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Enzymatic activity of adenylate cyclase toxin from *Bordetella pertussis* is not required for hemolysis

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Adenylate cyclase (AC) toxin from *Bordetella pertussis* enters cells to cause supraphysiologic increases in cAMP. AC toxin is also hemolytic. Substitution of Lys-58 with a methionine residue by site-directed mutagenesis of the structural gene for AC toxin, *cyaA*, and introduction of this mutation onto the *B. pertussis* chromosome results in an organism that synthesizes an enzyme-deficient AC toxin molecule. This mutant toxin molecule exhibits 1000-fold reduction in enzymatic activity relative to wild-type and has no toxin activity in J774 cells. The enzyme-deficient toxin molecule is not, however, impaired in its ability to lyse sheep red blood cells. In order to ascertain the importance of these two separate activities of AC toxin in vivo the enzyme-deficient organisms were used to infect infant mice. The hemolytic, enzyme-deficient mutant organisms are reduced in virulence relative to wild-type organisms after intranasal challenge indicating that, although the enzymatic activity of AC toxin does not contribute to hemolysis, it is this property of the toxin which is important for virulence of *B. pertussis*.

Bordetella pertussis virulence; Hemolysis

1. INTRODUCTION

Bordetella pertussis is the causative agent of whooping cough. Among the virulence factors elaborated by this organism is an adenylate cyclase (AC) toxin [1]. The AC toxin, which has an apparent mol. wt. of 216 kDa, enters eukaryotic cells and produces cAMP from endogenous ATP. Data from our laboratory and others demonstrate that purified AC toxin is also a hemolysin [2–4].

The *Bordetella* adenylate cyclase toxin shares extensive homology with *E. coli* hemolysin [5], and both toxins are thought to be important virulence factors in their respective hosts [1,6,7]. They produce osmotic lysis of red blood cells and are toxic to neutrophils. The *E. coli* hemolysin, however, possesses no associated enzymatic activity, and cell lysis which has immediate onset following toxin addition is thought to be due to creation of transmembrane pores [8]. Although pore formation appears to play a role in the hemolytic activity of AC toxin [3,9] there is no evidence for that event in neutrophils and monocytes where cellular dysfunction is attributed to target cell cAMP accumulation [10,11]. Furthermore, studies of the kinetics of intoxication and hemolysis by AC toxin reveal immediate onset of intracellular cAMP accumulation but a lag of 45–60 min for detection of hemolysis [3]. These observations raise the question of whether AC enzymatic activity may contribute to or cause the hemolytic process by generation of cAMP, depletion of ATP or other unrecognized secondary events. In order to address these issues an enzyme-deficient AC toxin mutant has been constructed and evaluated.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains and growth conditions*

E. coli strains were maintained at −20°C as glycerol stocks, and grown on glucose minimal medium plates and in liquid LB medium. Strains MM294 (pRK2013) and HB101 (pSS1129) were used for conjugation. MM294 (pRK2013) was grown on L-agar with 50 μg/ml kanamycin, and HB101 (pSS1129) was grown on L-agar with 10 μg/ml gentamycin and 50 μg/ml ampicillin. *B. pertussis* strain SM001 (streptomycin-resistant derivative of BP338) was the recipient bacteria for conjugation. SM001 was grown on Bordet Gengou (BG) plates or in Stainer-Scholte liquid medium supplemented with 30 μg/ml nalidixic acid and 300 μg/ml streptomycin.

2.2. *Mutagenesis*

Site-directed mutagenesis was performed as described by Kunkel [12] using the Muta-gene M13 in vitro Mutagenesis Kit from Bio-Rad and the procedure of Au et al. [13].

2.3. *Conjugation and recombination*

Plasmid pSS1129, a derivative of pRTP1, was constructed by Stibitz et al. [14] to facilitate the exchange of mutant *B. pertussis* DNA for wild-type DNA on the *B. pertussis* chromosome. Plasmid pSS1129, containing a fragment of mutant *cyaA*, was mobilized by conjugation of plasmid RK2013 into *Bordetella* SM001 as described previously by Barry et al. [15]. After 6 h the mixture was transferred onto BG plates containing 30 μg/ml nalidixic acid and 10 μg/ml gentamycin to select for colonies containing the plasmid integrated into the chromosome.

Bacteria from the first selection were then grown on BG plates with streptomycin and nalidixic acid to identify bacteria which had excised the plasmid from the chromosome. The nalidixic acid/streptomycin-resistant colonies were then screened for loss of enzymatic activity as described in results.

2.4. *DNA manipulations*
Restriction endonuclease digestions were performed according to the specifications of the manufacturers. Ligations were performed at 16°C overnight in total volume of 10 μl including fragment, vector and T4 DNA ligase. Transformations were performed according to standard protocols.

2.5. *Purification of adenylate cyclase toxin*
Adenylate cyclase toxin was purified as described previously [16].

2.6. *Adenylate cyclase enzyme assay*
Enzymatic activity was measured by conversion of [α-^{32}P]ATP to [^{32}P]cAMP as described previously [17].

2.7. *Adenylate cyclase toxin activity in nucleated cells*
Adenylate cyclase toxin activity was determined as described previously [17] by quantitation of intracellular cAMP in J774 cells treated with preparations containing the toxin.

2.8. *Adenylate cyclase toxin activity in red cells*
Adenylate cyclase toxin activity was measured in sheep erythrocytes as described previously [3] using cAMP determination by automated radioimmunoassay [18].

2.9. *SDS-PAGE and Western blot*
Electrophoresis of protein samples was performed according to Laemmli on 1.5 mM-thick SDS-polyacrylamide gels [19], transferred to nitrocellulose sheets and incubated with anti-AC toxin monoclonal antibody, 9D4 [16,17], using the method of Towbin et al. [20].

2.10. *Hemolysis assay*
Hemolysis assays were performed using sheep erythrocytes as target cells and the method of Clerc et al. [21] with minor modifications described previously [3].

2.11. *Infant mouse model*
Intra-nasal challenge and bacterial colonization studies were both performed on 6-day-old infant mice as described previously [22] using SM001 and BPLM58IE at the indicated inocula. To determine survival of the bacteria in the lung, mice infected with 500 colony forming units (cfu) were killed by methoxyflurane inhalation or cervical dislocation at 5-day intervals and the lungs were removed. Colony counts were determined by suspending the lungs in 5 ml of Stainer-Scholte broth, disrupting them for 60 s in a model 80 Stomacher laboratory blender, and plating dilutions on BG agar. Severity of infection was expressed as cfu of *B. pertussis* per lung.

3. RESULTS

The AC toxin purified from *Bordetella pertussis* is a single polypeptide with a mol. wt. of 177 kDa by calculation from amino acid sequence [5] and by equilibrium sedimentation [24], but 200–216 kDa by SDS-PAGE [17,23]. The discrepancy between these techniques is felt to be due to incomplete denaturation under SDS-PAGE conditions [24]. We have described an accessory gene, *cyaC*, which encodes a protein hypothesized to catalyze a post-translational modification required for toxin and hemolysin activities but not enzymatic activity [15]. In previous studies other investigators have

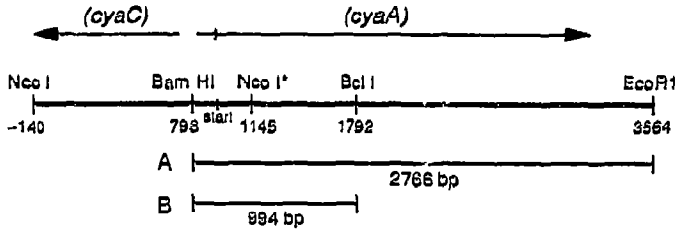

Fig. 1. Schematic representation of *cyaA* indicating fragments used for construction of enzyme-deficient mutants.

isolated and examined mutants in the enzymatic portion of the adenylate cyclase [13,25]. They employed material, however, from cloned genes expressed in *E. coli* which did not possess the *cyaC* gene and therefore lacked toxin and hemolysin activities. To generate an active toxin and hemolysin we introduced the enzyme mutation into the *Bordetella pertussis* chromosome and purified the protein from a strain that expressed all of the accessory genes.

Construction of an enzyme-deficient AC toxin is based on studies by Au et al. [13] and Glaser et al. [25] who have identified two of the amino acids involved in ATP catalysis. In those studies site-directed mutagenesis of Lys-58 to a glutamine or a methionine, or Lys-65 to a glutamine, reduced the enzymatic activity of AC toxin by >500-fold. As the first step in producing a mutant holotoxin the 2,776 bp *Bam*HI–*Eco*RI fragment (A, Fig. 1) was cloned into M13mp18, and site-directed mutagenesis was performed to substitute methionine for Lys-58, as described in section 2 and as previously described by Au et al. [13]. The advantage of these particular base changes is that an *Nco*I restriction endonuclease site is created. Consequently the products of the mutagenesis reaction, as well as the *B. pertussis* organisms resulting from mating and allelic exchange, can be screened for the presence of the *Nco*I site. A representative clone (M13mp18-LM58IE) with a new *Nco*I-site (*Nco*I*, Fig. 1) was identified and the presence of the predicted base changes was confirmed by Sanger dideoxy sequencing (data not shown).

The 994 base pair *Bam*HI–*Bcl*I fragment (B, Fig. 1) from M13mp18-LM58IE was then cloned into the *Bam*HI site of the plasmid pSS1129 (described in Materials and Methods). The mutation was introduced into *B. pertussis* by conjugation and homologous recombination. One hundred of the resulting transconjugants were screened for adenylate cyclase activity. When bacterial suspensions of primary subcultures were tested, ten had no AC activity but they were hemolytic on blood agar plates. Southern blot analysis was performed using chromosomal DNA from two of the enzyme-deficient colonies as well as a wild-type strain, confirming that the enzyme-deficient bacteria contain the newly created *Nco*I site while wild-type organisms do not (data not shown).

Once the position of the mutation in *cyaA* had been verified AC toxin was purified for functional studies from the resultant strain, BPLM581E, as described in section 2. The residual enzymatic activity of the mutant toxin is activated by calmodulin, indicating that the calmodulin-binding site has not been disturbed and enabling the mutant protein to be purified by calmodulin–Sepharose chromatography. Calmodulin affinity-purified AC toxin from the wild-type parent and BPLM581E organisms were visualized by Coomassie staining after SDS-PAGE. As seen in Fig. 2A, the preparations appear identical. The 216 kDa band from each organism reacts on Western blot with anti-AC toxin monoclonal antibody 9D4, indicating that they represent the purified holotoxin molecules (Fig. 2B). The degradation products observed only on Western blot reflect minor differences which are not explained. Characterization of the enzyme and toxin activities of AC toxin from SM001 and BPLM581E demonstrates that the enzyme specific activity of BPLM581E AC toxin is reduced 1000-fold relative to wild-type (wild-type SM001, 488 ± 51 μmol cAMP formed/min/mg in the presence of 1 μM calmodulin vs. BPLM581E, 0.412 ± 0.2). Furthermore AC toxin from BPLM581E has no detectable toxin activity in J774 cells, which are very sensitive to wild-type AC toxin (SM001, 10, 150 ± 3300 pmol cAMP/mg J774 cells protein vs. BPLM581E, 15.2 ± 1.2 with basal cAMP in untreated cells 13.9 ± 0.8).

To determine if enzymatic activity and resultant cAMP production are essential for hemolysis, the time–courses of lysis of red cells treated with wild-type or mutant toxin were compared. The data in Fig. 3 demonstrate that the AC toxin from BPLM581E is as hemolytic as wild-type toxin (A) yet does not elicit cAMP increases in erythrocytes (B). Since the onset of hemolysis with wild-type toxin is not more rapid than with BPLM581E and the rates are equivalent it appears that the presence of the enzymatic activity does not contribute to hemolysis. These results demonstrate directly that adenylate cyclase enzymatic activity is not required for the hemolytic activity of AC toxin.

In order to determine whether AC enzymatic activity is required for virulence of the intact organism we tested BPLM581E for lethality in the infant mouse model which has been used previously for evaluation of *B.*

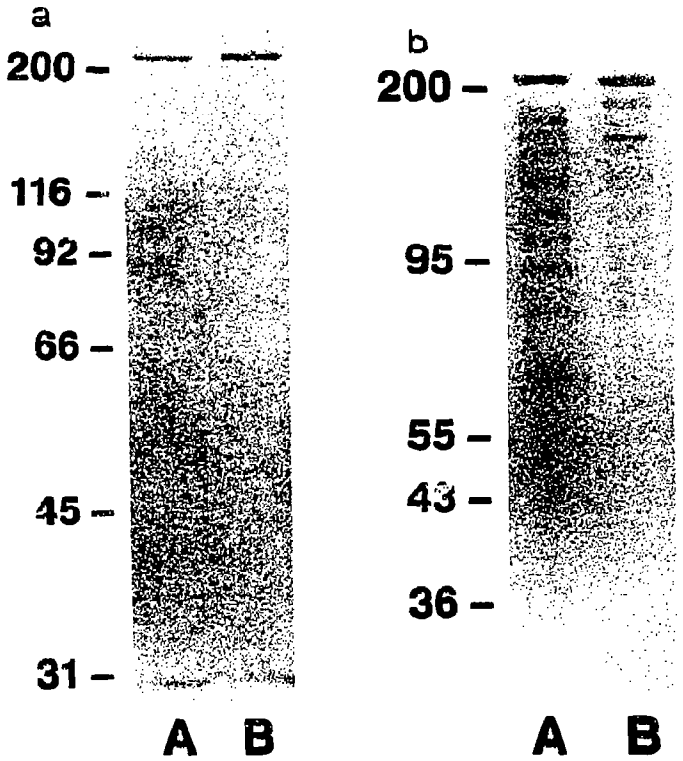

Fig. 2. AC toxin was purified from BPLME581E and SM001 as described [17] previously. Calmodulin–Sepharose-purified AC toxins from the two strains (2.8 μg) were separated by 7.5% SDS–polyacrylamide gels. The gels were stained with Coomassie blue (A) or electroblotted onto PVDF and probed with the monoclonal antibody, 9D4 (B). Lanes A contain toxin from SM001 and lanes B contain toxin from BPLM581E.

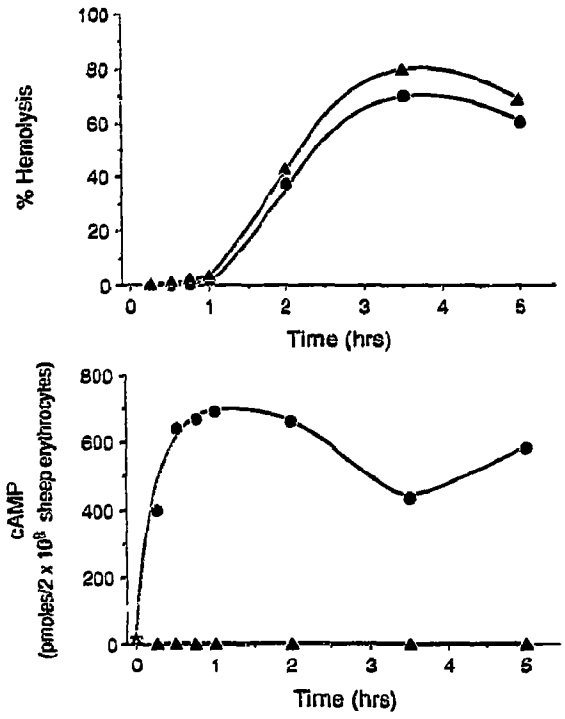

Fig. 3. Time-course of hemolysis and cyclic AMP accumulation elicited by AC toxins from SM001 and BPLM581E. (A) Calmodulin–Sepharose-purified AC toxin (75 μg/ml) from SM001 (●) or BPLME581E (▲) was incubated with 2×10^8 erythrocytes at 37°C and hemolysis was calculated at each point as described in section 2. (B) Aliquots of the same preparations from SM001 (●) and BPLM581E (▲) were incubated with 2×10^8 erythrocytes, and cAMP levels were determined as described in section 2. *B. pertussis* adenylate cyclase toxin activity represents the sum of intracellular cAMP and extracellular cAMP (from the supernatant of lysed cells) at each time point. An asterisk indicates the basal value for cAMP (2.8 pmol/2×10^8 sheep erythrocytes at time 0).

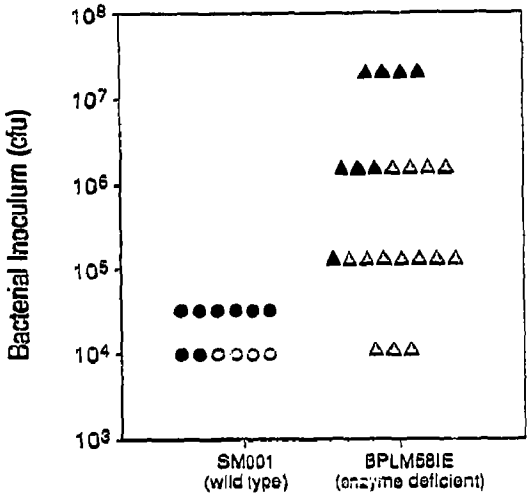

Fig. 4. Mortality of suckling mice as a function of bacterial inoculum. SM001 (●, ○) and BPLM58IE (▲, △) were prepared as described and used for intranasal inoculation of suckling mice. Closed symbols represent mice that died by 28 days; open symbols indicate survivors.

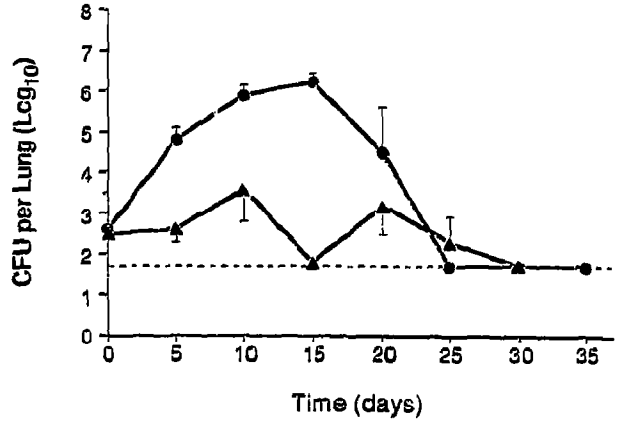

Fig. 5. Lung bacterial colony counts at five-day intervals during infection with SM001 (●) or BPLM58IE (▲). Suckling mice were challenged intranasally with 50 organisms on day 0 and groups sacrificed at 5-day intervals. Quantitation of infection was carried out as described in section 2 and expressed as mean log cfu/lung ± S.E.M. Limit of detection of the assay was 500 organisms, indicated by a hatched line. With the exception of the 30-day point for SM001 (2 mice surviving to sacrifice) all points represent mean values from 3–6 animals.

pertussis virulence factors [7,22]. Six-day-old Balb/cByJ mice were challenged intranasally with the indicated quantities of *B. pertussis* organisms (Fig. 4). At a dose of 5×10^4 wild-type organisms, 6/6 of the mice died. A 20-fold higher dose of enzyme-deficient organisms (1×10^6 cfu), however, killed only 3/7 of the mice. To elicit 100% mortality mutant organisms (BPLM58IE) at a dose of 3.5×10^7 cfu were required. These data demonstrate that the enzyme-deficient mutant is less virulent than wild-type *B. pertussis* and comparable to BP238, a mutant which is totally lacking adenylate cyclase toxin protein, as described in previous studies [7].

The ability of the bacteria to persist in infant mice was also examined. When mice were inoculated with 500 cfu, wild-type organisms multiplied within the lung and reached a maximal colony count of $>10^6$ organisms on day 15. The magnitude of infection with BPLM58IE is several logs lower in cfu yet both organisms were cleared by 25–30 days after inoculation (Fig. 5). These data suggest that adenylate cyclase enzymatic activity is important but not essential for colonization in the mouse lung, as well as establishment of lethal infection when mice are challenged with *Bordetella* organisms.

4. DISCUSSION

The kinetic studies comparing AC toxin-mediated hemolysis with intoxication [3,4] suggested that hemolysis might be a consequence of the enzymatic activity of the toxin because high levels of cAMP accumulate in the cell before lysis occurs. Under those circumstances adenylate cyclase toxin activity might cause or contribute to hemolysis through ATP depletion and/or cAMP accumulation. Two observations indicated that cAMP accumulation is probably not the cause of hemolysis: (i) exposure of stored cells to AC toxin results in lower cAMP levels and more hemolysis than in freshly drawn red cells (Ehrmann et al. unpublished data); (ii) addition of 8-bromo-cAMP does not cause hemolysis [2]. Consequently it was postulated that perhaps AC enzymatic activity contributed to hemolysis through ATP depletion. Construction of an enzyme-deficient AC toxin molecule has resolved the issue of whether enzymatic activity is responsible for hemolysis with resultant data demonstrating equivalent rates and magnitudes of hemolysis between wild-type and enzyme-deficient AC toxin.

Since enzymatic activity does not cause AC toxin-mediated hemolysis, what is the mechanism of this lytic event? Data from osmotic protection experiments suggest that AC toxin forms a pore in the erythrocyte membrane [3,9]. AC toxin interacts with target membranes within seconds [3,26] yet there is no evidence to indicate that a functional pore forms immediately because hemolysis does not begin until at least 45 min later. It may be that pore formation is the rate limiting step for hemolysis. Two possible explanations for this delay in hemolysis are: (i) aggregation or oligomerization of the toxin is required for development of a functional pore; or (ii) that the toxin must be processed in the membrane before a pore is formed. If proteolysis of the toxin results in 'opening' of the pore and subsequent hemolysis, one might find lower molecular weight forms of AC toxin in the red cell membranes. Rogel et al. [4] however, have used SDS-PAGE to examine red cell membranes exposed to iodinated AC toxin and found only the high molecular weight form (216 kDa) of the toxin. It is still possible that proteolysis is occurring and that SDS-

PAGE may not be sufficiently sensitive to detect small changes in molecular weight of large proteins. Another explanation is that aggregation of the toxin is required for hemolysis. There is no experimental evidence to support this hypothesis at present but the possibility is being explored with electron microscopy and more detailed kinetic studies. Nevertheless it appears that some processing step, which does not require AC enzymatic activity, occurs after AC toxin interacts with the red cell membrane and before hemolysis occurs.

Not only is AC toxin which has been purified from the enzyme-deficient mutant useful as a reagent to explore the role of enzymatic activity in hemolysis, it is also essential for determination of the role of AC activity in vivo. The results from the lethal challenge of infant mice show that the enzyme-deficient mutant is less virulent than wild-type. The mutant BPLM58IE, which synthesizes the enzyme-deficient toxin but retains hemolysin activity, is comparable in virulence to BP348 [7], a mutant which produces no detectable AC toxin molecule. These two organisms exhibit similar LD_{50} values, suggesting that the enzymatic activity and not the hemolytic activity is important in this test of virulence. Another organism, BPDE386, a *cyaC* gene mutant which is non-hemolytic but has full enzymatic activity, has also been shown to be less virulent than wild-type organisms for infant mice [15]. The gene product of *cyaC* is postulated to catalyze a post-translational modification of the toxin rendering it capable of interacting with target cells. The results of these studies suggest that full virulence of *B. pertussis* organisms is dependent upon possession of an AC toxin with enzymatic activity and the ability to interact with target cell membranes.

In addition to determination of doses required for lethal infection the bacteria were tested for their ability to survive in the mouse lung. Both wild-type and BPLM58IE were cleared by 25–30 days. Colony counts in the lungs, however, were higher in the mice infected with the wild-type strain. These results suggest that AC enzymatic activity may affect the magnitude but not the duration of infection, and are consistent with the in vitro studies which suggested that the AC enzymatic activity helps the organism evade immune effector cells by interfering with bacterial killing mechanisms such as superoxide production [10,11].

In contrast to *E. coli* hemolysin, which appears to be a virulence factor as a consequence of its hemolytic activity [6,8], it appears that AC enzymatic activity, more than hemolytic activity, is important in vivo. Since *E. coli* hemolysin and AC toxin share sequence homology and some structural similarity [3,5,25] it was postulated that the mechanisms of hemolysis and virulence might be the same. Although both toxins may form pores in red cells [3,5,8] *E. coli* hemolysin lyses cells immediately while AC toxin displays a lag period. It was believed that a contribution of AC enzymatic activity to hemolysis might provide an explanation for the difference in kinetics of hemolysis between the two toxins, yet it now appears that AC enzymatic activity has no role in hemolysis. Whether there is any structural role for the N-terminal catalytic domain (even when inactive) in hemolysis remains to be determined. Thus, it is reasonable to conclude that the hemolytic activity of the AC toxin contributes little or nothing to the virulence of *B. pertussis* in the absence of enzymatic activity. Further study of the mechanism of AC toxin-mediated hemolysis will surely enhance our understanding of the way in which AC toxin and other bacterial toxins interact with target cell membranes.

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