The Eukaryotic Host Factor 14-3-3 Inactivates Adenylate Cyclase Toxins of *Bordetella bronchiseptica* and *B. parapertussis*, but Not *B. pertussis*

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**ABSTRACT** *Bordetella pertussis*, *Bordetella bronchiseptica*, and *Bordetella parapertussis* share highly homologous virulence factors and commonly cause respiratory infections in mammals; however, their host specificities and disease severities differ, and the reasons for this remain largely unknown. Adenylate cyclase toxin (CyaA) is a homologous virulence factor that is thought to play crucial roles in *Bordetella* infections. We herein demonstrate that CyaAs function as virulence factors differently between *B. bronchiseptica/B. parapertussis* and *B. pertussis*. *B. bronchiseptica* CyaA bound to target cells, and its enzyme domain was translocated into the cytosol similarly to *B. pertussis* CyaA. The hemolytic activity of *B. bronchiseptica* CyaA on sheep erythrocytes was also preserved. However, in nucleated target cells, *B. bronchiseptica* CyaA was phosphorylated at Ser375, which constitutes a motif (RSXpSXP [pS is phosphoserine]) recognized by the host factor 14-3-3, resulting in the abrogation of adenylate cyclase activity. Consequently, the cytotoxic effects of *B. bronchiseptica* CyaA based on its enzyme activity were markedly attenuated. *B. parapertussis* CyaA carries the 14-3-3 motif, indicating that its intracellular enzyme activity is abrogated similarly to *B. bronchiseptica* CyaA; however, *B. pertussis* CyaA has Phe375 instead of Ser, and thus, was not affected by 14-3-3. In addition, *B. pertussis* CyaA impaired the barrier function of epithelial cells, whereas *B. bronchiseptica* CyaA did not. Rat infection experiments suggested that functional differences in CyaA are related to differences in pathogenicity between *B. bronchiseptica/B. parapertussis* and *B. pertussis*.

**IMPORTANCE** *Bordetella pertussis*, *B. bronchiseptica*, and *B. parapertussis* are bacterial respiratory pathogens that are genetically close to each other and produce many homologous virulence factors; however, their host specificities and disease severities differ, and the reasons for this remain unknown. Previous studies attempted to explain these differences by the distinct virulence factors produced by each *Bordetella* species. In contrast, we indicated functional differences in adenylate cyclase toxin, a homologous virulence factor of *Bordetella*. The toxins of *B. bronchiseptica* and presumably *B. parapertussis* were inactivated by the host factor 14-3-3 after phosphorylation in target cells, whereas the *B. pertussis* toxin was not inactivated because of the lack of the phosphorylation site. This is the first study to show that 14-3-3 inactivates the virulence factors of pathogens. The present results suggest that pathogenic differences in *Bordetella* are attributed to the different activities of adenylate cyclase toxins.

**KEYWORDS** 14-3-3, *Bordetella*, CyaA

*Bordetella pertussis* causes whooping cough, a contagious respiratory disease that is recently resurgent despite high vaccination coverage (1, 2). *Bordetella pertussis* is often called “classical *Bordetella,*” together with closely related *Bordetella parapertussis*...
Bordetella bronchiseptica, which also cause respiratory infections in various host animals with severe to mild coughing. A genomic analysis indicated that B. pertussis and B. parapertussis independently evolved from a B. bronchiseptica-like ancestor (3). Classical Bordetella is known to share many virulence factors, including adenylate cyclase toxin (CyaA), dermonecrotic toxin (DNT), and filamentous hemagglutinin, but not pertussis toxin, which is specific to B. pertussis. The molecular actions of these major virulence factors have been extensively examined. However, the pathogenesis of Bordetella infections has yet to be elucidated in detail (4). Furthermore, disease-causing propensities and host specificities apparently differ among classical Bordetella (5). B. pertussis is a strictly human pathogen that causes acute and severe respiratory infections with paroxysmal coughing and vomiting. B. parapertussis infects sheep and humans and is associated with a milder pertussis-like disease. B. bronchiseptica exhibits the broadest host range of mammals and leads to chronic and often asymptomatic infections. Limited information is currently available on how these three species exhibit different characteristics as pathogenic bacteria despite sharing many homologous virulence factors.

CyaA, one of the virulence factors commonly produced by classical Bordetella, is considered to play a key role in the establishment of infection (5). The toxin consists of two functional modules: the N-terminal adenylate cyclase domain (ACD), which is activated by eukaryotic calmodulin and causes supraphysiological cyclic AMP (cAMP) accumulation after being translocated into the cytosol of target cells, and the C-terminal RTX (repeats-in-toxin) domain, which is responsible for binding to target cells and organizing cation-selective toxin pores on the cell membrane (6). The toxin affects myeloid cells expressing CD11b/CD18 and subverts host immunity by inhibiting phagocytosis, chemotaxis, and superoxide production and modulating dendritic cell maturation and inflammatory cytokine/chemokine production (7–13). CyaA also lyse erythrocytes and induces apoptosis in macrophages (14–16). Moreover, previous studies showed that epithelial cells may be targets of CyaA (17, 18).

Since CyaA is highly conserved among classical Bordetella, its function and role have been considered to be similar in bacterial infections. However, we herein demonstrated that B. bronchiseptica CyaA, in contrast to B. pertussis CyaA, exhibited only weak toxicity in nucleated cells. After being translocated into target cells, B. bronchiseptica CyaA was phosphorylated in the cytosol and associated with the eukaryotic host factor 14-3-3, which resulted in the abrogation of adenylate cyclase activity. To the best of our knowledge, this is the first study to demonstrate that a virulence factor of pathogens is inactivated by 14-3-3 in target cells. Our results also suggest that a difference in cytotoxic effects between B. pertussis CyaA and B. bronchiseptica CyaA influences the pathogenicity of each Bordetella species in a rat infection model.

**RESULTS**

CyaA of B. pertussis, but not CyaA of B. bronchiseptica or B. parapertussis, induces morphological changes and cAMP accumulation in L2 cells. We previously reported that B. pertussis CyaA caused cell rounding in L2 cells through its adenylate cyclase activity (19): anti-CyaA serum neutralized cell-rounding activity of the culture supernatant of B. pertussis, indicating that CyaA (B. pertussis CyaA) is the sole cause of cell rounding. In contrast, we found that the culture supernatants of B. bronchiseptica and B. parapertussis, which contain CyaA, did not alter cell morphology (Fig. 1A and B; see Fig. S1A in the supplemental material). Ten strains each of B. pertussis and B. bronchiseptica that were maintained in the laboratory were similarly examined for their cell-rounding activities. Although the culture supernatants of B. pertussis strains caused cell rounding, a result that is consistent with previous findings (19), the culture supernatants of all the B. bronchiseptica strains tested did not (data not shown). These results indicate that a discrepancy in CyaA toxicity was commonly observed between B. pertussis and B. bronchiseptica but was not due to some unusual strains. When L2 cells were treated with purified preparations of recombinant CyaAs, similar results were obtained: B. pertussis CyaA induced cell rounding in a dose-dependent manner, while
B. bronchiseptica CyaA did not at any of the concentrations tested (Fig. 1C). As previously reported (19), B. pertussis CyaA K58Q, which is defective in adenylate cyclase activity, did not cause cell rounding. We then examined the enzyme activities of B. pertussis CyaA and B. bronchiseptica CyaA to generate cAMP from ATP in vitro. Both CyaAs similarly produced cAMP, indicating that the enzyme activity of B. bronchiseptica CyaA is intact (Fig. 2). In contrast, when cultured cells were treated with the toxins, a marked increase in intracellular cAMP was observed only in B. pertussis CyaA-treated cells (Fig. 2). These results indicate that B. bronchiseptica CyaA is enzymatically active in vitro, but it does not appear to exhibit cytotoxicity that is mediated by the accumulation of intracellular cAMP.

Phe375 is the key residue for the cytotoxicity of CyaA. B. bronchiseptica CyaA and B. pertussis CyaA show 98% homology with 36 amino acid replacements in the overall sequence. In order to identify the crucial structural differences responsible for the different cytotoxic activities of B. bronchiseptica CyaA and B. pertussis CyaA, we initially attempted to localize the region of B. pertussis CyaA necessary for cell-rounding activity. We examined chimeric CyaAs, in which the RTX domains were mutually exchanged between B. pertussis CyaA and B. bronchiseptica CyaA, for their cell-rounding activities (Fig. 3A). B. pertussis-B. bronchiseptica chimeric CyaA with the B. bronchiseptica-derived RTX domain caused cell rounding as well as B. pertussis CyaA did, whereas B. bronchiseptica CyaA and B. bronchiseptica-B. pertussis chimeric CyaA with the B. pertussis-derived RTX domain did not (Fig. 3B), indicating that cell-rounding activity is dependent on the region, including the adenylate cyclase domain (ACD) and transmembrane domain (TMD) of B. pertussis CyaA.

In the region including ACD and TMD (1 to 1,000 amino acids [aa]), there were 6 of the 36 amino acid replacements between B. pertussis CyaA and B. bronchiseptica CyaA (Fig. 3A and S2A). Although B. bronchiseptica CyaA has been reported to carry an N-terminal extension of 34 amino acid residues, whereas B. pertussis CyaA does not (3),
we experimentally confirmed that this was not the case and that the translation start site of *B. bronchiseptica* CyaA was identical to that of *B. pertussis* CyaA (Fig. S2B). In order to identify which amino acid residue is crucial for the cytotoxicity of *B. pertussis* CyaA, we generated recombinant CyaAs by replacing the amino acid residue of the chimeric *B. pertussis*-*B. bronchiseptica* CyaA with that of *B. bronchiseptica* CyaA at each position and then examined their cell-rounding activities (Fig. 3C and S2A). The recombinant CyaAs F375S and V800A, the amino acids of which were exchanged with those of *B. bronchiseptica* CyaA at amino acid positions 375 and 800, respectively, failed to cause cell rounding, whereas the activities of other recombinant CyaAs were intact. We generated another series of recombinant CyaAs by exchanging the amino acid at position 375 or 800 between *B. bronchiseptica* CyaA and *B. pertussis* CyaA and again examined cell-rounding activity. The *B. pertussis* CyaA mutants F375S and V800A both lost their cell-rounding activities (Fig. 3D), indicating that Phe375 and Val800 are essential for the cell-rounding activity of *B. pertussis* CyaA. In contrast, the replacement of Ser with Phe at position 375 made *B. bronchiseptica* CyaA active on cells, while the mutant CyaA A800V remained inactive. These results indicate that the amino acid at position 375 is responsible for the different cytotoxic activities of CyaAs between *B. pertussis* and *B. bronchiseptica*: CyaA with Phe375 (*B. pertussis* CyaA) is active on cells, whereas the activity of CyaA with Ser375 (*B. bronchiseptica* CyaA) was almost negligible. The results of *in vitro* infection assays supported these results: wild-type *B. pertussis* and the *B. bronchiseptica* mutant strain producing *B. bronchiseptica* CyaA_S375S markedly increased intracellular cAMP levels in L2 and J774A.1 cells, whereas wild-type *B. bronchiseptica* and the *B. pertussis* mutant strain producing *B. pertussis* CyaA_F375S barely increased intracellular cAMP (Fig. S3). We checked the amino acid residues at position 375 of CyaAs, the sequences of which are deposited in the genome database of the National Center for Biotechnology Information (NCBI), and found that all *B. pertussis* (543 deposited data sets) and *B. bronchiseptica* (87 deposited data sets) carried Phe375
and Ser375, respectively. In contrast, Val800 was conserved in *B. pertussis*, whereas Ala800 was not conserved in *B. bronchiseptica*: 43 out of 87 deposited data sets of *B. bronchiseptica* CyaA showed Ala800, whereas the others showed Val800. These results indicate that the 375th amino acid replacement is phylogenetically conserved, and Val800 appears to be involved in the cytotoxic activity of *B. pertussis* CyaA for unknown reasons. Thus, we focused on the amino acid residue at position 375.

*B. bronchiseptica* CyaA is not defective in each intoxication step on target cells. In order to elucidate the reason why *B. bronchiseptica* CyaA does not exert cytotoxic activity, we examined purified preparations of *B. bronchiseptica* CyaA, *B. pertussis* CyaA, *B. bronchiseptica* CyaA<sub>S375F</sub>, and *B. pertussis* CyaA<sub>F375S</sub> for actions in each intoxication step (Fig. 4A). Following their addition to a culture of L2 cells, *B. pertussis* CyaA and *B. bronchiseptica* CyaA<sub>S375F</sub> increased intracellular cAMP levels, whereas *B. bronchiseptica* CyaA and *B. pertussis* CyaA<sub>F375S</sub> did not, which is consistent with the results of the cell-rounding assay (Fig. 3D and 4A). However, the in vitro enzyme activities of these CyaA preparations were intact (Fig. 2 and 4A). The actions of CyaA on target cells are at least divided into the following steps (6): (i) binding to cells; (ii) translocation of the enzyme domain into the cytosol; (iii) formation of toxin pores to make the plasma membrane permeable; and (iv) exertion of enzymatic activity. There-

![Image](https://mbio.asm.org/mbio.asm.org/July/August_2018/Volume_9_Issue_4/e00628-18)

**FIG 3** Identification of the amino acid residues crucial for the cytotoxic activity of *B. pertussis* CyaA. (A) Schematic representation of chimeric CyaAs. CyaA is composed of an adenylate cyclase domain (ACD), hydrophobic transmembrane domain (TMD), and RTX domain in order from the N terminus to the C terminus. Asterisks indicate the positions of six amino acid replacements in the ACD-TMD region between *B. pertussis* (Bp) and *B. bronchiseptica* (Bb) CyaA (see also Fig. S2 in the supplemental material). The numbers indicate the amino acid positions of the start and/or end of the domains. In the following experiments, *B. pertussis* Tohama I and *B. bronchiseptica* RB50 were used as the sources for CyaA genes, and constructed genes were introduced into *B. bronchiseptica* RB50 ∆cyaA, as described in Text S1. (B to D) Percentages of rounded L2 cells treated with the culture supernatants of wild-type (wt) *Bordetella* and derivatives: *B. pertussis* (Bp), *B. bronchiseptica* (Bb) or *B. bronchiseptica* ∆cyaA complemented with wild-type or each chimeric cyaA (B), *B. bronchiseptica* cyaA complemented with each *B. pertussis*-*B. bronchiseptica* chimeric cyaA with or without a point mutation at the position of the amino acid replacement (C), *B. bronchiseptica* cyaA complemented with *B. pertussis* or *B. bronchiseptica* cyaA with or without the mutation at position 375 or 800 (D). The sample from *B. bronchiseptica* ∆cyaA transformed with the empty vector was used as mock infection. Each CyaA in the culture supernatant was detected by immunoblotting with anti-CyaA antiserum Values are means plus SD (n = 4).
Therefore, we examined CyaA preparations for each intoxication step. A flow cytometric analysis showed that all CyaA preparations similarly bound to L2 cells (Fig. 4B). The formation of toxin pores and translocation of the enzyme domain have been reported to occur in an unrelated manner after toxin binding (20). All CyaA preparations showed equivalent hemolytic activities on sheep erythrocytes (Fig. 4C). Translocation of the enzyme domain, as previously estimated by increases in cAMP levels in sheep erythrocytes (21), was confirmed for all CyaA preparations, although *B. pertussis* CyaA<sub>F375S</sub> and *B. bronchiseptica* CyaA were less efficient than *B. pertussis* CyaA and *B. bronchiseptica* CyaA<sub>S375F</sub> (Fig. 4D). *B. pertussis* CyaA has been reported to undergo proteolytic cleavage in the cytosol and liberate fragments of ~45 kDa, including the enzyme...
domain (22). After the treatment of J774A.1 cells with *B. bronchiseptica* CyaA and *B. bronchiseptica* CyaA<sub>F375S</sub>, we detected 46-kDa fragments in the cytosol using the 3D1 antibody, which recognizes the enzyme domain (Fig. 5A and B). Similar results were obtained in experiments with *B. pertussis* CyaA and *B. pertussis* CyaA<sub>F375S</sub> (data not shown). These results demonstrate that *B. bronchiseptica* CyaA and *B. pertussis* CyaA<sub>F375S</sub> carrying Ser<sup>375</sup> as well as *B. pertussis* CyaA and *B. bronchiseptica* CyaA<sub>S375F</sub> carrying Phe<sup>375</sup> are not defective in any intoxication steps against target cells.

**CyaA with Ser<sup>375</sup> interacts with 14-3-3 after phosphorylation.** In the above-described results of immunoblotting (Fig. 5B for *B. bronchiseptica* CyaA and *B. bronchiseptica* CyaA<sub>S375F</sub>), we noted that the fragment containing ACD of *B. bronchiseptica* CyaA and *B. pertussis* CyaA<sub>F375S</sub> showed slightly slower mobility on SDS-polyacrylamide gels than *B. pertussis* CyaA and *B. bronchiseptica* CyaA<sub>S375F</sub>, implying that S<sup>375</sup> in the fragment underwent some covalent modification in the cytosol of target cells. Mass spectrometric analyses revealed that 46-kDa fragments of *B. bronchiseptica* CyaA and *B. pertussis* CyaA<sub>F375S</sub>, which were composed of at least ~399 (~R<sup>399</sup>) amino acid residues covering the enzyme domain, were phosphorylated at Ser<sup>375</sup> (Fig. 5C).

In order to examine whether the phosphorylation of Ser<sup>375</sup> influences the toxic action of CyaA on target cells, we prepared recombinant ACD peptides of *B. pertussis* CyaA (*B. pertussis* ACD) and *B. pertussis* CyaA<sub>F375S</sub> (*B. pertussis* ACD<sub>F375S</sub>) phosphorylated by pretreatment with protein kinase A (PKA) *in vitro* and examined them for adenylate cyclase activity. *B. pertussis* ACD and *B. pertussis* ACD<sub>F375S</sub>, which consist of the region from amino acids at positions 2 to 400, were both phosphorylated with PKA, as judged by mobility shifts in SDS-polyacrylamide gels with Phos-tag and mass spectrometry (Fig. S4). The *in vitro* adenylate cyclase activities of these ACDs were unchanged before and after phosphorylation, indicating that the phosphorylation of Ser<sup>375</sup> per se does not influence the enzyme activity of CyaA (Fig. 6A).

We then searched a phosphorylation-related motif in ACD carrying Ser<sup>375</sup> with Scansite 3 (http://scansite3.mit.edu/) and found that the region surrounding phosphorylated Ser<sup>375</sup> corresponded to a 14-3-3 binding motif (mode 1, RSXpS/pTXP, in which pS and pT represent phosphoserine and phosphothreonine, respectively, and X is any type of residue [Fig. 5C]) (23, 24). The 14-3-3 protein family comprises seven isoforms that are expressed ubiquitously in a wide range of eukaryotic organisms and are known to regulate phosphoserine/phosphothreonine-mediated intracellular signal transduction by interacting with a number of phosphorylated proteins (23, 25). Therefore, we examined the influence of 14-3-3 on the enzyme activity of *B. pertussis* ACD<sub>F375S</sub> phosphorylated with PKA and found that each isoform of the 14-3-3 protein, except for the α isoform, significantly inhibited the *in vitro* adenylate cyclase activity of phosphorylated *B. pertussis* ACD<sub>F375S</sub> to various extents (Fig. 6B). In contrast to phosphorylated *B. pertussis* ACD<sub>F375S</sub>, the activities of *B. pertussis* ACD and nonphosphorylated *B. pertussis* ACD<sub>F375S</sub> were not influenced by 14-3-3 (Fig. 6C). These results indicate that the inhibition of adenylate cyclase activity was dependent on the phosphorylation of Ser<sup>375</sup>. The immunoprecipitation assay revealed that 14-3-3 interacted with phosphorylated, but not nonphosphorylated, *B. pertussis* ACD<sub>F375S</sub>. The apparent interaction between *B. pertussis* ACD and 14-3-3 was not detected regardless of the phosphorylation state (Fig. 6D). Similar results were obtained when the cytosolic fraction of J774A.1 cells, which contained 14-3-3, was used instead of *Escherichia coli*-produced recombinant 14-3-3 (Fig. S5). These results indicate that the activity of CyaA with Ser<sup>375</sup> was inhibited by 14-3-3 of the target cells after the phosphorylation of Ser<sup>375</sup>.

**B. pertussis CyaA, but not B. bronchiseptica CyaA, disrupts epithelial barrier function.** CyaA targets CD11b-expressing myeloid cells, such as macrophages, neutrophils, and dendritic cells, and subverts the immune responses of hosts by inhibiting bactericidal activities and affecting cytokine secretion (8, 9, 11–13). The cAMP-elevating activity of CyaA was previously reported to up- and downregulate the induction of interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF-α), respectively (13, 17, 26). Therefore, we compared the effects of *B. pertussis* CyaA and *B. bronchiseptica* CyaA on...
FIG 5  Mass spectrometric analysis of the adenylate cyclase domain (ACD) liberated in the cytosol. (A) Schematic representation of CyaA. The regions recognized by the anti-CyaA monoclonal antibodies 3D1 and 9D4 are indicated by solid and dotted lines, respectively (56). (B) Immunoblotting of the cytosolic and membrane fractions of J774A.1 cells treated with B. bronchiseptica (Bb) CyaA and B. bronchiseptica CyaAS375F. Cell fractions were prepared as described in Materials and Methods and separated by SDS-PAGE after trichloroacetic acid (TCA) precipitation, followed by immunoblotting with an anti-CyaA monoclonal antibody (3D1 or 9D4), anti-GAPDH antibody, and anti-transferrin receptor (anti-TfR) antibody. The positions of N- and C-terminal fragments and the full-length CyaA are indicated by black and white arrowheads, respectively. Panels 1, CyaA preparations (2 ng/lane); panels 2, cytosol fractions (100 μg/lane); panels 3, membrane fractions (10 μg/lane). (C) Localization of phosphorylated amino acid residues in ACD isolated from the cytosolic fraction of J774A.1 cells treated with each CyaA. All CyaAs were phosphorylated at S393 (white arrowhead). The black arrowhead indicates the additional phosphorylation site that is specific to B. bronchiseptica (Bb) CyaA and B. pertussis (Bp) CyaA375F. The sequences of isolated peptides surrounding the phosphorylation site are shown at the bottom of the panel. Phosphorylated serines (S375 or S376) are shown on a gray background in the sequences with the numbers of the amino acid positions. The 14-3-3 binding motif was aligned below the sequences. X, any type of residue; pS, phosphorylated serine.
Inactivation of CyaA by 14-3-3

The expression of IL-6 and TNF-α in L2 and NR8383 (rat alveolar macrophage) cells using real-time quantitative reverse transcription-PCR but observed no significant differences between B. pertussis CyaA and B. bronchiseptica CyaA, which is consistent with previous findings (13, 17, 26). This may have been because a slight increase in intracellular cAMP by residual active B. bronchiseptica CyaA was sufficient to affect cytokine production (note that B. bronchiseptica CyaA shows residual activity at higher concentrations, as shown in Fig. 2). We examined the effects of the toxin on epithelial cells, which were reportedly sensitive to CyaA (17, 18). Since previous studies indicated that CyaA does not exert clear cytotoxic (cell killing) activity against epithelial cells (17), we investigated whether CyaA affects the organization of the epithelial barrier, which is one of the important roles of the epithelium. Rat tracheal-epithelial EGV-4T cells were allowed to organize the epithelial barrier, as confirmed by high trans-epithelial electrical resistance (TEER) values, and were then treated with CyaA preparations applied to the apical side. The TEER values of B. pertussis CyaA and B. bronchiseptica CyaA decreased over time, whereas the values of B. bronchiseptica CyaA and B. pertussis CyaA did not (Fig. 7A). Immunofluorescence microscopy revealed that the structure of tight junctions visualized by anti-ZO-1 antibody was destroyed by treatment with B. pertussis CyaA and B. bronchiseptica CyaA, but not by treatment with B. bronchiseptica CyaA and B. pertussis CyaA (Fig. 7B).

**FIG 6** Inactivation of phosphorylated B. pertussis ACD375S by 14-3-3. (A) *In vitro* enzyme activities of B. pertussis ACD (○) and B. pertussis ACD375S (○) with (+) or without (-) phosphorylation by the protein kinase A (PKA) treatment. Values are means ± SD (error bars) (n = 3), rACD, recombinant ACD. (B) Inhibition of the enzyme activity of phosphorylated B. pertussis ACD375S (p-Bp ACD375S) in the presence of the 14-3-3 proteins expressed in E. coli. p-Bp ACD375S (1 nM) was preincubated with 10 μl of the FLAG-14-3-3-containing E. coli fraction (~50 μg of total protein) at 37°C for 30 min before measuring enzyme activity in vitro. The expression of each isoform was confirmed by immunoblotting with an anti-FLAG antibody. Value are means plus SD (n = 3). The statistical significance of differences was analyzed by an unpaired t test. Values that are significantly different (p < 0.01) from the value for the control “vector” are indicated by an asterisk. (C) Phosphorylation-dependent inactivation of the enzyme activities of B. pertussis ACDs by 14-3-3. B. pertussis (Bp) ACD (1 nM) and B. pertussis ACD375S (1 nM) were phosphorylated by PKA as described in Materials and Methods and preincubated with 48 μg of the FLAG-14-3-3γ-containing E. coli fraction before measuring enzyme activity in vitro. Values are means plus SD (n = 3). The statistical significance of differences was analyzed by an unpaired t test and indicated by asterisks as follows: *, p < 0.05; **, p < 0.01. (D) Immunoprecipitation assay for the interaction between ACDs and 14-3-3γ. The reaction solutions in panel C were immunoprecipitated (IP) using an anti-ACD antibody (Ab), followed by immunoblotting (IB) with an anti-14-3-3 antibody and anti-CyaA antibody (3D1).
In addition, *B. pertussis* CyaA, but not *B. bronchiseptica* CyaA, partly caused cell death under these experimental conditions (Fig. 7C). Consistent results were also obtained in rat infection experiments, in which rats were inoculated intranasally with a low dose (500 to 700 CFU) of *B. bronchiseptica* producing *B. bronchiseptica* CyaA (wild-type *B. bronchiseptica* [Bb WT]) or *B. bronchiseptica* CyaA_{S375F} (Bb S375F) (Fig. 7D). On day 3 postinoculation, the tracheas, the epithelia of which were labeled with N-hydroxysuccinimide (NHS)-linked biotin that was introduced into the tracheal lumen, were excised and subjected to microscopy (Fig. 7D). The infection with wild-type (WT) *B. bronchiseptica* and *B. bronchiseptica* ΔcyaA did not disrupt the barrier function of the tracheal epithelium, as judged by the intact apical area labeled with biotin. In contrast, in rats infected with *B. bronchiseptica* S375F, the introduced biotin permeated the submucosal layers through the epithelium, indicating that *B. bronchiseptica* CyaA_{S375F} impaired the barrier function of the tracheal epithelium (Fig. 7D). As shown in Fig. 7E, all bacteria tested colonized the rat trachea to similar extents.
DISCUSSION

The CyaAs of classical Bordetella are highly homologous: B. pertussis CyaA shows 97.8 and 97.7% amino acid identities to B. bronchiseptica CyaA and B. parapertussis CyaA, respectively, while B. bronchiseptica CyaA shows 99.8% amino acid identity to B. parapertussis CyaA. Although most amino acid replacements between the CyaAs of classical Bordetella were found in the C-terminal region, which binds to the cell receptor and elicits protective immunity (27, 28), differences in the toxic activities of CyaAs were not reported (29, 30). In the present study, we demonstrated that the strength of the intracellular enzyme activity of B. bronchiseptica CyaA was markedly less than that of B. pertussis CyaA. B. bronchiseptica CyaA was enzymatically active in vitro but was inactivated by phosphorylation and a subsequent association with 14-3-3 in target cells. These results are not in conflict with the previous study showing an intracellular increase in cAMP levels after the treatment of cells with B. bronchiseptica CyaA (31), because our results also demonstrated that the intracellular enzyme activity of B. bronchiseptica CyaA was not completely abrogated (e.g., in J774A.1, THP-1, and EGV-4T cells in Fig. 2 and in J774A.1 cells in Fig. S3 in the supplemental material). In addition, we found that B. bronchiseptica CyaA increased intracellular cAMP in erythrocytes (Fig. 4D), and this may have been because of the weak expression of 14-3-3 proteins (32) or unfavorable conditions for phosphorylation in erythrocytes. The amount of CyaA that translocated into the cytosol may also influence the efficiency of inactivation by 14-3-3. Furthermore, differences in responses to B. bronchiseptica CyaA among various cell types may occur due to different cell conditions as described above.

Ser^{375} of B. bronchiseptica CyaA was the crucial phosphorylation site responsible for the 14-3-3 association. NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos-3.1/) (33, 34) predicted PKA and CDC2 as possible kinases to phosphorylate Ser^{375}. However, the types of kinases actually involved in the intracellular phosphorylation of Ser^{375} have not yet been identified, although PKA phosphorylated Ser^{375} of ACD in vitro. B. pertussis CyaA carries Phe^{375} instead of Ser^{375} and therefore is not phosphorylated or inactivated by 14-3-3. B. parapertussis CyaA, which has Ser^{375}, did not cause cell rounding in L2 cells, indicating that it is also inactivated in an identical manner to B. bronchiseptica CyaA. A previous study showed that B. pertussis CyaA was cytotoxic against macrophages, whereas B. parapertussis CyaA was not (35), which is consistent with the present results.

14-3-3 proteins bind to the phosphoserine/threonine-containing sequence motifs of various target proteins and modulate their functions in diverse manners as follows: alterations in the intracellular localization or ability of target proteins to interact with other partners, the direct augmentation or inhibition of target protein activity, the protection of target proteins from proteolysis or dephosphorylation, and serving as a scaffold to bridge two distinct target molecules (24, 36, 37). The region of CyaA corresponding to the 14-3-3 motif (Arg^{372}–Pro^{377}) is not directly involved in enzyme activity or the association with calmodulin (38). However, the binding of 14-3-3 may interrupt the interaction between ACD and calmodulin because this region presumably faces the calmodulin binding space according to the crystal structures of the ACD-calmodulin complex (38). Alternatively, 14-3-3 may interfere with the conversion of ACD from the inactive state to the active state in response to calmodulin binding because the switch region of CyaA (Val^{343}–Ala^{364}) responsible for catalytic activation is sterically in the vicinity of the Arg^{372}–Pro^{377} region (38). 14-3-3, which is known to regulate various biological processes, including mitogenic signal transduction, apoptotic cell death, and cell cycle control, has been reported to interact with hundreds of proteins, including Raf-1, tyrosine and tryptophan hydroxylases, Bcr, and Bad (25, 36, 37, 39–41).

Regarding the virulence factors of pathogens, a previous study reported that Pseudomonas aeruginosa ExoS required 14-3-3 to exert its ADP-ribosylating effects (42). However, B. pertussis/B. parapertussis CyaAs are the first examples of the virulence factors of pathogens that are inactivated by 14-3-3. Ser^{375} and Phe^{375} were found to be phylogenetically conserved in B. bronchiseptica/
B. parapertussis and B. pertussis, respectively, implying that the different toxicities of CyaAs may be related to the distinct pathogenesis of Bordetella. In the present study, we compared the adenylate cyclase-derived toxicities of B. bronchiseptica CyaA and B. pertussis CyaA in relation to bacterial pathogenesis and found that CyaA with Phe$^{375}$ (B. pertussis CyaA and B. bronchiseptica CyaA$_{5375S}$), but not with Ser$^{375}$ (B. bronchiseptica CyaA and B. pertussis CyaA$_{3375S}$) disrupted tracheal epithelial barrier function. Cholera toxin (CT) and Bacillus anthracis edema factor (EF) were also recently revealed to disrupt host cell barrier function by inhibiting Rab11-mediated endocytic recycling, which is essential for transporting junctional proteins at tight/adherens junctions (43, 44). These effects were mediated by increases in intracellular cAMP induced by CT and EF. The mechanism by which CyaA with Phe$^{375}$ disrupted the epithelial cell barrier may be similar to that by CT and EF. We also found that some cells in the confluent cell layer were killed by the treatment with CyaA with Phe$^{375}$ (Fig. 7C), indicating that a long stimulation of active CyaA may induce epithelial cell death, which may exacerbate the disruption of the epithelial barrier. Similar epithelial damage was observed in rat tracheas infected with B. bronchiseptica expressing B. bronchiseptica CyaA$_{5375F}$, but not B. bronchiseptica CyaA (Fig. 7D). These results imply that B. pertussis CyaA causes more extensive damage to the respiratory epithelium than B. bronchiseptica/B. parapertussis CyaA. This may explain the less severe clinical manifestations in B. bronchiseptica and B. parapertussis infections than in B. pertussis infections. However, it is important to note that CyaA plays a diverse role in Bordetella infections through several properties, such as adenylate cyclase activity, pore-forming activity, and potent immunogenicity (45–50), and only adenylate cyclase activity was disturbed by 14-3-3 in CyaAs of B. bronchiseptica and B. parapertussis. Furthermore, B. bronchiseptica CyaA modulated the production of IL-6 and TNF-α, similar to B. pertussis CyaA (data not shown), indicating that slight increases in cAMP are sufficient to induce biological effects in some cases. Thus, our results do not deny the roles of B. bronchiseptica CyaA as a virulence factor in B. bronchiseptica infection.

Although classical Bordetella share major virulence factors, they vary in disease severity and host specificity. The exact nature of this difference in pathogenesis remains unknown; however, previous studies pointed out distinct virulence factors produced by each Bordetella species, including pertussis toxin specific for B. pertussis, the type III secretion system apparently expressed in B. bronchiseptica, and the different lipopolysaccharide structures of each Bordetella species (45, 51–53). In addition to specific virulence factors, homologous ones may also play different roles in each Bordetella infection. We previously demonstrated that the expression level of DNT varied among classical Bordetella because of polymorphisms in the promoter region for the dnt gene (54). Most pig isolates of B. bronchiseptica carried the promoter types with increasing transcription activities, whereas human isolates of B. pertussis and B. parapertussis carried the least active promoter. These results appear to reflect the important role of DNT in the pathogenesis of pig infections with B. bronchiseptica. In the present study, we showed that CyaA may function differently as a virulence factor between B. bronchiseptica/B. parapertussis and B. pertussis infections. Studies on functional differences in homologous virulence factors, in addition to specific factors, may provide insights into the underlying reasons for the different pathogenicities and host specificities of classical Bordetella.

**Note.** After the manuscript had been completed, Hasan et al. reported the disruption of paracellular barrier function in human bronchial epithelial VA10 cells by B. pertussis CyaA (55). Further studies are warranted in order to evaluate the importance of epithelial barrier disruption in the pathogenesis of B. pertussis.

**MATERIALS AND METHODS**

Cultured cell lines, bacterial strains, gene constructions, and other commonly utilized methods are given in Text S1 in the supplemental material.

**Isolation of cytosolic and membrane fractions from CyaA-treated cells.** J774A.1 cells grown to confluence were treated with 0.5 μg/ml of each recombinant CyaA at 37°C for 1 h. After the cells were washed with cold Dulbecco-modified phosphate-buffered saline (D-PBS), the cells were scraped and
collected into a tube with prechilled homogenization buffer (25 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA [pH 7.4] containing protease inhibitor cocktail [Nacalai] and a mixture of a phosphatase inhibitor [PhosSTOP; Roche]). The cells were homogenized in a Potter-Elvehjem homogenizer and centrifuged at 1,000 x g for 30 min. The supernatant was collected into another tube, and the pellet was resuspended in fresh homogenization buffer and homogenized again. Homogenization and centrifugation were repeated four times, and the supernatant obtained each time was collected into a tube and centrifuged at 100,000 x g at 4°C for 30 min. The resulting supernatant was used as the cytosolic fraction. The pellet was resuspended in the homogenization buffer and used as the membrane fraction. Each fraction was validated by immunoblotting with an anti-transferrin receptor monoclonal antibody (Zymed) and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) polyclonal antibody (Santa Cruz).

Mass analysis of the intracellular 46-kDa fragment of CyaA. The cytosolic fraction of CyaA-treated J774A.1 cells was immunoprecipitated with the anti-ACD (adenylate cyclase domain) antibody, and the precipitated proteins were subjected to SDS-PAGE, followed by silver staining (silver staining MS kit; Wako) and separately by immunoblotting with the anti-ACD antibody after electrophoresis to polyvinylidene difluoride (PVDF) membranes. The position of ACD in the gel after SDS-PAGE was estimated on the basis of the relative position of ACD detected by immunoblotting, and the piece of the gel including ACD was reduced with 10 mM dithiothreitol (DTT), alkylated with 55 mM iodoacetamide, and digested with trypsin. The resultant peptides were analyzed by nanocapillary reversed-phase liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) using a C18 column on a nanoLC system (Advance; Michrom BioResources) coupled to a linear trap quadrupole (LTQ) Orbitrap Velos plus electron transfer dissociation (ETD) mass spectrometer (Thermo Fisher Scientific). Tandem mass spectra were acquired automatically by activating collision-induced dissociation (CID)/ETD fragmentation and searched against a nonredundant Bordetella database from NCBI using the MASCOT Server (Matrix Science). Precursor mass tolerance was set at 10 ppm and 0.8 Da for the Orbitrap and linear ion trap, respectively. Carbamidomethylation (C) was set as the static modification. Oxidized methionine (M), the acetylation of the N terminus, and the phosphorylation of serine, threonine, or tyrosine were set as dynamic modifications. A maximum of two missed cleavage sites were allowed.

Measurement of transepithelial electrical resistance and immunofluorescence microscopy of CyaA-treated EGV-4T cells. EGV-4T cells were seeded on a 24-well Transwell plate at 50,000 cells/well and allowed to grow for 5 days in medium, but not in an air-liquid interface until the transepithelial electrical resistance (TEER) reached 2 to 3 kΩ/well. Medium was replaced with fresh medium, and cells were incubated at 37°C for 30 min and then treated with 1 µg/ml of each recombinant CyaA for 24 h. TEER was measured with MilliCell-ERS (electrical resistance system) (Merck) every 2 h. After 24 h, the cells were fixed with 4% paraformaldehyde in D-PBS for 15 min, permeabilized with 0.5% Triton X-100 in D-PBS for 5 min, and blocked with 5% bovine serum albumin (BSA) in D-PBS at room temperature for 30 min. The cells were treated with an anti-ZO-1 monoclonal antibody (Invitrogen) for 1 h and then treated with an Alexa Fluor 488-labeled secondary antibody (Molecular Probes) and Hoechst 33258 for 30 min, mounted with the filter in Fluoromount (Diagnostic BioSystems), and subjected to microscopy with a confocal laser scanning microscope (Olympus Fluoview FV10i; Olympus). Cell death was detected by the LIVE/DEAD cell imaging kit (Molecular Probes) according to the manufacturer’s instructions.

Animal experiments. Three-week-old female Wistar rats (Japan SLC) were anesthetized with a mixture of medetomidine (Kyoritsu-seiyaku Co., Tokyo, Japan), midazolam (Teva Takeda Pharma, Nagoya, Japan), and butorphanol (Meiji Seika Pharma Co., Tokyo, Japan) at final doses of 0.3, 2.0, and 5.0 mg/kg of body weight, respectively, and intranasally inoculated with 500 to 700 CFU of B. bronchiseptica in 10 l. The nuclei were stained with Alexa Fluor 488 streptavidin (Thermo Fisher) and Hoechst 33258, and the sections were stained for biotin, D-PBS containing 5% fetal calf serum (FCS) and 0.3% Triton X-100, the sections were stained for biotin, and the nuclei were stained with Alexa Fluor 488 streptavidin (Thermo Fisher) and Hoechst 33258, respectively, and subjected to fluorescence microscopy as described above.

All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, and performed according to the Regulations on Animal Experiments at Osaka University.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00628-18.

TEXT S1, DOCX file, 0.1 MB.
FIG S1, EPS file, 2.3 MB.
FIG S2, EPS file, 0.6 MB.
FIG S3, EPS file, 0.4 MB.
FIG S4, EPS file, 0.7 MB.
REFERENCES

1. Mills KHG, Ross PJ, Allen AC, Wilk MM. 2014. Do we need a new vaccine to control the re-emergence of pertussis? Trends Microbiol 22:49–52. https://doi.org/10.1016/j.tim.2013.11.007.

2. Tan T, Dalby T, Forsyth K, Halperin SA, Heininger U, Hozbor D, Plotkin S, Conboy M, Weiss AA, Hewlett EL. 1987. Inhibition of Bordetella bronchiseptica for providing pABB-CRS2 and the expression plasmids for the 14-3-3 proteins. We are also grateful to Akio Abe for providing pABB-CRS2 and the B. parapertussis strains and to Peggy Cotter for providing the B. bronchiseptica strain.

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27. El-Azami-El-Idrissi M, Bauche C, Loucka J, Osicka R, Sebo P, Ladant D, Leclerc C. 2003. Interaction of Bordetella pertussis adenylate cyclase with CD11b/CD18: role of toxin acylation and the main integrin interaction domain. J Biol Chem 278:38514–38521. https://doi.org/10.1074/jbc.M304387200.

28. Betso F, Sebo P, Guiso N. 1995. The C-terminal domain is essential for protective activity of the Bordetella pertussis adenylate cyclase- hemolysin. Infect Immun 63:3309–3315.

29. Chenal-Francisque V, Caro V, Boursaux-Eude C, Guiso N. 2009. Genomic analysis of the adenylate cyclase-hemolysin C-terminal region of Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Res Microbiol 160:330–336. https://doi.org/10.1016/j.resmic.2009.03.006.

30. Park J, Zhang Y, Buboltz AM, Zhang X, Schuster SC, Ahuja U, Liu M, Miller JF, Sebaihia M, Bentley SD, Parkhill J, Harvill ET. 2012. Comparative genomics of the classical Bordetella subspecies: the evolution and exchange of virulence-associated diversity amongst closely related pathogens. BMC Genomics 13:545. https://doi.org/10.1186/1471-2164-13-545.

31. Henderson MW, Iatrousa CS, Sheets AJ, Williams CL, Benaron DJ, Donato GM, Gray MC, Hewlett EL, Cotter PA. 2012. Contribution of Bordetella filamentous hemagglutinin and adenylate cyclase toxin to suppression and evasion of interleukin-17-mediated inflammation. Infect Immun 80:2061–2075. https://doi.org/10.1128/IAI.00148-12.

32. Boston PF, Jackson P, Thompson RJ. 1982. Human 14-3-3 protein: radioimmunoassay, tissue distribution, and cerebrospinal fluid levels in patients with neurological disorders. J Neurochem 38:1475–1482. https://doi.org/10.1111/j.1471-4159.1982.tb07928.x.

33. Blom N, Gammeltoft S, Brunak S. 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol 294:1351–1362. https://doi.org/10.1006/jmbi.1999.3310.

34. Blom N, Sicheritz-Pontén T, Gupta R, Gammeltoft S, Brunak S. 2004. Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. Proteomics 4:1633–1649. https://doi.org/10.1002/pmic.200300771.

35. Bouchez V, Douché T, Dazas M, Delaplane S, Matondo M, Chamot-Rooke J, Guiso N. 2017. Characterization of post-translational modifications and cysteine properties of the adenylate-cyclase hemolysin produced by various Bordetella pertussis and Bordetella parapertussis isolates. Toxins 9:304–312. https://doi.org/10.3390/toxins903004.

36. Aitken A. 2006. 14-3-3 proteins: a historic overview. Semin Cancer Biol 16:162–172. https://doi.org/10.1016/j.semcancer.2006.03.005.

37. Tzivion G, Avruch J. 2002. 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. J Biol Chem 277:3061–3064. https://doi.org/10.1074/jbc.R100059200.

38. Guo Q, Shen Y, Lee V-S, Gibbs CS, Mkirsch M, Tang W-J. 2005. Structural basis for the interaction of Bordetella pertussis adenyl cyclase toxin with calmodulin. EMBO J 24:3190–3201. https://doi.org/10.1038/sj.emboj.7600800.

39. Ichimura T, Isobe T, Okuyama T, Takahashi N, Araki K, Kuwano R, Takahashi YA, Wang Z, Hang S, Pineton de Chambrun GP, McCole DF, Watnick P, Nizet V, Bier E. 2013. Cholera toxin disrupts barrier function by inhibiting exocytosis-mediated trafficking of host proteins to intestinal cell junctions. Cell Host Microbe 14:294–305. https://doi.org/10.1016/j.chom.2013.08.001.

40. Thorson JA, McGillivray SM, Cruz-Moreno B, van Sorge NM, Kuwano T, Bier E. 2010. Anthrax toxins cooperatively inhibit endocytic recycling by the Rab11/Sec15 exocyst. Nature 467:854–858. https://doi.org/10.1038/nature09446.

41. Guiso N. 2017. Bordetella adenylate cyclase-hemolysin toxins. Toxins 9:277. https://doi.org/10.3390/toxins9090277.

42. Novak J, Fabrik I, Linhartova I, Link M, Černý O, Stulik J, Sebo P. 2017. Phosphoproteomics of CAMP signaling of Bordetella adenylate cyclase toxin: a protein kinase-dependent activator of tyrosine and tryptophan hydroxylase. Mol Cell Proteomics 16:162–172. https://doi.org/10.1073/pnas.1613459111.

43. Novak J, Černý O, Osickova A, Linhartova I, Masin J, Bumba L, Sebo P, Osicka R. 2017. Structure-function relationships underlying the capacity of Bordetella adenylate cyclase toxin to disarm host phagocytes. Toxins 9:300–328. https://doi.org/10.3390/toxins9100300.

44. Guichard A, McGillivray SM, Cruz-Moreno B, van Sorge NM, Nizet V, Bier E. 2015. Bordetella pertussis adenylate cyclase-hemolysin toxins. Toxins 7:300–328. https://doi.org/10.3390/toxins703017.

45. Skopkova K, Tomalova B, Kanchev I, Rossmann P, Svedova M, Adkins I, Bíbora I, Tomala J, Masín J, Guiso N, Osicka R, Sedlacek R, Kvaro M, Sebo P. 2017. CAMP-elevating capacity of the adenylate cyclase toxin-hemolysin is sufficient for lung infection but not for full virulence of Bordetella pertussis. Infect Immun 85:e00937-16. https://doi.org/10.1128/IAI.00937-16.

46. Vojtova J, Kamanova J, Sebo P. 2006. Bordetella adenylate cyclase toxin: a swift saboteur of host defense. Curr Opin Microbiol 9:304–312. https://doi.org/10.1016/j.mib.2005.12.011.

47. Wang X, Gray MC, Hewlett EL, Maynard JA. 2015. The Bordetella adel- nylate cyclase repeat-in-toxin (RTX) domain is immunodominant and elicits neutralizing antibodies. J Biol Chem 290:3576–3591. https://doi.org/10.1074/jbc.M114.585281.

48. Allen AG, Thomas RM, Cadisch JT, Maskell DJ. 1998. Molecular and functional analysis of the lipopolysaccharide biosynthesis locus wib from Bordetella pertussis, Bordetella parapertussis and Bordetella bronchiseptica. Mol Microbiol 29:272–38. https://doi.org/10.1111/j.1365-2958.1998.00878.x.

49. Harvill ET, Preston A, Cotter PA, Allen AG, Maskell DJ, Miller JF. 2000. Multiple roles for Bordetella lipopolysaccharide molecules during respiratory tract infection. Infect Immun 68:6720–6728. https://doi.org/10.1128/IAI.68.12.6720-6728.2000.

50. Mattoo S, Yuk MH, Huang LL, Miller JF. 2004. Regulation of type III secretion in Bordetella. Mol Microbiol 52:1201–1214. https://doi.org/10.1111/j.1365-2958.2004.04053.x.

51. Hasan S, Kulkarni NN, Asbjarnarson A, Linhartova I, Osicka R, Gudmundsson GH. 2018. Bordetella pertussis adenylate cyclase toxin disrupts functional integrity of bronchial epithelial layers. Infect Immun 86:e00445-17. https://doi.org/10.1128/IAI.00445-17.

52. Blake J, Gray MC, Guo L, Sebo P, Hewlett EL. 1999. Epitope mapping of monoclonal antibodies against Bordetella pertussis adenylate cyclase toxin. Infect Immun 67:2090–2095.