student's *t*-test was used to compare the percentage of bac-

teria-associated AC activity between wild-type and mutant strains.

Binding of exogenous AC toxin to *B. pertussis*

Bordetella pertussis organisms were grown to an OD₆₅₀ of between 0.7 and 1.0. Bacteria were centrifuged at 6000 r.p.m. for 10 min, washed once in HBSS, and resuspended in HBSS to the starting volume to yield approximately 10⁹ bacteria per ml. Then, 10 µg ml⁻¹ of AC toxin was added to 10⁹ bacteria and incubated for 1 h at RT. Bacteria were centrifuged at 10 000 r.p.m. in a microfuge for 5 min, washed three times with 1 ml of HBSS, and resuspended in 1 ml of HBSS. Samples were frozen at -70°C for determination of AC enzymatic activity. The percentage of exogenous AC toxin that bound to whole bacteria was determined by the following equation:

$$\frac{[(\text{total AC enzymatic activity (exogenous + endogenous)} - \text{endogenous AC activity}) / \text{AC activity added to bacteria}] \times 100}{}$$

Overlay

To determine if AC toxin can interact with proteins on a solid matrix, we subjected purified PRN, FHA, BSA (1 µg each), PTX (2 µg), and ΔN549 (AC toxin truncate lacking the first 549 amino acids of AC toxin; 10 ng) to SDS-PAGE on 10% PA gels. Purified PRN, FHA and PTX were provided by Carine Capiou (GlaxoSmithKline). Proteins were transferred to nitrocellulose and membranes were incubated with 5 µg ml⁻¹ of full-length AC toxin in TBS-T plus 5% skimmed milk for 2 h at RT. After three washes in TBS-T for 15 min each, the membranes were subjected to immunoblot analysis as described earlier, using mAb 3D1, which recognizes wild-type AC toxin, but not ΔN549.

Immunoprecipitation

To determine if AC toxin and FHA interact in solution, AC toxin, FHA, and AC toxin + FHA were mixed end-over-end at 4°C for 2 h. AC toxin was then immunoprecipitated from these samples using mAb 9D4 bound to protein G-Sepharose (Gammabind G Sepharose, Pharmacia) as described (Bonifacino *et al.*, 2001). Briefly, 30 µl of 50% protein G-Sepharose bead slurry was combined with 1 µg of mAb 9D4 and mixed end-over-end for 2 h at 4°C. Antibody-coated beads were washed three times in 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100. Then, 10 µl of 10% BSA was added to the mAb-coated bead pellet along with 1 ml of PBS containing either 4.4 µg of AC toxin, 4.4 µg of FHA or 4.4 µg of both AC toxin and FHA, which had been incubated for 2 h at 4°C. This protein/antibody-coated bead mixture was then incubated for 1 h at 4°C mixing end over end. Beads were washed four times with 50 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 0.1% Triton X-100 and once with PBS, pH 7.4. Proteins were eluted from the beads with Laemmli buffer, subjected to SDS-PAGE on 10% PA gels, and transferred to nitrocellulose membranes. The

membranes were probed either with mAb 9D4 (for detection of AC toxin) or mAb X3C (for detection of FHA).

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