RETRACTED: The Mechanism of Pertussis Cough Revealed by the Mouse-Coughing Model

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ABSTRACT Pertussis, also known as whooping cough, is a contagious respiratory disease caused by the Gram-negative bacterium Bordetella pertussis. This disease is characterized by severe and uncontrollable coughing, which imposes a significant burden on patients. However, its etiological agent and the mechanism are totally unknown because of a lack of versatile animal models that reproduce the cough. Here, we present a mouse model that reproduces coughing after intranasal inoculation with the bacterium or its components and demonstrate that lipooligosaccharide (LOS), pertussis toxin (PTx), and Vag8 of the bacterium cooperatively function to cause coughing. Bradykinin induced by LOS sensitized a transient receptor potential ion channel, TRPV1, which acts as a sensor to evoke the cough reflex. Vag8 further increased bradykinin levels by inhibiting the AC1 esterase inhibitor, the major downregulator of the contact system, which generates bradykinin. PTx inhibits intrinsic negative regulation systems for TRPV1 through the inactivation of G, GTPases. Our findings provide a basis to answer long-standing questions on the pathophysiology of pertussis cough.

IMPORTANTThe Gram-negative bacterium Bordetella pertussis causes a respiratory disease called whooping cough, or pertussis. This disease is characterized by paroxysmal coughing, the mechanism of which has not been intensively studied because of a lack of versatile animal models that reproduce the cough. In this study, we present a mouse model that reproduces coughing after intranasal inoculation with the bacterium or its components. Using this model, we demonstrate that lipooligosaccharide, Vag8, and pertussis toxin of the bacteria cooperatively function to cause coughing. Our results also indicate that bradykinin, an inflammatory mediator, and TRPV1, an ion channel linked to nociceptive signaling, are host factors involved in the coughing mechanism.

KEYWORDS Bordetella pertussis, bradykinin, TRPV1, Vag8, cough, pertussis toxin

Pertussis, also referred to as whooping cough, is a highly contagious respiratory disease caused by the Gram-negative bacterium Bordetella pertussis (1–3). Generally, the disease can be prevented through vaccination; however, the number of pertussis cases is significantly increasing worldwide, hypothetically because of the rapid waning of immunity induced by recent acellular vaccines and adaptation of the bacterium to
escape vaccine-induced immunity (1, 2). Patients with the disease exhibit various clinical manifestations, including bronchopneumonia, pulmonary hypertension, hypoglycemia, leukocytosis, and paroxysmal coughing. Among these, paroxysmal coughing, which persists for several weeks and imposes a significant burden on infants, is the hallmark of pertussis. However, etiological agents and the mechanism of pertussis cough remain unknown.

*B. pertussis* is highly adapted to humans; therefore, convenient animal models that reproduce coughing induced by the bacterial infection are difficult to establish. Baboons were recently reported to replicate various pertussis symptoms, including paroxysmal coughing (4–6); however, it is difficult to obtain a sufficient number of these large primates for analytical experiments because of ethical and cost issues. Rats were reported to cough after inoculation of *B. pertussis* into airways (7–15); however, these studies did not explore the mechanism underlying coughing. Meanwhile, we reestablished the coughing model of rats infected with *Bordetella bronchiseptica*, which produces many common virulence factors shared with *B. pertussis*, and observed that BspR/BtrA, an anti-α factor, regulates the ability of *B. bronchiseptica* to cause coughing in rats (16). Additionally, our rat model also exhibited coughing in response to *B. pertussis* infection; however, the cough frequency was lower than that caused by *B. bronchiseptica*, and the cough production was not reproduced well. Therefore, we attempted to develop an alternative animal model for pertussis cough and focused on mice because of their multiple genetically modified mutants. Mice have not been used for analyses of *B. pertussis*-induced coughing because they have long been believed to be unable to cough (17–19). However, many recent reports have demonstrated that vagal sensory neurons, which are involved in the cough reflex, innervate the mouse airway (20–22). Mouse coughing in response to certain tussive stimuli has been detected and recorded by independent research groups (23–27). In the present study, we demonstrated that C57BL/6J mice developed coughs after inoculation with *B. pertussis*. Our mouse-coughing model further revealed that lipooligosaccharide (LOS), Vag8, and pertussis toxin (PTx) cooperatively function to produce coughing through the pathway from bradykinin (Bdk) generation to transient receptor potential vanilloid 1 (TRPV1) sensitization.

**RESULTS**

**C57BL/6 mice respond to *B. pertussis* infection by coughing.** We intranasally inoculated mice with *B. pertussis* in a manner similar to that in our previous study on rats (16) and observed that C57BL/6J mice coughed approximately 1 week after inoculation (Fig. 1; see Movie S1 in the supplemental material). The incidence of coughing varied, depending on the bacterial strains and mouse strains. *B. pertussis* 18323, a classical strain, markedly caused coughing, while another commonly studied strain, Tohama, did not. Clinical isolates caused various degrees of coughing. This effect did not correlate with their ability to colonize the trachea and lungs. The extent of cough production did not differ between the sexes of the mice (data not shown). Thus, we further explored the mechanism of pertussis cough using *B. pertussis* 18323 and male C57BL/6J mice.

**PTx contributes to cough production.** Previous studies using rat or baboon models of *B. pertussis* infection suggested the partial involvement of PTx in cough production based on the observation that infection with a PTx-deficient strain did not cause coughing (12) and that immunization with acellular pertussis vaccines containing pertussis toxoid protected the animals from coughing after the bacterial infection (8, 28). Therefore, we examined if PTx contributes to cough production by using the mouse model. A PTx-deficient strain (Δptx), which colonized the tracheas and lungs, although slightly less than the wild-type strain, did not cause coughing in mice (Fig. 2A and B). We previously observed that not only living *B. bronchiseptica* cells but also bacterial lysates caused coughing in rats (16). Similarly, in the present study, the lysates of wild-type *B. pertussis* caused coughing similar to the infection with living bacteria (Fig. 2C and D). In contrast, the lysates from neither the Δptx mutant nor the mutant producing
enzymatically inactive PTx (PTxED) caused coughing. The Dptx lysate complemented with purified PTx caused coughing to the same extent as the wild-type lysate. These results indicate that the enzyme (ADP-ribosylating) activity of PTx is required for cough production. However, contrary to our expectation, intranasal inoculation of purified PTx hardly caused coughing, implying that bacterial factors other than PTx are required for cough production.

**Vag8 and LOS along with PTx contribute to cough production.** To seek additional bacterial factors contributing to cough production, we examined various combinations of bacterial lysates and purified bacterial components in the mouse model. *B. pertussis* exhibits two distinct phenotypic phases, Bvg⁺ and Bvg⁻, in response to environmental alterations (29). In the Bvg⁺ phase, the bacteria produce a set of virulence factors, including PTx, adenylate cyclase toxin, dermonecrotic toxin, and the machinery and effectors of the type III secretion system. In contrast, in the Bvg⁻ phase, the bacteria shut down the expression of the virulence factors and, instead, express several factors specific to this phenotype. Therefore, in general, the Bvg⁺ phase is considered to represent the virulent phenotype of the organism. Consistently, the lysate from the Bvg⁻ phase-locked mutant (Bvg⁻ lysate) caused coughing, while that from the Bvg⁺
FIG 2  Involvement of PtX in *B. pertussis*-induced coughing. (A and B) Cough production in C57BL/6J mice inoculated with wild-type (18323) and Δptx strains of *B. pertussis*. SS medium without the bacteria was used for mock inoculation. The number of coughs was counted for 5 min/mouse/day for 9 days from days 6 to 14 postinoculation, and the total number is expressed (A). The number of bacteria recovered from the tracheas and lungs was counted on day 14 (B). (C to F) Cough production in mice inoculated with cell lysates from *B. pertussis* wild-type and mutant strains. Mice were intranasally inoculated with cell lysates of the *B. pertussis* 18323 wild type or indicated mutants with or without PtX (200 ng) on days 0 to 4 (arrows in panel C). The number of coughs was counted for 11 days from days 4 to 14 (C) and expressed as the sum from days 6 to 14 (D to F). PBS was used for mock inoculation. "Heat +" in panel F indicates cell lysates incubated at 56°C for 1 h. The experiments were repeated at least twice, and representative data are shown. Each of the horizontal bars and plots represents the mean ± SEM (A and C to F) or the geometric mean ± SD (B). One-way ANOVA with Dunnett's test to compare with 18323 (A and B) or mock inoculation (D and E) and with Tukey's test (F) was used. The number of mice in each test group is presented in parentheses (A and B) or as follows: n = 5 in panels C and D, n = 3 or 4 in panel E, and n = 4 in panel F.
phase-locked mutant (Bvg^- lysate) did not (Fig. 2E). The addition of PTx into the Bvg^- lysate only partially restored the cough production, compared to the wild-type lysate and the Δptx mutant lysate complemented with PTx (Fig. 2F). The wild-type lysate and Δptx lysate were obtained from the bacteria in the Bvg^+ phase. PTx alone did not cause coughing. Therefore, these results suggest that at least two distinct factors in addition to PTx are involved in cough production. One factor is present in the Bvg^- lysate, and the other is present in both the Bvg^- and Bvg^- lysates.

Heat treatment at 56°C for 1 h abrogated the cough induced by the wild-type lysate (Fig. 2F). The addition of PTx to the heat-treated lysate moderately but not completely restored the cough production, compared to the wild-type lysate and the Δptx lysate complemented with PTx. Therefore, we hypothesized again that at least two distinct factors in addition to PTx are involved in cough production, and one factor is heat labile and the other factor is heat stable.

To identify the two factors responsible for cough production in addition to PTx, we next examined various mutants of B. pertussis kept in our laboratory and observed that a mutant strain (Δvag8) that is deficient in the autotransporter protein Vag8 exhibited only a moderate ability to cause coughing, whereas another autotransporter mutant, with the mutation ΔbrkA, was fully active (Fig. 3A and B). Similarly, the Δvag8 lysate caused moderate coughing compared to the wild-type lysate (Fig. 3C). The addition of a recombinant Vag8 protein compensated for the activity of the Δvag8 lysate. Vag8 alone or in combination with PTx did not cause coughing (Fig. 3C). The Bvg^- lysate complemented with PTx and Vag8 caused coughing to the same extent as the wild-type lysate (Fig. 3E). These results revealed that Vag8 is the second factor contributing to cough production. Because Vag8 is heat labile and specific to the Bvg^+ phase bacteria, we narrowed the third factor to one that is heat stable and present independently of the Bvg phases. We considered LOS, which is a heat-stable and biologically active outer membrane component, as a probable candidate. The wild-type lysate in which LOS was eliminated with polymyxin B treatment exhibited a reduced ability to cause coughing (Fig. 3D). The addition of a purified LOS preparation restored the coughing.

When the lysate from E. coli DH5α was inoculated, instead of that from B. pertussis, in combination with PTx and Vag8, the mice exhibited coughing (Fig. 3E). Finally, the combination of PTx, Vag8, and LOS caused coughing to the same extent as the wild-type lysate (Fig. 3E and F). Thus, we concluded that LOS, PTx, and Vag8 cooperatively cause coughing in mice. Because LOS is essential for cough induction.

Cough-evoking pathways stimulated by LOS, Vag8, and PTx. The cough reflex involves vagal afferent nerves innervating the airways from the larynx to the proximal bronchi; however, how the afferent nerves are activated to evoke coughing is not fully understood. Nevertheless, it is accepted that transient receptor potential (TRP) ion channels on sensory nerve terminals participate in some reflex pathways of coughing (30, 31). TRP ion channels, which show a preference for Ca^{2+}, are modulated by cell signals from G protein-coupled receptors (GPCRs) that are activated by ligands such as Bdk, prostanoids, and tachykinins, including neurokinins and substance P (30–39).

Therefore, to examine whether some GPCRs and TRP channels are involved in B. pertussis-induced cough, we applied various antagonists for GPCRs (Bdk B1 and B2 receptors, neurokinin 1 to 3 receptors, and prostaglandin E2 receptor EP3) and TRP channels (TRPV1, TRPV4, and TRPA1) in our mouse model. Mice preadministered antagonists against the B2 receptor (B2R) and TRPV1 exhibited reduced levels of coughing in response to bacterial lysate; however, other reagents were apparently ineffective at the concentrations used (see Fig. S1A in the supplemental material). The inhibitory effects of the antagonists against B2R and TRPV1 were also observed in mice inoculated with LOS, PTx, and Vag8 (Fig. 4A). When both antagonists against B2R and TRPV1...
were simultaneously administered, the inhibitory effects were not additively augmented, suggesting that the pathway upstream of B2R to TRPV1 may neither diverge nor converge (Fig. S1B). TRPV1-decient mice, but not TRPA1-decient mice, were less responsive to the combination of LOS, PTx, and Vag8 (Fig. 4B); this observation is
FIG 4 Involvement of host factors B2R and TRPV1 in *B. pertussis*-induced coughing. (A) Effects of antagonists on cough reflex-related pathways in *B. pertussis*-induced coughing. Each mouse was inoculated with 20 or 60 nmol of antagonists.

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consistent with the results of the experiments using the antagonists. Thus, we considered LOS, PTx, and Vag8 to cooperatively stimulate the afferent nerves through a pathway from Bdk-B2R to TRPV1 (see Fig. 8 below). Bdk has been reported to enhance cough reflex in guinea pigs (40–42). Bdk concentrations in the bronchoalveolar lavage fluid (BALF) of mice increased 4 days after intranasal inoculation with LOS-PTx-Vag8 or the living bacteria (Fig. 4C and D).

**Roles of LOS.** Because the lipid A moiety of LOS was determined to be essential for cough production, we examined the role of the specific receptor for lipid A, Toll-like receptor 4 (TLR4), by using TLR4-deficient mice. *B. pertussis* colonized the respiratory organs of TLR4-deficient mice, similar to that in isogenic wild-type mice (Fig. 4F); however, it caused low cough production in the deficient mice (Fig. 4E). Similar results were obtained with intranasal inoculation of the mice with the bacterial lysates or the combination LOS-PTx-Vag8 (Fig. 4E). The increase in Bdk levels in the BALF of TLR4-deficient mice inoculated with LOS-PTx-Vag8 was considerably lower than that of wild-type mice (Fig. 4G). These results suggest that LOS stimulates Bdk generation via interaction with TLR4. In addition to Bdk, proinflammatory cytokines such as interleukin-β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α) showed a slight increase in wild-type mice, but not in TLR4-deficient mice inoculated with LOS-PTx-Vag8 (see Fig. S2A in the supplemental material). Furthermore, increases in these cytokines were not observed without inoculation of LOS (Fig. S2B), indicating that LOS leads to slight inflammation via TLR4 under these experimental conditions. Bdk is a potent inflammatory mediator that is released from high-molecular-weight kininogen (HK) through the proteolytic activity of plasma kallikrein (PK). To corroborate the role of Bdk in cough production, we generated HK-deficient (*Kng1−/−*) mice, which do not produce Bdk, and subjected them to the cough analysis. The *Kng1−/−* mice were less responsive to infection with *B. pertussis* or inoculation of the bacterial lysate than isogenic wild-type mice (Fig. 5A and B), demonstrating that Bdk participates in a cascade leading to *B. pertussis*-induced cough.

**Roles of Vag8.** Bdk, HK, and PK comprise the kallikrein-kinin system in the plasma contact system, which is initiated and accelerated by two proteases, factor XII (FXII) and plasma prekallikrein (PPK) (43–45) (see Fig. 8 below). Certain stimuli convert zymogen FXII into an active enzyme FXIIa. FXIIa cleaves PPK to generate PK, which releases Bdk by cleaving HK. In turn, PK cleaves FXII to release FXIIa, providing a positive-feedback cycle. FXIIa and PK are inhibited by C1 esterase inhibitor (C1-Inh), the major regulator of the kallikrein-kinin system, which is present in human plasma (46). This inhibitory action of C1-Inh was reportedly inhibited by Vag8 (47, 48). If this is the case, Vag8 is likely to exacerbate the cough response of mice by upregulating the Bdk level through the inhibition of C1-Inh activity. Indeed, the Bdk levels in the BALF of mice inoculated with LOS and PTx were increased upon the administration of Vag8 (Fig. 5C). These results are consistent with the results described above, showing that Vag8 increased the number of coughs induced by the Δvag8 lysate or the combination of LOS and PTx (Fig. 5C and D). This exacerbating effect of Vag8 on cough production was not observed in *Kng1−/−* mice (Fig. 5D). In addition, the truncated fragment of Vag8 ranging from amino acid positions 102 to 479 (ΔC479), which is unable

**FIG 4 Legend (Continued)**

against Bdk receptors (B1 and B2), TRPV1 and TRPA1, or PBS (300 μL) prior to inoculation with combinations of PTx, LOS, and Vag8. The sum of coughs from days 6 to 14 is shown. (B) Cough production in mice deficient in TRP ion channels. The sum of coughs from days 6 to 14 is shown. (C and D) Bdk concentrations in the BALF of mice. Mice were inoculated with the combination of PTx, LOS, and Vag8 (C) or *B. pertussis* 18323 (D), and the concentrations of Bdk in BALF on days 1, 4, and 10 (C) or 4 (D) were determined. (E and F) Cough production in mice deficient in TLR4. The mice were inoculated with *B. pertussis* 18323 (left panel), the cell lysate of the bacteria (center panel), or the combination of PTx, LOS, and Vag8 (right panel). The sum of coughs from days 6 to 14 is shown (E). The number of bacteria recovered from the tracheas and lungs was counted on day 14 postinoculation of the experiment of the left panel (F). (G) Bdk concentrations in the BALF of mice. The concentration of Bdk was determined on day 4. The experiments were performed at least twice, and representative data are shown. Each horizontal bar represents the mean ± SEM (A, C, D, and G, n = 5; B, n = 7 to 10; E, n = 4 to 5) or geometric mean ± SD (F, n = 4 to 5). One-way ANOVA with Dunnett’s test to compare with PBS (A) or wild-type mice (B), two-way ANOVA with Sidak’s test (C and G), and an unpaired t test (D to F) were used for statistical analyses.
FIG 5 Role of Vag8 in cough production. (A, B, and D) Cough production of wild-type (WT) and Kng1−/− mice. Mice were inoculated with *B. pertussis* 18323 (A), the cell lysate of the bacteria (B), or combinations (D) of PTx, LOS, and Vag8. The sum (Continued on next page)
to bind and inactivate C1-Inh, did not increase the number of coughs in mice inoculated with LOS and PTx; in contrast, other types of truncated Vag8, which retain the ability to inhibit C1-Inh increased the number of coughs, similar to Vag8 (49) (Fig. 5E to G). These results indicate that Vag8 exacerbates the cough response of mice by upregulating the Bdk level through the inhibition of C1-Inh activity.

Role of PTx in Bdk-induced sensitization of TRPV1. The above results indicate that LOS and Vag8 cooperatively raise the Bdk level. We next explored the role of PTx, which is known to ADP-ribosylate the heterotrimeric GTPases of the Gi family and inhibit intracellular signaling mediated by Gi, GTPases (50). Bdk is known to sensitize TRPV1 through binding to B2R, a member of GPCRs, to evoke coughing in animals (30, 31, 42, 51–54). In addition, previous reports demonstrated that PTx enhanced several actions of Bdk (55, 56). Therefore, we examined the role of PTx in the effect of Bdk on TRPV1 by using the whole-cell patch-clamp technique in HEK293T cells expressing B2R and TRPV1 (Fig. 6A and B). Capsaicin (Cap), a ligand for TRPV1, induces a TRPV1-dependent current, which is desensitized in the second Cap application (57–60) (Fig. 6A; the top panel). When the cells were treated with Bdk before the second application, the Cap-induced currents were not reduced but enhanced in the second application, as reported previously (42) (Fig. 6A, the second panel). We observed that this enhancement was further augmented in cells pretreated with PTx, but not in those pretreated with PTxEd (Fig. 6A, the fourth and the bottom panels). Without Bdk, PTx did not influence the desensitization induced by the second Cap application (Fig. 6A, the third panel). A similar exacerbating effect of PTx was confirmed by independent experiments using dorsal root ganglion (DRG) neurons isolated from mice (Fig. 6D and E): Cap-induced (TRPV1-dependent) increase of intracellular Ca2+ was enhanced by Bdk. In PTx-treated DRG neurons, the Bdk-induced enhancement of intracellular Ca2+ accumulation was further augmented.

We further investigated the mechanism by which PTx exacerbates the Bdk-induced sensitization of TRPV1. As illustrated in Fig. 8, the sensitization of TRPV1 by Bdk is considered to be mediated by the protein kinase C (PKC)-dependent pathway, which is activated via B2R-coupled Gq/11 (38, 51, 62). In contrast, the desensitization (tachyphylaxis) of TRPV1, which partly results from calcineurin-induced dephosphorylation of the channel, is reversed through phosphorylation by the CAM-dependent protein kinase (PKA) (57–59). We confirmed that the Bdk-induced sensitization of TRPV1 was inhibited by calphostin C (CalpC), a PKC inhibitor, but not by H-89, a PKA inhibitor (Fig. 6C; see Fig. S3B in the supplemental material). Exacerbation of the Bdk-induced sensitization of TRPV1 by PTx was prevented by both inhibitors (Fig. 6C; Fig. S3C). Considering that the PKC inhibitor reduced the Bdk-induced sensitization of TRPV1 and that PTx inactivates Gi, GTPases, leading to PKA activation through an increase in the intracellular CaM level (50), we concluded that H-89 is likely to inhibit PTx-related events. To verify this, we examined HEK293T cells expressing B2R and a PKA-insensitive mutant of TRPV1 (TRPV1mut), whose Ser117 and Thr371, which are phosphorylated by PKA (57, 59), were substituted for with Ala (Fig. 6B; Fig. S3A). In these cells, the effects of PTx were abrogated. These results indicate that PTx exacerbates Bdk-induced sensitization of TRPV1 through an increase in intracellular CAM levels, followed by PKA-mediated phosphorylation of TRPV1. B2R has been reported to couple with Gq/11, Gs, and Gi.
FIG 6 Role of PTx in Bdk-induced sensitization of TRPV1. (A) Current responses of HEK293T cells expressing B2R and wild-type TRPV1 to repetitive applications of Cap and an intervening application of Bdk. Representative traces of Cap-evoked currents are

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GTPases (38, 63–67). Our results indicate that Bdk simultaneously stimulates Gq/11+ and Gαi-dependent pathways through B2R as if it simultaneously applies the accelerator and brakes to TRPV1. We conclude that PTx reverses only the inhibitory effect of Bdk on TRPV1 by uncoupling Gq GTPases from B2R, thereby exacerbating the Bdk-induced sensitization mediated by Gαi GTPases (see Fig. 8 below). The involvement of Gq GTPases, which stimulate adenylate cyclase and subsequently PKA, in these events is probably negligible, since H-89 did not influence Bdk-induced sensitization in the absence of PTx (Fig. 6C).

Another possible role of PTx in the antinociceptive system. Coughs, as well as pain sensation, are initiated by nociceptive stimuli that act on nociceptors, including Aδ and C fibers of the sensory nerves, where TRP channels are located. In pain sensation, nociceptive signals are modulated by the descending antinociceptive system conducted by serotonergic and noradrenergic neurons (68, 69). In addition, because of the similarities between the neural processing systems for pain- and cough-related inputs, several researchers have recently provided evidence for the existence of possible neural pathways that modulate the processing of the cough reflex, as well as pain sensation (70–73). The modulating systems for respiratory reflexes are suggested to involve the α2-adrenergic receptor, which is coupled with Gi GTPases, the target molecule for PTx (70, 71). If PTx inhibits such modulating systems to suppress the cough reflex, coughing will be further exacerbated. We examined this possibility by using a recently reported experimental system in which noradrenaline (NA), a ligand for the α2-adrenergic receptor, reduced the TRPV1 activity of rat DRG neurons (74, 75). Consistent with previous studies (74, 75), in HEK293T cells expressing TRPV1, B2R, and α2A-adrenergic receptor (ADRA2A), NA inhibited Cap-stimulated TRPV1 activity; this inhibitory effect was abolished through pretreatment of the cells with PTx but not with PTxED (Fig. 7B; Fig. S3D). In addition, NA also inhibited the Bdk-induced sensitization of TRPV1, and this inhibitory effect of NA was reversed through pretreatment with PTx (Fig. 7A and B). Consistent results were obtained using the calcium imaging technique with DRG neurons: no DRG neurons that responded to NA were found after PTx treatment, indicating that the inhibitory effect of NA on the Bdk-induced enhancement of Cap-dependent intracellular Ca2+ increase was abrogated by PTx (Fig. 7C and D).

Differences among bacterial strains and mouse strains in cough production. As shown in Fig. 1B to D, the frequency of coughing in mice varied according to the inoculated bacterial strains. We tried to address why different bacterial strains caused different degrees of coughing. The amounts of major virulence factors, including PTx and Vag8, produced by these strains were similar (see Fig. S4A in the supplemental material). The sequence variation type (76) of PTx produced by the strains was irrelevant to the ability to develop coughing (see Table S3 in the supplemental material). Single amino acid substitutions were identified in Vag8 between 18323 (Val231) and the other strains (Ala231). The amount of LOS in 18323, as measured by the Limulus test, was greater than that in the other strains (Fig. S4B). However, the levels of Bdk in the BALF were apparently uncorrelated with the sequence variation of Vag8 and the amount of LOS per bacterial cell (Fig. S4B to D). The living cells or lysate of the Tohama strain hardly caused coughing, as mentioned above; however, LOS, PTx, and Vag8 from

FIG 6 Legend (Continued)

shown. Scale at the left bottom of each trace indicate 1 nA and 10 s on the ordinate and abscissa, respectively. Horizontal bars below the reagent names indicate the incubation period of each reagent. (B and C) Ratio of the second peak to the initial peak of the current densities induced by Cap. The cells expressing TRPV1 or TRPV1mut were preincubated with or without PTx or the enzymatically inactive derivative of PTx (PTxED) for 24 to 30 h prior to the recording (A to C). The cells were stimulated with Bdk and subsequently Cap in the presence of 1 μM CalpC and/or H-89 (C). Values represent the means ± SEM from three independent cells. (D) Intracellular calcium levels of DRG cells changed in response to repetitive applications of Cap. Isolated DRG cells pretreated with or without PTx or PTxED were subjected to calcium imaging with transient applications of Cap (1 μM for 1 min) and Bdk (100 nM for 3 min). Each line represents a single cell isolated from DRG. Nine to 10 independent cells were tested for each experiment. The results for Cap-sensitive neurons are strongly colored. (E) Fluorescence intensity ratios of the second peak to the initial peak increased in response to Cap. A series of experiments were conducted once (B to E). Values represent the means ± SEM from three independent cells. One-way ANOVA with Tukey’s test (B) and with Dunnett’s test to compare with Bdk+ (C, left), Bdk−/PTx+ (C, right), or Bdk−/PTx− (E) was used for statistical analyses.
FIG 7  Reversal of the inhibitory effect of NA on Cap-evoked and Bdk-sensitized TRPV1 by PTx. (A and B) Current responses of HEK293T cells expressing B2R, TRPV1, and ADRA2A to repetitive applications of Cap and intervening applications of Bdk in the presence or absence of NA. The cells were treated with PTx or PTxED or untreated prior to the whole-cell patch-clamp recording. Representative traces of Cap-evoked currents (A) and the ratio of the second peak to the initial peak of current densities induced by Cap (B) are shown. Scales at the left bottom of each trace indicate 1 nA and 10 s on the ordinate and abscissa, respectively. Horizontal bars below the reagent names indicate the incubation period of each reagent (A). A series of experiments were conducted once. Plotted data in panel B represent means ± SEM from three independent cells. One-way ANOVA with Dunnett’s test was used to compare with Bdk / NA / PTx− (left) or Bdk / NA / PTxED− (right). (C) Intracellular calcium levels of DRG cells changed in response to repetitive applications of Cap (1 μM, 1 min) and an intervening application of Bdk (100 nM, 3 min) in the presence of NA (100 nM, 5 min). Temporal changes in the calcium levels are shown. Each line (Continued on next page)
Tohama caused coughing (Fig. 3F). In addition, PTx from Tohama compensated for the ability of the lysate of the 18323 Δptx strain to cause coughing (Fig. S4E). In contrast, PTx from both Tohama and 18323 did not compensate for the ability of the lysate of the Tohama Δptx strain to cause coughing. From these results, we consider that the bacteria may produce another substance to disturb LOS-PTx-Vag8 action, and the varied levels of such substances may result in the varied ability of the bacterial strains to induce cough.

We additionally observed that, unlike C57BL/6J mice, the BALB/c mice did not develop coughing after infection with 18323 strain or inoculation with LOS-PTx-Vag8 (see Fig. S5A and C in the supplemental material). The bacterial colonization level in the BALB/c mice was not lower than that in the C57BL/6J mice (Fig. S5B). The levels of Bdk in the BALF of BALB/c mice inoculated with the living bacteria or the bacterial components were higher than those in the uninoculated mice (Fig. S5D). The exacerbating effect of Vag8 on the increase in Bdk level was also observed in the BALB/c mice (Fig. S5E). However, the degree of increase in Bdk in the BALB/c mice was lower than that in the C57BL/6J mice. A previous report demonstrated that the BALB/c mice exhibit less thermal pain sensitivity than the C57BL/6 mice because of lower expression levels of TRPV1 in some subsets of sensory neurons (77). If this is the case for the vagus nerve, which is responsible for the cough reflex, it may be possible that the BALB/c mice hardly develop pertussis-induced coughing because of low Bdk production in response to LOS-PTx-Vag8, the low expression level of TRPV1, or both.

DISCUSSION
In this study, we have presented the mouse-coughing model, which enables us to evaluate the extent of coughs evoked by *B. pertussis* under certain conditions. By using this model, we demonstrate that LOS, Vag8, and PTx of *B. pertussis* cooperatively function to produce coughing in mice as follows (Fig. 8). (i) In step 1, LOS stimulates Bdk generation by the kallikrein-kinin system through interaction with TLR4. (ii) In step 2,
Vag8 accelerates Bdk generation by inhibiting C1-Inh, which is the major negative regulator of the kallikrein-kinin system. (iii) In step 3, Bdk sensitizes TRPV1, which is regulated by the phosphorylation states mediated by both PKC and PKA; phosphatases (PPases) such as calcineurin desensitize TRPV1 through dephosphorylation. Sensitization of TRPV1 by Bdk depends on B2R-Gi-mediated PKC activation. Additionally, Bdk stimulates the inhibitory pathway of TRPV1 through B2R-Gi-mediated inhibition of adenylate cyclase (AC) and subsequently PKA. Hewitt and Canning previously speculated that LOS-induced Bdk might be involved in the pertussis cough (78). This speculation is partially consistent with the coughing mechanism that we demonstrated in the present study. (iv) In step 4, PTx reverses the B2R-Gi-mediated inhibition and exacerbates B2R-Gq-mediated sensitization of TRPV1. Consequently, TRPV1 remains in the sensitized state and readily increases nervous excitation to evoke coughing. The sensitized state of TRPV1 continues until G, which is ADP-ribosylated by PTx, is replaced by intact ones. (v) In addition, in step 5, PTx possibly inhibits predicted negative regulation systems for the cough reflex that are relayed by Gq/11 and G12/13, such as NA-stimulated α2-adrenergic receptors. The negative regulation systems for the cough reflex are yet to be clearly defined; however, step 5 deserves consideration because it may explain the long-lasting coughing in pertussis. Through the above-mentioned mechanism, host animals can be triggered by normally innocuous stimuli leading to uncontrolled coughing. This situation is similar to that of tetanus, in which spastic paralysis is caused by innocuous stimuli such as light and sound, because of inhibition of inhibitory neurotransmission of motor neurons.

The causative agent of pertussis cough has long been subject to debate. One theory postulated an unidentified “cough toxin” that is shared by the classical Bordetella species B. pertussis, B. parapertussis, and B. bronchiseptica. This idea originated from the fact that infections with the classical Bordetella species commonly exhibit characteristic paroxysmal coughing in host animals, including humans. According to this idea, the “cough toxin” is not PTx, which is not produced by B. parapertussis or B. bronchiseptica (79). In contrast, another theory proposed that PTx significantly contributes to pertussis cough, which is supported by previous observations that rats and baboons that are experimentally infected with PTx-deficient B. pertussis do not exhibit coughing (12) and that immunization with pertussis toxoid protected animals from coughing but not from bacterial colonization (8, 28). Our results demonstrate that PTx is necessary but not sufficient to cause coughing. LOS (lipid A) as the ligand for TLR4 is common among Gram-negative bacteria. Vag8 of B. pertussis is 97.3% identical to the Vag8 proteins of B. parapertussis and B. bronchiseptica, which themselves are completely identical. Therefore, lipid A and Vag8 are possibly the “cough toxins” shared by the classical Bordetella species. B. parapertussis and B. bronchiseptica, but not B. pertussis, may produce another virulence factor that corresponds to PTx, which modulates the activity of the ion channels that evoke action potentials for the cough reflex. Identification of such a factor may provide insight into the mechanism of cough production by classical Bordetella species other than B. pertussis.

Our model provides a basis for the development of causal therapeutic methods: antagonists for B2R or TRPV1, which are considered potential antitussives (30, 34, 42), were effective against B. pertussis-induced cough. The benefit of these agents in pertussis cases needs to be determined. Meanwhile, cough production was markedly reduced but not completely eliminated in Tlr4−/−, Kng1−/−, or Trpv1−/− mice, indicating that the TLR4-Bdk-TRPV1 pathway is not the only pathway that evokes pertussis cough. LOS (lipid A) is essential for cough production; however, the mechanism through which it triggers Bdk generation remains unknown. Tlr4−/− mice exhibited slight coughing in response to LOS-PTx-Vag8, probably because LOS marginally induces Bdk or other cough mediators in a TLR4-independent manner. The former possibility is supported by previous studies demonstrating that lipopolysaccharide and lipid A induce Bdk generation in a mixture of FXII, PPK, and HK (80, 81). The latter possibility is supported by the present study, which demonstrated that Kng1−/− mice exhibited slight coughing in response to inoculation with the bacterium or bacterial components. In addition to TRPV1, other ion channels,
including TRPV4, TRPA1, TRPM8, and the purinergic P2X3 receptor, are reportedly involved in signals that evoke the cough reflex (30). Additionally, arachidonic acid metabolites, such as prostaglandins and hydroxyeicosatetraenoic acids, and tachykinins, including neuropeptides and substance P, are known to mediate the activation of TRP ion channels (30–38, 52, 53, 82, 83). In the present study, we excluded TRPV4, TRPA1, neuropeptide receptors, and the prostaglandin E2 receptor EP3 through experiments using Tipa1−/− mice and antagonists against these ion channels and receptors. However, our results using antagonists are not conclusive, as their efficacy depends on the administration route, timing, and concentrations. In addition, the cough reflex observed in various diseases is evoked via a network or cross talk of events involving mediators and ion channels that are not fully understood. Furthermore, the physiology of the cough reflex is not necessarily identical among animal species (18, 84). Thus, further work is required for understanding the complete mechanism of pertussis cough and applying our results to human cases.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *B. pertussis* strains 18323 (85) and Tohama (86) were maintained in our laboratory. The *B. pertussis* clinical strains BP140, BP141, BP142, and BP144 were provided by K. Kamakura (National Institute of Infectious Diseases). *B. pertussis* was grown on Bordet-Gengou agar (Becton, Dickinson) plates containing 1% Hipolypepton (Nihon Pharmaceutical), 1% glucose, 1.5% defibrinated horse blood, and 10 µg/mL cefotaxime (BG plate). The bacteria recovered from colonies on BG plates were suspended in Stainer-Scholte (SS) medium (87) to yield an optical density at 650 nm (OD650) value of 0.2 and incubated at 37°C for 12 to 14 h with shaking. The obtained bacteria were used as Bvg−-phase bacteria. Unless otherwise specified, *B. pertussis* in the Bvg− phase was obtained by cultiva-

**Construction of bacterial mutant strains.** Mutant strains derived from *B. pertussis* strains 18323 and Tohama were constructed by double-crossover homologous recombination as described previously (89, 90). The primers, plasmids, and constructed mutant strains used in this study are listed in Table S1 and Table S2 in the supplemental material. For the generation of the 18323 Δptx, Δvag8, and ΔbrkA mutants and Tohama Δptx mutant, ~1-kbp DNA fragments of the up- and downstream regions of the ptx operon and vag8 and brkA genes were amplified by PCR using genomic DNA from *B. pertussis* 18323 as the template with the primers ptx-U-S and ptx-U-AS, ptx-D-S and ptx-d-AS, vag8-U-S and vag8-U-AS, vag8-D-S and vag8-d-AS, brkA-U-S and brkA-U-AS, and brkA-D-S and brkA-D-AS, respectively. An ~0.7-kbp DNA fragment of the chloramphenicol resistance (Cmr) gene was also amplified by PCR using pPK212-8 (Addgene) as the template with the primer Cmr-U-S and Cmr-AS. The PCR products of the up- and downstream regions of each gene were ligated to theSmal site of pABB-CR2-Gm by using the In-Fusion HD cloning kit (TaKaRa Bio). The resultant plasmids (pABB-CRS2-Gm, was introduced into *E. coli* DH5α and transconjugated into the *B. pertussis* stains 18323 and Tohama by triparental conjugation with helper strain E.coli HB101 harboring pRK2013 (88) provided by K. Minamisawa (Tohoku University). The growth media were supplemented with antibiotics when necessary at the following concentrations: ampicillin, 50 µg/mL; gentamicin, 10 µg/mL; kanamycin, 50 µg/mL.

A mutant strain of *B. pertussis* 18323 that produces an enzymatically inactive PTx (PTxED) was con-

**Mice.** Six- to 10-week-old male C57BL/6J, C57BL/6N, or BALB/c mice were used (Clea Japan and Japan SLC). Tlr4−/− mice (93) were purchased from Oriental Bio Service, and wild-type C57BL/6J mice were used as controls. Trpv1−/−, Trpa1−/−, and Trpv1+/−/Trpa1−/− mice were generated as previously reported (94–96). The original strains were backcrossed for more than eight generations with C57BL/6N mice (97), and wild-type C57BL/6N mice were used as controls. Kng1−/− mice were generated by delet-

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gene were 5′-GACCTCAGAATCTAAATAG-3′ and 5′-ACAGGGCAGGGTGCCACA-3′, respectively. The treated zygotes were then cultured to the two-cell stage and transplanted into the oviducts of 0.5-day pseudopregnant ICR females. The founder generation was obtained by natural delivery or cesarean section. The pups obtained were genotyped by PCR using the primers Kng1-check-S1, Kng1-check-S2, and Kng1-check-AS (Table S1) and subsequently confirmed by Sanger sequencing. The lack of Kng1 protein, including HK in murine plasma was confirmed by immunoblotting with rabbit anti-Kng1 (Sigma-Aldrich) and goat anti-rabbit IgG-horseradish peroxidase (HRP) (Jackson Immunoresearch). Wild-type littermates (C57BL/6J) were used as controls for Kng1−/− mice. The Kng1−/− mouse line was deposited at the Riken Bioresource Research Center and Center for Animal Resources and Development, Kumamoto University.

Inoculation into mice with bacteria, bacterial components, and antagonists. Mice were anesthetized with a mixture of medetomidine (Kyoritsu Seiyaku), midazolam (Teva Takeda Pharma), and butorphanol (Meiji Seika Pharma) at final doses of 0.3, 2, and 5 mg/kg body weight, respectively, and intranasally inoculated with 5 × 10^5 CFU of B. pertussis or its components in 50 μL of SS medium by using a micropipette with a needle-like tip. The number of bacteria was confirmed by counting the colonies after cultivation of the inocula on BG plates.

For the preparation of bacterial lysates, B. pertussis cultivated in the SS medium was collected by centrifugation at 8,000 × g for 10 min. The bacteria resuspended in phosphate-buffered saline (PBS) were disrupted by 5 rounds of 2-min sonication with a Bioruptor (Cosmo Bio). The sonicated suspensions were centrifuged at 12,000 × g for 5 min, and the cell lysates were obtained after filtration of the supernatants through a 0.22-μm-pore filter (Millex GV, Merck Millipore). The obtained cell lysates were intranasally inoculated daily for 5 days at 50 μg/μL into mice that were anesthetized with isoflurane by using an anesthetizer (MK-A110; Muromachi Kikai). LOS, Vag8, and PTx were inoculated in a similar way to the bacterial cell lysate at 4 × 10^6 endotoxin units [EU], 500 ng, and 200 ng in 50 μL, respectively. Antagonists of B1R (Des-Arg⁹-[Leu⁵]-bradykinin; Peptide Institute), B2R (Icatibant; Peptide Institute), TRPV1 (Capsazepine; Wako Pure Chemical Industries), TRPA1 (HC-030031; Wako Pure Chemical Industries), TRPV4 (HC-067047; Sigma-Aldrich), NK1 (Spantide; Peptide Institute), NK2 (GR159897; R&D Systems), NK3 (SB222200; Sigma-Aldrich), and EP3 (L-798106; Sigma-Aldrich) were intraperitoneally injected at 20 or 60 nmol/300 μL daily for 5 days into mice immediately before intranasal inoculation of B. pertussis cell lysates or PTx, LOS, and Vag8.

Cough analysis. The numbers of murine coughs from days 4 to 14 postinoculation were measured as previously described, with slight modifications (16). Mice were isolated individually in disposable clear plastic cages, which were laid on a soundproof sheet and covered with plastic cardboard. Coughing was recorded by a video camera (Canon HD IVIS HF21; Canon) equipped with two monaural microphones (AT9903; Audio-Technica Co.) every day for 5 min. The recorded data were processed with Adobe Premiere Pro CS5.5 (Adobe Systems Incorporated) on a computer and displayed as videos along with sound waveforms (23) recorded by a whole-body plethysmograph (Fig. 1A), which consists of a differential pressure transducer (Biopac Systems, SS40L), a detection device (Biopac Systems, MP36R), and the analysis software AcqKnowledge 5.0 (Biopac Systems). The coughing postures of mice (headtossing and open mouth with click-like sounds) were captured by characteristic sound waveforms and the coughing postures of mice (headtossing and open mouth with click-like sound) and enumerated by an observer who did not know detailed information regarding the experiments. In independent experiments, coughs of mice were confirmed by the typical airflow waveforms (23) recorded by a whole-body plethysmograph (Fig. 1A), which consists of a custom-made chamber, a differential pressure transducer (Biopac Systems, SS40L), a detection device (Biopac Systems, MP36R), and the analysis software AcqKnowledge 5.0 (Biopac Systems): on average, 86.4% ± 5.1% (mice inoculated with the bacterial lysate) and 96.7% ± 1.9% (mice inoculated with LOS-PTx-Vag8) of coughs judged by the observer corresponded to those judged on the basis of plethysmography (Fig. 1A). Because our plethysmograph system was not capable of handling a large number of mice, the cough count in each experiment was manually performed by video observation. On the indicated days postinoculation, mice were euthanized with pentobarbital, and their airway tissues, such as nasal septa, tracheas, and lungs, were aseptically excised, minced, and homogenized in Dulbecco’s phosphate-buffered saline (PBS) with a BioMasher (Nippi) and a Polytron PT1200E (Kinematica), respectively. The resultant tissue extracts were serially diluted with PBS and spread on BG plates. The bacteria on the plates were cultivated after overnight cultivation of the inocula on BG plates.

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Cells for whole-cell patch-clamp recordings. HEK293T cells that were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma-Aldrich or Wako) supplemented with 10% fetal bovine serum (FBS) (BioWest) at 37°C under 5% CO2 in air were seeded on a 35-mm dish (Iwaki) at 5 × 105 cells/well, grown overnight, and transfected with pRC/CMV-mB2R (100), pcDNA-hTRPV1 (100), pcDNA-hTRPV1G1158D227A (DNASTAR, ID 619830), and/or pGreen-Lantern 1 (101), which is a humanized green fluorescent protein (GFP) expression vector, using Lipofectamine reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. pcDNA-hTRPV1G1158D227A was generated from pcDNA-hTRPV1 by site-directed mutagenesis to replace Ser117 and Thr227 with Ala by using a PrimeSTAR Mutagenesis Basal kit (TaKaRa Bio) with the primer sets S117A-S and S117A-AS, and T227A-S and T227A-AS (Table S1). After incubation for 3 to 4 h, the cells were reseeded on 12-mm coverslips (Matsunami Glass), further incubated for 2 days, and subjected to whole-cell patch-clamp recordings.

Whole-cell patch-clamp recordings. HEK293T cells were prepared as described above. Whole-cell patch-clamp recordings were performed in a GFP-positive single cell that had been treated with PTx or PTxED at 10 ng/mL for 24 to 30 h prior to the recordings, as previously described with slight modifications (39). The standard bath solution (10 mM HEPES buffer [pH 7.4] containing 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, and 10 mM glucose) was replaced with a Ca2+ -free bath solution, in which CaCl2 of the standard bath solution was replaced with 5 mM EGTA, immediately prior to the recording. Electrodes were filled with pipette solution (10 mM HEPES buffer [pH 7.4]) containing 140 mM KCl and 5 mM EGTA). Currents generated across the cells, which were treated with 10 nM capsaicin, 100 nM bradykinin, and 100 nM noradrenaline (Sigma-Aldrich), 1 μM calphostin C (Merck Millipore), and/or 1 μM H-89 (Merck Millipore) for arbitrary periods of time, were recorded with an Axopatch 200B amplifier (Molecular Devices), filtered at 5 kHz with a low-pass filter, and digitized with Digidata 1440A (Axon Instruments). The membrane potential was clamped at −60 mV, and voltage ramp pulses from −100 to +100 mV (0.5 s) were applied every 5 s. Data were acquired with pCLAMP 10 (Axon Instruments), and the plots were generated with OriginPro 2016 (OriginLab). The current densities (pA/pF) of the peak currents induced by the first and second capsaicin treatments were calculated as the quotient of the current amplitude (pA) divided by the whole-cell capacitance (pF).

Calcium imaging. DRG cells were isolated from 7-week-old male C57BL/6J mice that were euthanized with pentobarbital, as described previously (96). For each mouse, the DRG cells were suspended in 0.5 mL of Earle’s balanced salts solution containing 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin, 1% GlutaMAX supplement (Thermo Fisher Scientific), and MEM vitamin solution (Sigma-Aldrich), seeded on a 35-mm glass base dish, and incubated at 37°C under 5% CO2 for 24 to 30 h with or without PTx or PTxED. For the calcium imaging, DRG cells were incubated with Hanks’ balanced salt solution (Sigma-Aldrich) plus 20 mM HEPES (pH 7.4) (HBSS-HEPES) containing 4 μM Fluo-4 AM (Dojindo), 1.25 mM probenecid, and 0.04% cremophor EL at room temperature for 30 min, and then washed three times with HBSS-HEPES to remove extracellular Fluo-4 AM. The Fluorolabeled cells were treated with 1 μM capsaicin, 100 nM bradykinin, and 100 nM noradrenaline at the appropriate time points, and their fluorescence images were captured at 2-s intervals using a fluorescence microscope (Olympus BX51). The relative fluorescence intensities of independent cells in each fluorescence image were quantified with Metamorph 7.6 (Molecular Devices). The decreased fluorescence peak values of the fluorescence intensity induced by the first and second capsaicin treatments were calculated by subtracting the fluorescence value obtained immediately before the initial capsaicin treatment from that of the maximum fluorescence peak.

Measurement of bradykinin and cytokine levels in BALF. Six- to 8-week-old mice were intranasally inoculated with the living bacteria or PTx, LOS, and/or Vag8, as described above. On the indicated days after the first inoculation, the mice were euthanized with pentobarbital, and their tracheas were exposed by incising the necks in the median line. A catheter (Surflo Flash 20G; Terumo) was inserted into the exposed trachea, 0.5 mL of PBS was slowly injected, and the bronchoalveolar lavage fluid (BALF) was thoroughly aspirated. The BALF was centrifuged at 12,000 g for 5 min, and the supernatant was collected. The concentrations of bradykinin and cytokines in the supernatant were measured with a mouse bradykinin enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource), a mouse IL-1β/IL-1F2 DuoSet ELISA (R&D Systems, code DY406-05), a mouse IL-6 DuoSet ELISA (R&D Systems, code DY406-05), and a mouse NF-κB DuoSet ELISA (R&D Systems, code DY410-05).

Other methods. PTx was purified from culture supernatants of B. pertussis strains 18323 (wild type) and 18323 ΔptxRuv, Δmbl, and Tohama, as previously reported (102). Rabbit anti-PTx, anti-FtsZ, anti-adenylate cyclase toxin, and anti-lamin B antibodies were obtained by immunization of rabbits and rats with purified PTx and a purified preparation of recombinant antigens according to our previous method (103). Rabbit anti-pertactin (code orb242449) was purchased from Biorbyt, Ltd. The protein concentrations of the test materials used in this study were measured using a Micro BCA (bicinchoninic acid) protein assay kit (Thermo Fisher Scientific). The synthesized lipid A of E. coli (code no. 24005-5) was purchased from Peptide Institute, Inc. For immunoblotting, the target proteins were visualized by enhanced chemiluminescence using Immobilon Western (Merck Millipore) and a LAS-4000 mini-chemical image analyzer (GE Healthcare) or Amersham imager 600 UV (GE Healthcare).

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

Statistical analysis. An unpaired t test or one-way analysis of variance (ANOVA) with Dunnett’s or Tukey’s multiple-comparison test was performed to evaluate differences between test groups. Two-way ANOVA was applied to the experiments that include two different categorical variables. Prism
(GraphPad Software) was used for all analyses. In each figure, P values of <0.1 are shown above brackets between the test groups in question.

Data availability. Sequence data of ptx and vag8 of B. pertussis clinical strains BP140, BP141, BP142, and BP144 have been deposited in the DDBJ under the accession numbers shown in Table S3.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 0.1 MB.
FIG S2, EPS file, 0.2 MB.
FIG S3, EPS file, 0.7 MB.
FIG S4, EPS file, 0.5 MB.
FIG S5, EPS file, 0.2 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, DOCX file, 0.02 MB.
MOVIE S1, MOV file, 8 MB.

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Y. Hiramatsu, K. Suzuki, and N. Onoda performed the main experiments. T. Nishida, H. Ikeda, and J. Kamei analyzed mouse coughing using a plethysmograph. T. Satoh, S. Derouiche performed the main experiments. S. Derouiche performed the electrophysiological study. Y. Hiramatsu, M. Tominaga, and Y. Horiguchi outlined the study and wrote the manuscript, with contributions from the other authors.

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