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Targeted mutations that ablate either the adenylate cyclase or hemolysin function of the bifunctional cyaA toxin of *Bordetella pertussis* abolish virulence

(point mutant/pertussis pathogenesis/component vaccines)

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ABSTRACT **_Bordetella pertussis_, the causative agent of whooping cough, secretes several toxins implicated in this disease. One of these putative virulence factors is the adenylate cyclase (AC) toxin that elevates intracellular cAMP in eukaryotic cells to cytotoxic levels. This toxin is a bifunctional protein comprising both AC and hemolysin (HLY) enzymatic domains. The gene encoding the AC toxin (_cyaA_) is expressed as part of an operon that includes genes required for secretion or activation of the toxin. Because of this genetic organization, it is difficult to create _B. pertussis_ mutants of _cyaA_ that are ablations of a single enzyme function by conventional means, such as transposon mutagenesis. Therefore, to clarify the role of individual toxin functions in the virulence of _B. pertussis_, we have used site-directed or deletion mutagenesis and genetic recombination to specifically target the _cyaA_ gene of _B. pertussis_ to produce mutants that lack only the AC or HLY activity of this toxin. A point mutant of _B. pertussis_ with abolished AC catalytic activity was >1000 times less pathogenic to newborn mice than wild-type bacteria, directly demonstrating the importance of the AC toxin in pertussis virulence. Similarly, an in-frame deletion mutant of _B. pertussis_ that lacks HLY is equally avirulent, supporting observations that the HLY domain plays a critical role in AC toxin entry into cells. Furthermore, the genetically inactivated AC toxin produced by the point mutant is antigenically similar to the native toxin, suggesting that this strain may be useful in the development of pertussis component vaccines.**

The human pathogen *Bordetella pertussis* produces several unique biologically active factors that are thought to play a role in the pertussis disease process. Despite the availability of pertussis vaccines, concern about adverse reactions has compromised their use, resulting in an increased incidence of the disease (1–3). Thus, there is considerable interest in defining the contribution of these putative virulence factors to the pathogenesis of pertussis and to immunoprotection from the disease.

Early studies with transposon mutants of *B. pertussis* suggested that at least two of these bacterial factors, pertussis toxin and adenylate cyclase (AC) toxin, are crucial for virulence in animal models of pertussis (4, 5). Like pertussis toxin, the AC toxin of *B. pertussis* causes dramatic increases of intracellular cAMP concentrations in a number of eukaryotic cell types (6–8). Molecular analysis of the structural gene (*cyaA*) encoding the AC toxin has shown that it is a 200-kDa bifunctional protein comprising a calmodulin (CaM)-sensitive AC catalytic domain at the N terminus and a hemolysin (HLY) domain at the C terminus (9, 10). The mechanism by which the AC domain of the toxin enters target cells is not clear but appears to require the HLY domain of the toxin (11) by a pathway independent of receptor-mediated endocytosis (12, 13).

As genetic analysis of *B. pertussis* has progressed, it has become apparent that several of its accepted virulence factors, including AC toxin, pertussis toxin, and filamentous hemagglutinin, are expressed within operons that include genes required for secretion or activation of their respective factors (10, 14–16). For example, immediately downstream of the *cyaA* structural gene are genes involved in secretion of the AC toxin (*cyaB*, *cyaD*, and *cyaE*), and at least one other gene (*cyaC*) located upstream and in opposite orientation to *cyaA* is required for activation of the AC toxin to a hemolytic and cell-invasive form (10, 16). Given this genetic organization, it is crucial to reexamine the classification of these bacterial proteins as virulence factors based on observations of the diminished virulence of *B. pertussis* transposon mutants in animal models of pertussis. Because of the polar nature of transposon mutagenesis (17), transposon insertion within an operon has the potential to reduce or disrupt the expression of adjacent genes encoding less well characterized functions. This has been shown to be the case for at least three widely used transposon mutants of *B. pertussis*. In two filamentous hemagglutinin$^-$ strains (BP353 and BP354) and one HLY$^-$ strain (BP349), transposon insertions map outside the structural gene encoding its respective virulence factor, resulting in mutants with complex phenotypes that are not simple ablations of a single gene product (10, 15). Furthermore, it is difficult to determine with certainty whether transposon mutants are less virulent because of their observed effects on a given phenotype or because of some hidden effect on an uncharacterized downstream gene product.

To avoid the problems intrinsic to transposon mutants and to clarify the role of individual toxin functions in the virulence of *B. pertussis*, we have used homologous recombination to create specific *B. pertussis* mutants, expressing mutations in *cyaA* that target and ablate either the AC or HLY function of this toxin. We report here that an AC$^-$ point mutant of *B. pertussis* is avirulent to newborn mice, directly demonstrating that the AC toxin plays a major role in the pathogenesis of pertussis. Furthermore, in contrast to earlier observations in transposon mutants, an HLY$^-$ strain of *B. pertussis* proved to be as avirulent to newborn mice as the AC$^-$ strain, indicating that some function of the HLY domain of the AC toxin is required for pertussis virulence.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *B. pertussis* Nalr11 is a nalidixic acid-resistant and streptomycin-resistant mutant of

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Abbreviations: AC, adenylate cyclase; CaM, calmodulin; HLY, hemolysin; BSA, bovine serum albumin; cfu, colony-forming unit(s).

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the virulent phase I Tohama strain. *Escherichia coli* SM10 (18) was used for conjugative transfer of mutant *cyaA* fragments carried on the gene replacement vector pSS1129 into *B. pertussis*. Vector pSS1129 [generously provided by Scott Stibitz (Federal Drug Administration, Bethesda, MD)] is identical to gene replacement vector pRTP1 (19), except for the insertion of a gentamicin-resistance cassette.

Construction of *cyaA* Mutants. Mutagenesis was performed on a 2.7-kilobase (kb) *Bam*HI–*Eco*RI fragment of *cyaA* subcloned in pUC19 and expressed in *E. coli* (20). A mutant *cyaA* fragment carrying a selectable marker approximately equidistant between the two regions of *cyaA* to be specifically mutated was created by insertion of a 1.6-kb *Bam*HI kanamycin-resistance cassette from pUC4-KIXX (Pharmacia) into the *Bcl* I site of *cyaA* (Fig. 1). Oligonucleotide-directed mutagenesis to substitute methionine for lysine at position 58 of *cyaA* was performed as described (20) to produce the mutant *cyaA* fragment used for the creation of the AC^- point mutant strain A2-6 (Fig. 1). Excision of a 1047-base-pair *pfl*MI fragment of *cyaA* and religation produced the mutant *cyaA* fragment used for the creation of the HLY^- deletion mutant strain 32-5 which lacks amino acids 469–817 of the cyaA toxin.

All of the mutant *cyaA* fragments were subsequently excised from pUC19 and ligated in the appropriate orientation into a recombinant pSS1129 vector carrying an additional 2-kb *Bam*HI fragment from directly upstream of *cyaA*. Mutant strains of *B. pertussis* were created by conjugative transfer of these recombinant pSS1129 vectors and selection for genetic recombination, according to the method of Stibitz *et al.* (19). Allelic exchange of the AC^- or HLY^- mutant *cyaA* gene was accomplished in a two-step process to ensure that genetic recombination did not occur outside *cyaA* (Fig. 1). The chromosomal *cyaA* was first marked with a selectable phenotype by homologous recombination of the kanamycin-resistance insertion mutation into *B. pertussis*, creating an AC^- and HLY^- strain (S7c2). In the second cycle, the marked chromosomal gene was replaced with either the AC^- point mutation or the HLY^- deletion mutation; recombination at the appropriate site was confirmed by phenotype analysis and either Southern (21) or immunologic blot analysis.

Hemolysis Assays. Hemolytic phenotype was determined using a modification of a microwell assay (22) on Bordet–Gengou blood/agar plates. Wells of 2 mm in diameter were made in blood/agar plates with sterile Pasteur pipettes. The wells were inoculated with 5 μl of an overnight liquid culture of selected strains of *B. pertussis*, and the plates were maintained at 37°C for 3–4 days. Hemolytic activity was detected as a zone of clearing surrounding the central well.

AC Assay. AC activity was measured by the method of Salomon *et al.* (23) in an assay mixture containing 20 mM Tris·HCl (pH 7.4), 1 mM [α-^{32}P]ATP, 5 mM $MgCl_2$, 1 mM EDTA, 0.1% bovine serum albumin (BSA), and 2.4 μM CaM.

Virulence Testing in Newborn Mice. Bacteria from 2- to 3-day-old cultures on Bordet–Gengou agar were harvested and suspended in sterile Stainer–Scholte medium (24) at an OD_{650} of 0.2 unit [equivalent to approximately 10^7 colony-forming units (cfu) per 20-μl inoculum]. Intranasal inoculations of 6- to 7-day-old BALB/c mice with 20 μl of this bacterial stock or an appropriate dilution were performed as described by Weiss and colleagues (4, 5). All animal experimentation and care was conducted in accordance with guidelines established by the University of Washington Animal Care Committee.

RESULTS

Identification and Characterization of an AC^- Mutant of *B. pertussis*. We (20) and other researchers (25) have identified point mutations at Lys^{58} of the *B. pertussis* AC toxin that abolished the AC catalytic activity of the cyaA toxin expressed in *E. coli*. Therefore, we returned a single $Lys^{58} \rightarrow$ Met point mutation to the chromosome of *B. pertussis* for the purpose of directly testing the role of the AC toxin in virulence. This substitution was accomplished in two cycles of genetic recombination as diagrammed in Fig. 1. The chromosomal *cyaA* was first marked with a 1.6-kb kanamycin-resistance cassette to produce an insertion mutant that was AC^- and HLY^-; the resistance cassette was in turn replaced by homologous recombination with the point mutation. The expected gain and loss of the extra 1.6 kb of coding sequence within a 2-kb *Eco*RV restriction fragment was confirmed by Southern blot analysis of bacterial DNA from each step in this process.

The phenotypes of the resulting *B. pertussis* strains were screened to ascertain which had undergone homologous recombination in the desired region, by selecting clones that regained hemolytic activity (i.e., lytic zones surrounding colonies grown on blood/agar; Fig. 2) and lost the kanamycin resistance of the insertion mutant intermediate. Potential positive clones were subsequently grown in Stainer–Scholte medium supplemented with BSA at 2 mg/ml to optimize secretion of the 200-kDa AC toxin (11). Conditioned medium

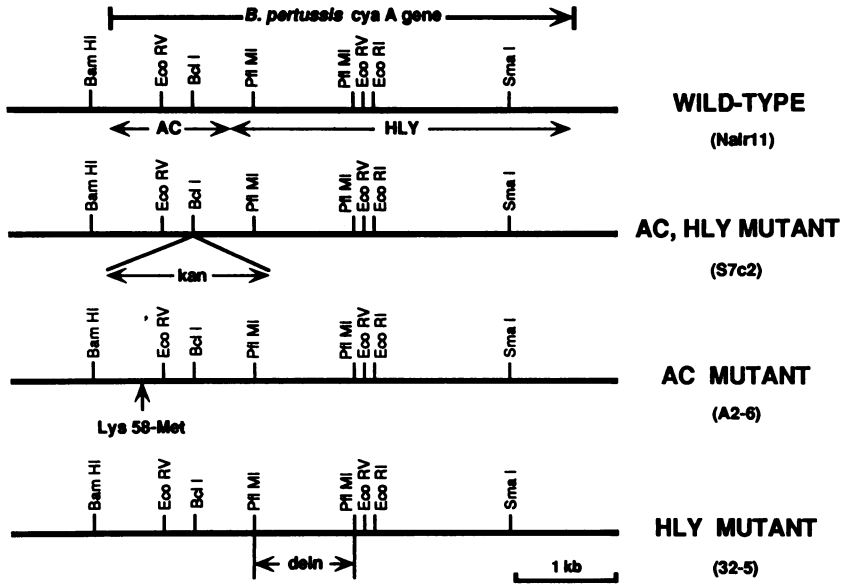

FIG. 1. *B. pertussis* strains. Wild-type *B. pertussis* Nalr11 is a streptomycin- and nalidixic acid-resistant variant of the Tohama strain of *B. pertussis*. Mutants of *B. pertussis* were produced by mutagenesis of *cyaA* in *E. coli* and allelic exchange in *B. pertussis* by using the gene replacement vector pSS1129. Genetic recombination of a mutant *cyaA* encoding a kanamycin-resistance insertion mutation (kan) created the AC^-, HLY^- *B. pertussis* S7c2. The AC^- mutant A2-6 was produced by homologous recombination of a mutant *cyaA* encoding a Lys → Met point mutation at position 58 of the AC domain of the toxin. The HLY^- mutant 32-5 was produced by homologous recombination of a mutant *cyaA* carrying an in-frame deletion mutation that eliminated amino acids 469–817 in the HLY domain of the AC toxin.

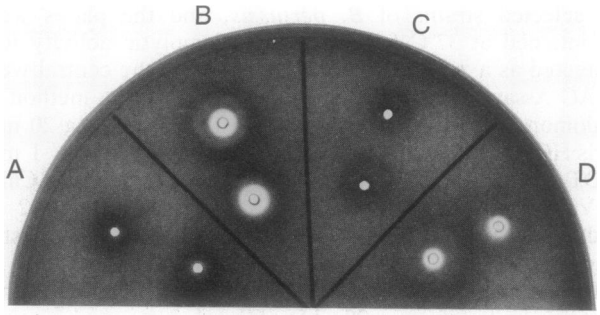

FIG. 2. Hemolytic phenotype of wild-type and mutant *B. pertussis*. The hemolytic phenotype was determined using a microwell assay on blood/agar plates. Duplicate wells were inoculated with the following *B. pertussis* strains. (*A*) AC$^-$, HLY$^-$ strain S7c2. (*B*) Wild-type Nalr11. (*C*) HLY$^-$ mutant 32-5. (*D*) AC$^-$ point mutant A2-6.

from wild-type or from mutant *B. pertussis* cultures was assayed for CaM-sensitive AC activity at several points in the bacterial growth curve. At all time points examined, the *B. pertussis* point mutant (A2-6) showed secreted AC activity that was less than 0.2% of that observed for the wild-type strain (Fig. 3). Similar results were obtained when the cell-associated AC activity of the mutant and wild-type strains was examined (data not shown). Although the observed HLY activity of strain A2-6 argues that no global disruption of *vir* regulation of toxin expression occurred during the creation of this mutant, we confirmed this fact by demonstrating that the point mutant also expressed wild-type levels of islet-activating protein catalyzed ADP-ribosylation of the inhibitory guanine nucleotide binding protein G_i (M.K.G. and D.R.S., unpublished data).

As implied by the demonstration of hemolytic activity of the point mutant, immunoblot analysis confirmed that strain A2-6 secretes a 200-kDa cyaA toxin at levels that are fairly comparable to those observed for the parental wild-type strain (Fig. 4). Furthermore, antibodies raised to the 200-kDa cyaA toxin recognized both the native protein from wild-type *B. pertussis* and the mutant toxin from strain A2-6, suggesting that the two proteins are sufficiently similar antigenically to show immunologic cross-reactivity.

Identification and Characterization of a HLY$^-$ Mutant of *B. pertussis*. To ablate only the HLY function of the AC toxin without affecting expression of AC activity or other operon genes involved in toxin processing, the HLY domain of the

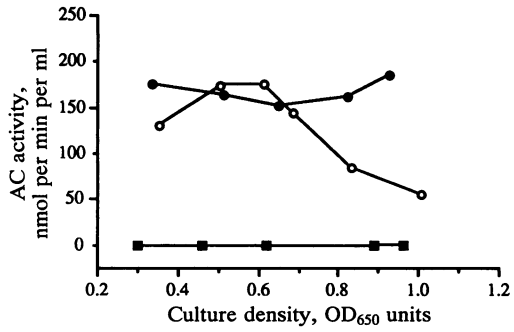

FIG. 3. Secreted AC activity of wild-type and mutant *B. pertussis*. Wild-type Nalr11 (○), AC$^-$ mutant A2-6 (■), or HLY$^-$ mutant 32-5 (●) *B. pertussis* were inoculated into 50-ml cultures of Stainer–Scholte minimal medium supplemented with BSA at 2 mg/ml and grown at 37°C (200 rpm) for 48 h. At the indicated culture densities (OD_{650} units), 1 ml of the culture was removed and microcentrifuged for 15 min at 4°C. AC activity present in the supernate was subsequently measured by the method of Salomon *et al.* (23) by using [α-^{32}P]Mg-ATP as a substrate and excess CaM as an activator. Strains are as indicated in Fig. 1.

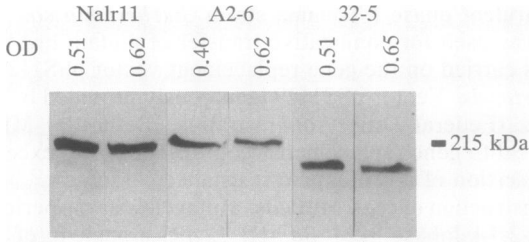

FIG. 4. Immunoblot analysis of full-length cyaA toxin secretion from wild-type and mutant *B. pertussis*. Conditioned medium was prepared from wild-type and mutant *B. pertussis* strains grown to approximately equivalent optical densities in Stainer–Scholte medium supplemented with BSA as in Fig. 3. The secreted proteins present in 40 μl of the conditioned medium were separated by SDS/PAGE (26) and subsequently immunoblotted with antigen-purified rabbit antiserum raised to the native 200-kDa cyaA toxin (kindly provided by Theresa Serwold-Davis, University of Washington, Seattle). Immunoreactive bands were visualized with an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin secondary antibody. OD_{650} values are shown.

AC toxin was disrupted by an in-frame deletion mutation that was first introduced into *cyaA* subcloned in *E. coli* and then returned to the *B. pertussis* genome by homologous recombination (Fig. 1). Similar deletions in this region of *cyaA* abolish the hemolytic and cell-entry properties of the AC toxin but leave the AC catalytic activity of the toxin intact (11).

B. pertussis clones that had undergone genetic recombination flanking the region of the deletion mutation were identified by their phenotype and by immunoblot analysis. Potential positive clones did not produce lytic zones on blood/agar plates (Fig. 2) and had lost their kanamycin resistance after the final round of genetic recombination. Selected clones were subsequently grown in liquid culture under conditions that optimized secretion of the full-length AC toxin (11) to analyze the AC activity and size of the cyaA toxin that they produced. At all stages of bacterial growth that were examined, the HLY deletion mutant strain 32-5 produced comparable or higher levels of AC catalytic activity in the culture medium than did wild-type Nalr11 *B. pertussis* (Fig. 3). Immunoblot analysis confirmed that both strains secreted similar levels of the full-length cyaA toxin and that the smaller size of the toxin produced by strain 32-5 was consistent with the amount of coding sequence eliminated by the deletion mutation (Fig. 4). Despite its high AC catalytic activity, the toxin produced by strain 32-5 did not increase intracellular cAMP levels in eukaryotic cells (M.K.G., C. S. Wong, and D. J. Oldenburg, unpublished data). This phenomenon has been observed for other deletion mutations in this region of *cyaA* (11).

Virulence Properties of Wild-Type, AC$^-$, or HLY$^-$ *B. pertussis*. To compare the virulence characteristics of the AC$^-$ or HLY$^-$ mutant relative to wild-type *B. pertussis*, we examined the lung infection and survival rates of newborn mice challenged with intranasal inoculations of the bacterial strains. This animal model has been used extensively to characterize the virulence of *B. pertussis* transposon mutants (4, 5) and to identify potential immunoprotective antigens that may modify the course of the disease (27–29). In contrast to wild-type *B. pertussis*, the AC$^-$ point mutant A2-6 is unable to establish a lethal infection in newborn mice at the highest administered dose (10^7 cfu) of bacteria tested (Table 1). Furthermore, the AC$^-$ point mutant was associated with a greater survival frequency among inoculated animals than the survival frequency observed for animals inoculated with 1000 times lower doses of wild-type *B. pertussis*. This observation directly demonstrates that the AC toxin is a crucial virulence factor in this model of pertussis. The lack of

Table 1. Virulence of wild-type or mutant *B. pertussis* to newborn BALB/c mice

Strain	Dose, cfu	No. tested	No. of survivors	Lung colonization at post-exposure days 25/40/50
Nalr11 (wild type)	1×10^4	9	8 (89)	ND/ND/+
	1×10^5	9	0	
	1×10^6	9	1 (11)	ND/ND/+
A2-6 ($Lys^{58} \rightarrow$ Met)	1×10^3	9	9 (100)	+/+/−
	1×10^5	9	9 (100)	+/+/+
	1×10^7	9	9 (100)	+/+/−
32-5 (HLY^-)	2×10^4	8	8 (100)	+/+/+
	2×10^5	9	9 (100)	+/+/+
	2×10^6	9	9 (100)	+/+/+
	2×10^7	5	5 (100)	+/+/+

Number of bacteria (cfu) delivered in an intranasal inoculum volume of 20 μl of sterile Stainer–Scholte medium is shown as the dose. Number of survivors to day 25 post-exposure is shown; the percent survival is in parentheses. +, Lung colonies detected in survivors at the indicated day after exposure; −, lung colonies not detected in survivors at the indicated day after exposure; ND, not determined.

virulence of the point mutant does not appear to be due to an intrinsic lack of viability of this bacterial strain, as we have been unable to detect any differences in growth characteristics between A2-6 and wild-type *B. pertussis* under several different *in vitro* growth conditions. Moreover, despite the inability of the AC^- mutant to establish a lethal respiratory infection in newborn mice, animals inoculated with A2-6 show persistent lung colonization with viable bacteria up to 40 or 50 days after exposure (Table 1).

Similarly, the HLY^- *B. pertussis* mutant was unable to establish a lethal infection in newborn mice at doses up to 2×10^7 cfu. Animals challenged with the HLY^- mutant showed a higher survival frequency than animals inoculated with doses of wild-type *B. pertussis* more than 1000 times lower. This observation is identical to the virulence data obtained for the AC^- point mutant of *B. pertussis*, suggesting that the role of the cyaA toxin in pertussis pathogenesis requires functional components of both the AC and the HLY domains of the molecule. Again, the pattern of persistence of HLY^- bacteria in the lungs of inoculated animals was indistinguishable from the wild-type organism, indicating that the lack of virulence of strain 32-5 cannot be attributed to a simple lack of bacterial viability.

DISCUSSION

The availability of targeted mutants in either the AC or HLY function of the *B. pertussis* cyaA toxin made it possible to directly examine the role of these entities in the virulence of this pathogen. The present data directly demonstrate that the cyaA toxin is critical for *B. pertussis* virulence and that the HLY and AC structural determinants of the toxin are equally important for pertussis pathogenesis. The latter observation is not consistent with early studies of the virulence of transposon mutants of *B. pertussis*. Whereas a transposon mutant (strain BP348) lacking both AC and HLY functions of the cyaA toxin was avirulent, a HLY^- transposon mutant with reduced AC activity (strain BP349) was only moderately less virulent than wild-type *B. pertussis* to newborn mice (5). Subsequent mapping of the transposon in BP349 to 2 bases downstream of the stop codon for *cyaA* (10) supports the conjecture that this transposon insertion disrupts the expression of *cyaB* and possibly other downstream genes involved in secretion of the toxin. Thus, it is likely that the moderately lower virulence of strain BP349 is due to a dosage effect on secretion of the cyaA toxin rather than to an ablation of hemolytic activity of the toxin. This example illustrates the difficulty of assessing contributions to virulence based on comparisons between phenotypically complex transposon mutants.

In addition to its role in pathogenesis, recent evidence suggests that the AC toxin may be an important immunoprotective antigen in prevention of or recovery from pertussis (27–30). Anti-AC toxin antibodies have been shown to prevent the development of lethal hemorrhagic alveolitis in mice challenged with virulent *B. pertussis* (27–29). The AC toxin is cytotoxic to phagocytic leukocytes *in vitro* (6) and may contribute to evasion of host immune defenses, permitting initial lung colonization by the bacterium (31). Furthermore, antibodies to the AC toxin are common in sera from patients diagnosed with pertussis, suggesting a potential role for these antibodies in immunomodulation of the disease in humans (30).

The observation that there is antigenic relatedness between the cyaA toxin produced by the AC^- point mutant and the native toxin from wild-type *B. pertussis* is not unexpected, because the two toxins differ at only a single residue within a primary structure of 1706 amino acids. Thus, the point mutant could provide a source of genetically inactivated cyaA toxin for inclusion in acellular pertussis component vaccines. Current cellular pertussis vaccine administration is associated with occasional severe side effects, some of which may be due to persistence of active bacterial toxins in the vaccines (32–34). The first generation of acellular pertussis vaccines, based predominantly on inactivated pertussis toxin or pertussis toxin and filamentous hemagglutinin, lessen the severity of disease in clinical trials but provide only incomplete protection against pertussis infection (35, 36). Therefore, the *B. pertussis* AC toxin, which has been implicated in the establishment of pertussis infection in animal models of the disease (31), may be a good candidate for inclusion in future generations of acellular vaccines.

Furthermore, the *in vivo* evidence that HLY is required for *B. pertussis* virulence supports *in vitro* observations that the HLY domain of *cyaA* contributes to toxin entry into target cells (11, 16) and suggests that it is not AC activity *per se* but the invasive characteristic of the AC toxin that is crucial to the pertussis disease process. It also seems likely that it is not the ability to lyse erythroid cells that is of significance in the progress of this disease, but rather the cell entry properties that the HLY domain confers on the AC catalytic unit that is critical for virulence (37). Further elucidation of the pathogenic and immunogenic properties of these targeted mutants may provide insight into potential strategies for protection from pertussis, a disease that remains a worldwide health concern.

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