



# Transcriptional Downregulation of a Type III Secretion System under Reducing Conditions in *Bordetella pertussis*

Masataka Goto,<sup>a</sup> Tomoko Hanawa,<sup>b</sup> Akio Abe,<sup>a</sup>  Asaomi Kuwae<sup>a</sup>

<sup>a</sup>Laboratory of Bacterial Infection, Graduate School of Infection Control Sciences, Kitasato University, Tokyo, Japan

<sup>b</sup>Department of Infectious Diseases, Kyorin University School of Medicine, Tokyo, Japan

**ABSTRACT** *Bordetella pertussis* uses a type III secretion system (T3SS) to inject virulence proteins into host cells. Although the *B. pertussis* T3SS was presumed to be involved in host colonization, efficient secretion of type III secreted proteins from *B. pertussis* has not been observed. To investigate the roles of type III secreted proteins during infection, we attempted to optimize culture conditions for the production and secretion of a type III secreted protein, BteA, in *B. pertussis*. We observed that *B. pertussis* efficiently secretes BteA in ascorbic acid-depleted (AsA<sup>-</sup>) medium. When L2 cells, a rat lung epithelial cell line, were infected with *B. pertussis* cultured in the AsA<sup>-</sup> medium, BteA-dependent cytotoxicity was observed. We also performed an immunofluorescence assay of L2 cells infected with *B. pertussis*. Clear fluorescence signals of Bsp22, a needle structure of T3SS, were detected on the bacterial surface of *B. pertussis* cultured in the AsA<sup>-</sup> medium. Since ascorbic acid is known as a reducing agent, we cultured *B. pertussis* in liquid medium containing other reducing agents such as 2-mercaptoethanol and dithioerythritol. Under these reducing conditions, the production of type III secreted proteins was repressed. These results suggest that in *B. pertussis*, the production and secretion of type III secreted proteins are downregulated under reducing conditions.

**IMPORTANCE** The type III secretion system (T3SS) of *Bordetella pertussis* forms a needlelike structure that protrudes from the bacterial cell surface. *B. pertussis* uses a T3SS to translocate virulence proteins called effectors into host cells. The culture conditions for effector production in *B. pertussis* have not been investigated. We attempted to optimize culture medium compositions for producing and secreting type III secreted proteins. We found that *B. pertussis* secretes type III secreted proteins in reducing agent-deprived liquid medium and that BteA-secreting *B. pertussis* provokes cytotoxicity against cultured mammalian cells. These results suggest that redox signaling is involved in the regulation of *B. pertussis* T3SS.

**KEYWORDS** *Bordetella*, type III secretion system

The genus *Bordetella* is made up of Gram-negative bacteria and has been subdivided into 16 subspecies to date (1). *Bordetella pertussis* is a causative agent of whooping cough, also known as pertussis. *B. pertussis* strictly adapts to humans, and it lacks an environmental reservoir (2). A variety of virulence factors have been identified in *Bordetella*, e.g., filamentous hemagglutinin (3), adenylate cyclase toxin (4), and pertactin (5). These virulence factors are regulated by a *Bordetella* two-component regulatory system, BvgAS. In addition to these virulence factors, the type III secretion system (T3SS) of *B. pertussis* is also regulated by BvgAS (6, 7).

A T3SS that forms a needlelike structure is conserved among many Gram-negative bacteria. To subvert hosts, bacteria use a T3SS to inject type III secreted proteins into host cells (8). Such type III secreted proteins are called effectors. Three types of effectors have been identified in *Bordetella*: BteA (9) (also referred to as BopC [10]), BopN (11, 12),

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Address correspondence to Asaomi Kuwae, [kuwae@iisci.kitasato-u.ac.jp](mailto:kuwae@iisci.kitasato-u.ac.jp).

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**TABLE 1** Medium compositions

Compound	Amt (g/liter) in:	
	SS medium	LCA medium
Basal media		
Sodium glutamate	10.7	10.7
L-Proline	0.24	0.24
NaCl	2.5	2.5
KCl	0.2	0.2
KH <sub>2</sub> PO <sub>4</sub>	0.5	0.5
MgCl <sub>2</sub> ·6H <sub>2</sub> O	0.1	0.1
CaCl <sub>2</sub>	0.02	0.02
Tris	6.1	6.1
Casamino Acids	10	0.5
Dimethyl- $\beta$ -cyclodextrin	1	1
Supplements		
L-Cysteine-HCl	4	4
FeSO <sub>4</sub> ·7H <sub>2</sub> O	1	1
L-Ascorbic acid	40	2
Niacin	0.4	0.4
L-Glutathione, reduced	15	15

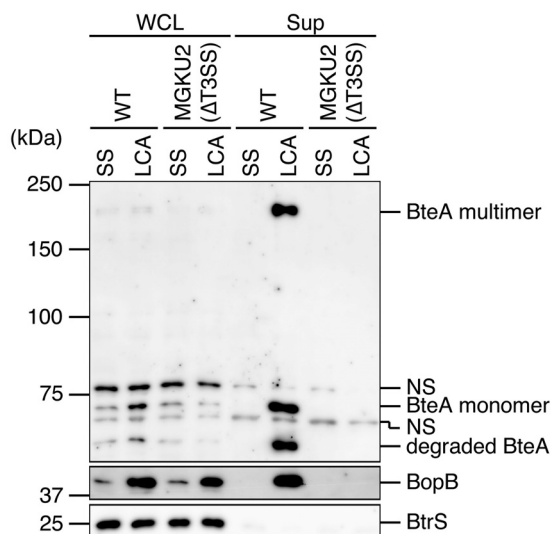
and BspR (13) (also referred to as BtrA [14, 15]). BteA is 658 amino acids in length and is localized to lipid rafts in host cells via its N-terminal region (16). BteA induces necrotic cell death in several types of cultured mammalian cells (9, 10), and BteA translocation into host cells is promoted by the functioning of BopN (11). BspR is a negative regulator for type III secreted proteins (13) and is translocated into nuclei of cultured mammalian cells (17). BspR has been suggested to be an anti-sigma factor against BtrS that functions as a positive regulator for type III secreted proteins (15). Some type III secreted proteins are called translocators rather than effectors. Three types of translocators have been identified in *Bordetella*, i.e., BopB (18), BopD (19), and Bsp22 (20), and they function as the path for effectors delivered into host cells. The complex of BopB and BopD forms the pores on the host membrane (19), and Bsp22 forms a filament-like structure and is associated with the T3SS needle and with a pore-forming component, BopD (21).

The optimal culture conditions for the production and secretion of BteA have not been established. To investigate the roles of BteA and other type III secreted proteins during *B. pertussis* infection, we investigated culture conditions for the maximal production and secretion of type III secreted proteins in *B. pertussis* strain Tohama I.

## RESULTS

**Type III secreted proteins are secreted from *B. pertussis* cultured in LCA medium.** Cyclodextrin solid medium (CSM) is reported as a synthetic medium for clinical isolation of *B. pertussis* (22). We sought to determine whether *B. pertussis* grown on this agar secretes type III effectors, and we prepared a liquid medium, designated "low-Casamino Acids (LCA)" medium, by removing the agar from CSM contents (Table 1). *B. pertussis* was cultured in Stainer-Scholte (SS) or LCA medium, and then the whole-cell lysate (WCL) and supernatant fraction (Sup) samples were subjected to Western blotting with antibodies against BteA (an effector, a type III secreted protein) and BopB (a translocator, a type III secreted protein).

BteA and BopB signals were detected in the WCL and Sup of the wild-type Tohama I cultured in LCA medium (Fig. 1). As reported previously, BteA forms SDS-resistant multimers, and in our present study, we also detected the signals at around 200 kDa. In contrast, the BteA and BopB signals were faintly detected in the WCL samples of SS medium, and no BteA and BopB signals were detected in the Sup samples of MGKU2 (a T3SS-inactive strain) or the wild-type cultured in SS medium (Fig. 1). These results suggest that *B. pertussis* secretes type III secreted proteins in LCA medium.



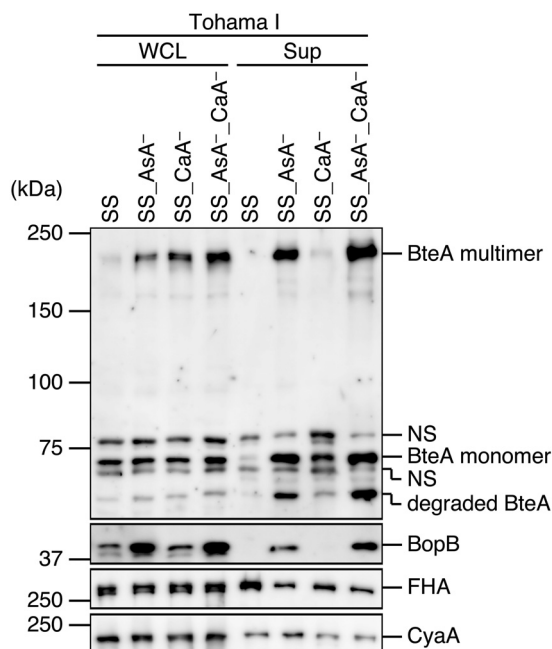
**FIG 1** BteA and BopB production by *B. pertussis* Tohama I cultured in LCA medium. Tohama I and MGKU2 (a T3SS-inactive strain) were cultured in SS or LCA medium. Whole-cell lysates (WCL) and supernatant fraction (Sup) samples were separated by SDS-PAGE and analyzed by Western blotting with antibodies against BteA (top), BopB (middle), or BtrS (bottom). BtrS, an RNA polymerase sigma factor (15), was used as the unsecreted protein control. BteA forms SDS-resistant multimers (10). NS, nonspecific signals. Loaded WCL and Sup samples were prepared from equal volumes of bacterial cultures. Experiments were performed at least three times, and representative data are shown.

**Ascorbic acid downregulates the production and secretion of type III secreted proteins in *B. pertussis*.** Although the ingredients of the LCA and SS media are the same, the amounts of ascorbic acid and Casamino Acids in LCA medium are lower than those in SS medium (Table 1). To determine which substance is involved in the production and secretion of type III secreted protein, we cultured *B. pertussis* in SS medium, ascorbic acid-deprived SS medium (SS<sub>AsA</sub><sup>-</sup>), Casamino Acids-deprived SS medium (SS<sub>CaA</sub><sup>-</sup>), or both ascorbic acid- and Casamino Acids-deprived medium (SS<sub>AsA</sub><sup>-</sup><sub>CaA</sub><sup>-</sup>). WCL and Sup samples were subjected to Western blotting with antibodies against BteA and BopB (Fig. 2).

The BteA multimer signal intensities in the WCL samples from the SS<sub>AsA</sub><sup>-</sup>, SS<sub>CaA</sub><sup>-</sup>, and SS<sub>AsA</sub><sup>-</sup><sub>CaA</sub><sup>-</sup> media were stronger than those from the SS medium (Fig. 2). A faint BteA signal was detected for the Sup samples from SS medium (Fig. 2). BteA signals were evident in the Sup samples from the SS<sub>AsA</sub><sup>-</sup> and SS<sub>AsA</sub><sup>-</sup><sub>CaA</sub><sup>-</sup> media (Fig. 2). Again, BopB signals were evident in both WCL and Sup samples from the SS<sub>AsA</sub><sup>-</sup> and SS<sub>AsA</sub><sup>-</sup><sub>CaA</sub><sup>-</sup> media (Fig. 2).

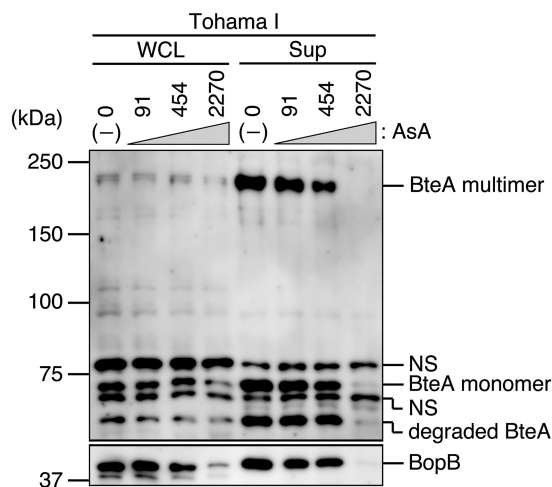
We also investigated the potential involvement of ascorbic acid or Casamino Acids in Bvg-regulated virulence factors, e.g., filamentous hemagglutinin (FHA) and CyaA. WCL and Sup samples were subjected to Western blotting with antibodies against FHA and CyaA (Fig. 2). The results revealed that there were no significant differences in the FHA or CyaA signal intensities in the WCL and Sup samples from the media used here (Fig. 2). Collectively, these results suggest that ascorbic acid has a specific influence on the production and secretion of BteA and BopB in *B. pertussis*.

Standard SS medium contains ascorbic acid at a final concentration of 2,270  $\mu$ M. To further explore whether ascorbic acid affects the production and secretion of type III secreted proteins, we cultured *B. pertussis* in SS medium in the presence of ascorbic acid at a final concentration of 91, 454, or 2,270  $\mu$ M. WCL and Sup samples were prepared from each bacterial culture and analyzed by Western blotting (Fig. 3). The BteA signal was detected in both WCL and Sup samples from SS medium at 0 to 454  $\mu$ M ascorbic acid, but the BteA signal from the SS medium at 2,270  $\mu$ M ascorbic acid was faint or absent (Fig. 3). The BopB signals showed a similar pattern (Fig. 3). These results indicate that *B. pertussis* produces and secretes type III secreted proteins at low ascorbic acid concentrations.



**FIG 2** Effects of ascorbic acid and Casamino Acids on Bvg-regulated virulence factors. Tohama I was cultured in SS medium or ascorbic acid-deprived SS medium (SS\_ASA<sup>-</sup>), Casamino Acids-deprived SS medium (SS\_CaA<sup>-</sup>), or ascorbic acid- and Casamino Acids-deprived SS medium (SS\_ASA<sup>-</sup>\_CaA<sup>-</sup>). WCL and Sup samples were separated by SDS-PAGE and analyzed by Western blotting with antibodies against BteA, BopB, FHA, or CyaA. NS, nonspecific signals. Loaded WCL and Sup samples were prepared from equal volumes of bacterial cultures. Experiments were performed at least three times, and representative data are shown.

**BteA and BopB are secreted from other *B. pertussis* clinical isolates cultured in SS\_ASA<sup>-</sup> medium.** To determine whether *B. pertussis* clinical isolates secrete type III secreted proteins at low ascorbic acid concentrations, we cultured four strains of *B. pertussis* (Table 2) in SS medium or ascorbic acid-deprived medium (SS\_ASA<sup>-</sup>). The prepared WCL and Sup samples were analyzed by Western blotting (Fig. 4). BteA signals were evident in the Sup samples of clinical isolates BP350 and BP351 from the SS\_ASA<sup>-</sup>



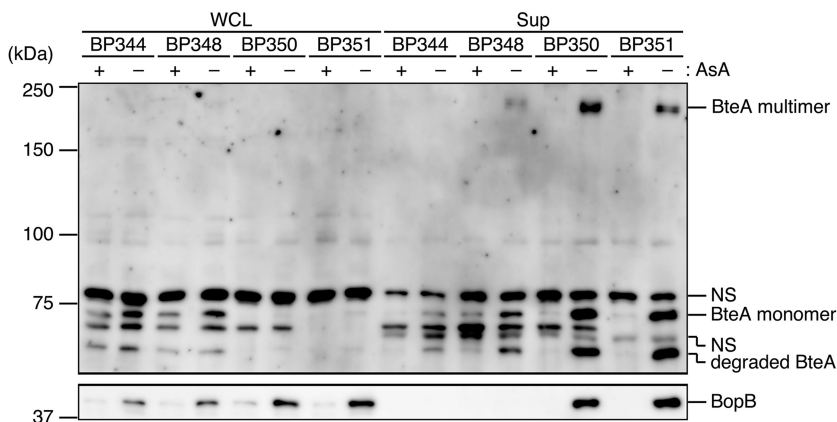
**FIG 3** Effects of ascorbic acid on type III secreted proteins. Whole-cell lysates and secreted proteins prepared from Tohama I cultured in SS medium in the presence of ascorbic acid at the final concentration of 91, 454, or 2,270 μM were separated by SDS-PAGE and then analyzed by Western blotting with antibodies against BteA or BopB. NS, nonspecific signals. Loaded WCL and Sup samples were prepared from equal volumes of bacterial cultures. Experiments were performed at least three times, and representative data are shown.

**TABLE 2** Bacterial strains and plasmids used in the study

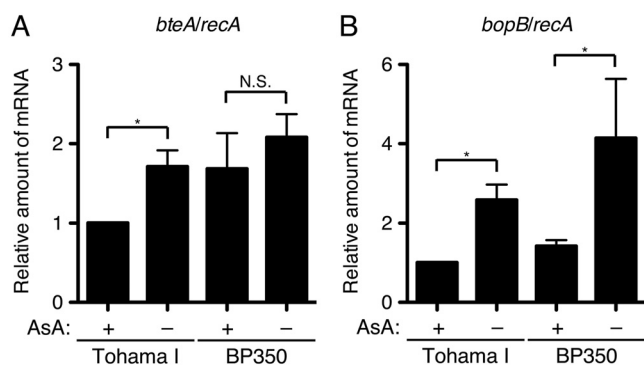
Strain or plasmid	Relevant genotype or description	Reference or source
<b>Strains</b>		
<i>B. pertussis</i>		
Tohama I	Clinical isolate	34
BP344	Clinical isolate	35
BP348	Clinical isolate	35
BP350	Clinical isolate	35
BP351	Clinical isolate	35
MGKU1	Tohama I nalidixic acid-resistant strain	This study
MGKU2	T3SS-inactive strain	This study
MGKU3	BteA-deficient strain	This study
MGKU4	BspR-deficient strain	This study
<i>E. coli</i>		
DH10B	Host strain for pDONR201	Invitrogen
Sm10λpir	Host strain for pABB-CRS2	18
<b>Plasmids</b>		
pDONR201	DNA cloning vector, Km <sup>r</sup>	Invitrogen
pABB-CRS2	Suicide vector for conjugation, Amp <sup>r</sup>	38
pABB-ΔbscN	pABB-CRS2, ΔbscN gene	18
pABB-ΔbopC	pABB-CRS2, ΔbopC (ΔbteA) gene	10
pMGKU401	pDONR201, bspR gene	This study
pMGKU402	pDONR201, ΔbspR gene	This study
pMGKU403	pABB-CRS2, ΔbspR gene	This study

medium (Fig. 4). Again, BopB signals were evident in the Sup samples of BP350 and BP351 from SS<sub>AsA</sub><sup>-</sup> medium. These results suggest that some clinical strains, e.g., BP350 and BP351, also secrete type III secreted proteins at low ascorbic acid concentrations.

**Translocator BopB is upregulated at the transcriptional level in *B. pertussis* cultured in SS<sub>AsA</sub><sup>-</sup> medium.** To investigate the gene expressions of type III secreted proteins under ascorbic acid-starved conditions, we prepared total RNA from *B. pertussis* cultured in SS or SS<sub>AsA</sub><sup>-</sup> medium. The cDNA samples reverse transcribed from the total RNA samples were subjected to a quantitative reverse transcription-PCR (qRT-PCR) analysis to quantify the relative amounts of *bteA* and *bopB* mRNA. The results demonstrated that the relative amount of *bteA* mRNA of Tohama I in SS<sub>AsA</sub><sup>-</sup> medium was increased compared to that in SS medium (Fig. 5A). However, the relative amount of *bteA* mRNA of BP350 in the SS<sub>AsA</sub><sup>-</sup> medium was not significantly different from that



**FIG 4** Effects of ascorbic acid on the secretion of BteA and BopB in clinical isolates cultured in SS<sub>AsA</sub><sup>-</sup> medium. The *B. pertussis* clinical isolates BP344, BP348, BP350, and BP351 were cultured in SS (+) or SS<sub>AsA</sub><sup>-</sup> (-) medium. WCL and Sup samples were separated by SDS-PAGE and analyzed by Western blotting with antibodies against BteA (top) or BopB (bottom). NS, nonspecific signals. Loaded WCL and Sup samples were prepared from equal volumes of bacterial cultures. Experiments were performed at least three times, and representative data are shown.



**FIG 5** Results of the RT-PCR analysis for mRNA levels of *bteA* and *bopB* from *B. pertussis* cultured under ascorbic acid-starved conditions. Total RNA was prepared from Tohama I or BP350 cultured in SS or SS<sub>AsA</sub><sup>-</sup> medium and subjected to a quantitative RT-PCR analysis. The histograms show the relative amounts of *bteA* (A) and *bopB* (B) mRNA normalized by housekeeping gene *recA* mRNA. Error bars are the standard errors of the means (SEMs) from triplicate experiments. \*,  $P < 0.05$ ; N.S., no significant difference. Experiments were performed at least three times, and representative data are shown.

in the SS medium (Fig. 5A). The relative amounts of *bopB* mRNA of Tohama I and BP350 in SS<sub>AsA</sub><sup>-</sup> medium were increased compared to those in SS medium (Fig. 5B).

These results suggest that BopB is upregulated at the transcriptional level in Tohama I and BP350 at low ascorbic acid concentrations. BteA is also upregulated at the transcriptional level in Tohama I at low ascorbic acid concentrations but not in BP350.

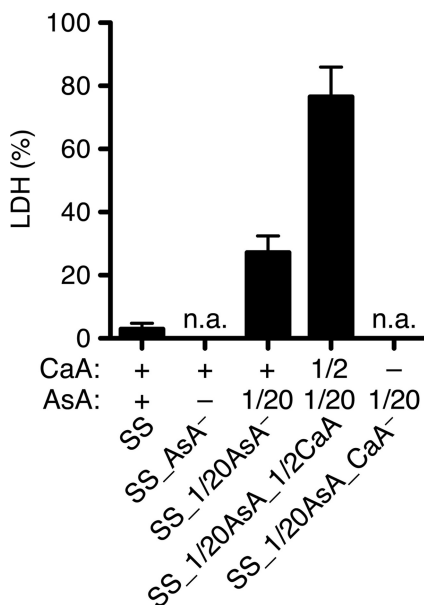
***B. pertussis* induces cytotoxicity against cultured mammalian cells.** A *B. pertussis* BspR-deficient strain was reported to induce cytotoxicity against cultured mammalian cells (15). In the present study, we identified better culture conditions for the production and secretion of BteA in *B. pertussis* than those provided with standard SS medium. We thus investigated whether wild-type *B. pertussis* provokes cytotoxicity against cultured mammalian cells under these conditions. L2 cells, a rat lung epithelial cell line, were infected at a multiplicity of infection (MOI) of 500 for 3 h in several modified SS media, shown in Fig. 6. The amount of Casamino Acids and/or ascorbic acid was reduced to one-half (1/2CaA) and 1/20 (1/20AsA), respectively, of that in SS medium. We also used the SS media without Casamino Acids and/or ascorbic acid. The amounts of lactate dehydrogenase (LDH) released into the extracellular medium were then measured.

The results revealed that released LDH was detected from L2 cells infected with Tohama I in the SS<sub>1/20AsA</sub> or SS<sub>1/20AsA\_1/2CaA</sub> medium (Fig. 6). The SS<sub>1/20AsA</sub> medium and SS<sub>1/20AsA\_1/2CaA</sub> medium showed no cytotoxicity. On the other hand, the L2 cells incubated in the SS<sub>AsA</sub><sup>-</sup> or SS<sub>1/20AsA\_CaA</sub><sup>-</sup> medium were injured by the medium itself. Therefore, these media were not useful for this assay. Taken together, these results demonstrate that we successfully observed cytotoxicity of mammalian cells induced by wild-type *B. pertussis*.

**The T3SS of *B. pertussis* cultured in SS<sub>AsA</sub><sup>-</sup> medium is active during infection against cultured mammalian cells.** As described above, we observed cytotoxicity of *B. pertussis* against cultured cells. To confirm that *B. pertussis* indeed secretes type III secreted proteins during infection, we infected L2 cells with *B. pertussis* in SS or SS<sub>1/20AsA\_1/2CaA</sub> medium at an MOI of 125 for 3 h. After infection, Bsp22 (a translocator and a component of the filament-like structure) and F-actin were stained with anti-Bsp22 and rhodamine-phalloidin, respectively.

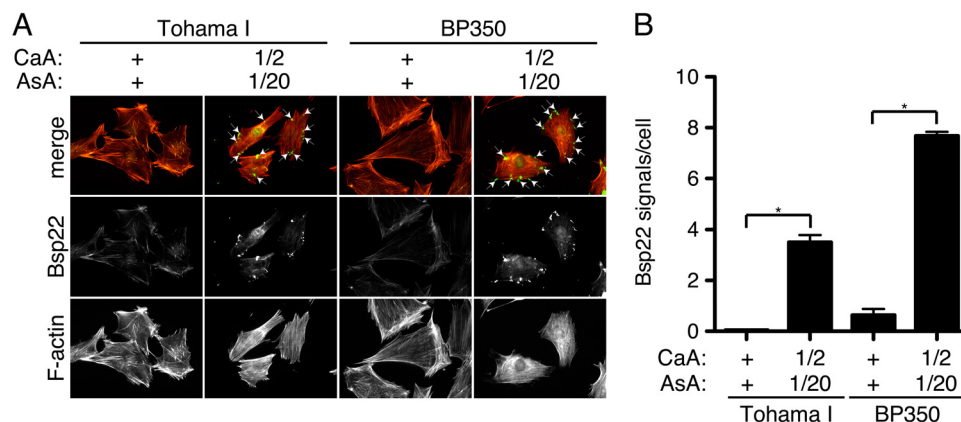
The results showed that the amounts of Bsp22 signals (Fig. 7A, green) on L2 cells infected with Tohama I and BP350 cultured in SS<sub>1/20AsA\_1/2CaA</sub> medium were greater than those in SS medium (Fig. 7). Thus, we demonstrated that *B. pertussis* T3SS is activated during the infection of cultured mammalian cells under appropriate conditions.

**The cytotoxicity induced by wild-type *B. pertussis* depends on the T3SS.** It has been reported that *Bordetella bronchiseptica* induces BteA-dependent necrotic cell



**FIG 6** Cytotoxic activity of *B. pertussis* cultured in SS\_ASA<sup>-</sup> medium. L2 cells were infected with Tohama I cultured in SS medium, SS\_ASA<sup>-</sup> medium, SS medium containing 1/20 as much ascorbic acid as SS medium (SS\_1/20AsA), SS medium containing 1/20 as much ascorbic acid and one-half as much Casamino Acids as SS medium (SS\_1/20AsA\_1/2CaA), or SS medium containing 1/20 as much ascorbic acid as SS medium and no Casamino Acids (SS\_1/20AsA\_CaA<sup>-</sup>) at an MOI of 500 for 3 h. +, 1/2, and 1/20 show the amounts of indicated substances that are equal to, one-half of, and 1/20 of those in SS medium, respectively. The amounts of LDH released into the extracellular medium from infected cells are shown, and the relative cytotoxicity (percent) was determined as described in Materials and Methods. Error bars are the SEMs from triplicate experiments. n.a., no available result. Experiments were performed at least three times, and representative data are shown.

death against cultured mammalian cells (10). To investigate whether the cytotoxicity provoked by wild-type *B. pertussis* also depends on BteA function, we infected L2 cells with MGKU1 (a Tohama I nalidixic acid-resistant strain), MGKU2 (a T3SS-inactive strain), MGKU3 (a BteA-deficient strain), or MGKU4 (a BspR-deficient strain that allows the constitutive activation of T3SS) in SS\_1/20AsA\_1/2CaA medium at an MOI of 125 or 250 for 3 h. The amounts of LDH released into extracellular medium were measured.



**FIG 7** Immunofluorescent staining of Bsp22 on L2 cells infected with *B. pertussis*. (A) L2 cells were infected with Tohama I or BP350 in the indicated media at an MOI of 125 for 3 h. +, 1/2, and 1/20 indicate the amounts of indicated substances that are equal to, one-half of, and 1/20 of those in SS medium, respectively. After fixation, cells were stained with anti-Bsp22 antibody (green) and rhodamine-phalloidin (red). Arrows indicate the Bsp22 (green) signal. (B) Bsp22 signals per cell were counted under a fluorescence microscope. At least 120 cells were randomly chosen. Error bars indicate the SEMs from triplicate experiments. \*,  $P < 0.05$ . Experiments were performed at least three times, and representative data are shown.

As a result, LDH was not detected in the medium of L2 cells infected with MGKU2 (Fig. 8A and B). The amount of LDH detected in the medium of L2 cells infected with MGKU3 was significantly smaller than that in the medium of L2 cells infected with MGKU1 (Fig. 8A and B). The amount of LDH detected in the medium of L2 cells infected with MGKU4 was significantly greater than that in the medium of L2 cells infected with MGKU1 at an MOI of 125 (Fig. 8A) but not at an MOI of 250 (Fig. 8B).

WCL and Sup samples were prepared from each strain, separated by SDS-PAGE, and then analyzed by Western blotting with anti-BteA antibody (Fig. 8C). BteA signals were detected in the WCL samples with MGKU1, MGKU2, and MGKU4 but not with MGKU3 (Fig. 8C). BteA signals were detected in the Sup samples with MGKU1 and MGKU4 but not with MGKU2 or MGKU3 (Fig. 8C). These results suggest that the cytotoxicity induced by wild-type *B. pertussis* (Fig. 6) is dependent on T3SS activity and is partially dependent on BteA.

**The secretion of type III secreted proteins in *B. pertussis* is downregulated under reducing conditions.** Ascorbic acid is known as a reducing agent. To determine whether the secretion of type III secreted proteins is downregulated under reducing conditions, we cultured Tohama I in ascorbic acid-deprived SS medium (SS<sub>AsA</sub><sup>-</sup>) (Fig. 9, none) and in SS<sub>AsA</sub><sup>-</sup> medium containing the reducing agent ascorbic acid (AsA), 2-mercaptoethanol (2-ME), or dithioerythritol (DTE). The prepared WCL and Sup samples were analyzed by Western blotting with anti-BteA, anti-BopB, and anti-FHA antibodies (Fig. 9). The BteA and BopB signal intensities from both WCL and Sup samples were decreased in the presence of each of the reducing agents (Fig. 9). In contrast, the FHA signal intensities from WCL and Sup samples were not affected by the reducing agents. The growth of bacteria cultured in SS medium with reducing agents was not significantly different from that of the bacteria cultured in SS medium (data not shown). These results suggest that the production and secretion of type III secreted proteins in *B. pertussis* are downregulated under reducing conditions.

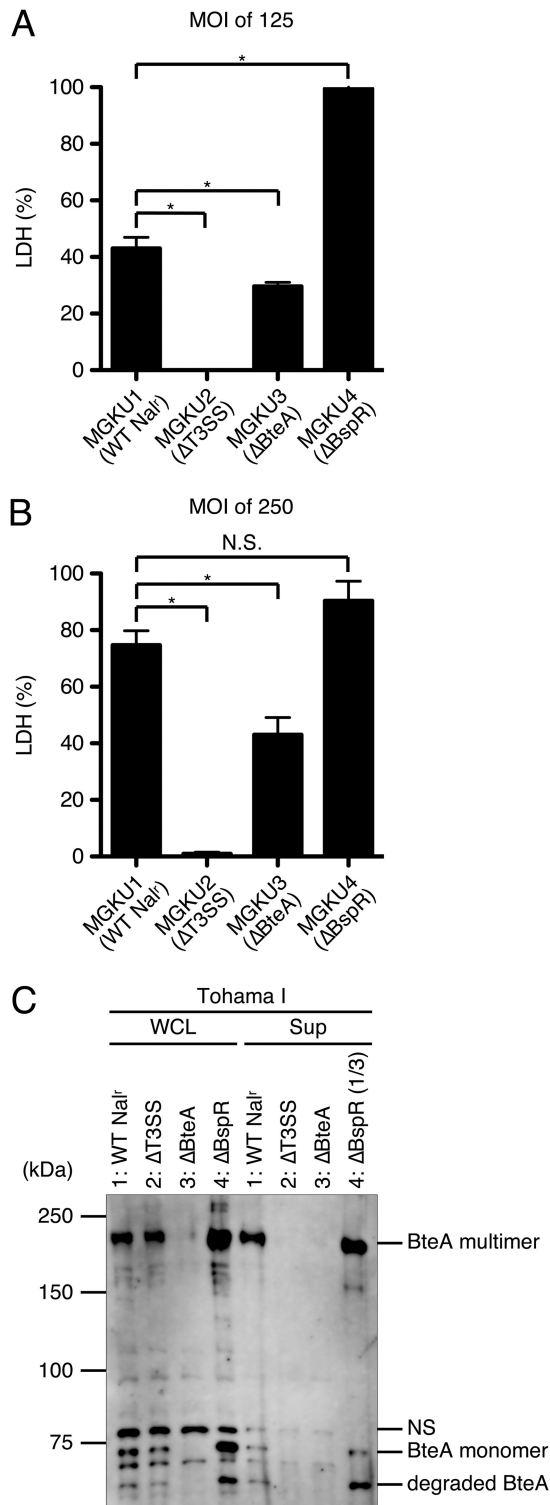
## DISCUSSION

In this study, *B. pertussis* increased the production and secretion of type III secreted proteins, BteA and BopB, when cultured in ascorbic acid-deprived SS medium (SS<sub>AsA</sub><sup>-</sup>). We successfully produced cytotoxicity against mammalian cells by the infection of wild-type Tohama I cultured in SS<sub>1/20AsA\_1/2CaA</sub> medium. *B. pertussis* decreased the production and secretion of type III secreted proteins when cultured in SS<sub>AsA</sub><sup>-</sup> medium containing reducing agents such as 2-ME or dithioerythritol (DTE). These results suggest that redox signaling is involved in regulation of the T3SS in *B. pertussis*.

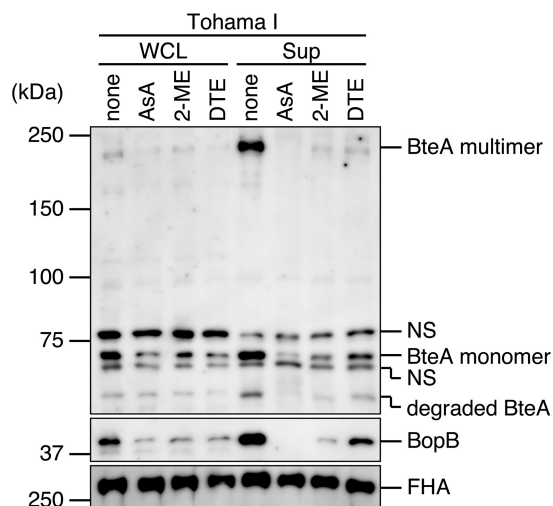
It has been reported that no secretion of the type III secreted proteins BteA and Bsp22 was detected from Tohama I (23) and that the secretion of the type III secreted proteins BteA and BopD was detected from a nonvaccine-type strain, BP157. However, BP157 showed no cytotoxicity against L2 cells, J774 mouse macrophage-like cells, and HeLa cells (24). In another report, glutamate limitation upregulated production of the type III secreted proteins (25). Thus, the requirement of a type III secretion system (T3SS) for virulence in *B. pertussis* remains unclear. On the other hand, a BscN-deficient mutant in which the T3SS was inactive colonized mouse lungs to a significantly lower degree than the wild type (23). In addition, cytotoxicity against HeLa cells was observed by infection with a BspR (a negative regulator for type III secreted proteins)-deficient *B. pertussis* strain (15). These results suggest that the T3SS has a significant role in the virulence of *B. pertussis*.

The production and the relative amounts of *bopB* mRNA of Tohama I cultured in SS<sub>AsA</sub><sup>-</sup> medium were significantly increased compared to those of Tohama cultured in SS medium, but this was not the case for BteA mRNA (Fig. 2 and 5). It was reported that *bteA* and *bopB* genes are upregulated by BtrS (a sigma factor for type III secreted proteins) and downregulated by BspR (13, 15). It has also been reported that the amount of *bteA* mRNA was increased 2-fold in a BspR-deficient strain compared to that in the wild type, whereas the amount of *bopB* mRNA was increased by 18-fold





**FIG 8** Cytotoxic activities of *B. pertussis* mutants. The results of LDH assays at an MOI of 125 (A) or 250 (B) are shown as a histograms. Error bars indicate the SEMs from triplicate experiments. \*,  $P < 0.05$ . Experiments were performed at least three times, and representative data are shown. (C) MGKU1 (Tohama I nalidixic acid-resistant strain [1: WT Nal<sup>r</sup>]), MGKU2 (T3SS-inactive strain [2:  $\Delta$ T3SS]), MGKU3 (BteA-deficient strain [3:  $\Delta$ BteA]), or MGKU4 (BspR-deficient strain [4:  $\Delta$ BspR]) was cultured in SS<sub>1</sub>/20AsA<sub>1</sub>/2CaA medium. WCL and Sup samples were separated by SDS-PAGE and analyzed by Western blotting with anti-BteA antibody. We loaded a 3-fold-smaller amount of MGKU4 supernatant fraction on the SDS-PAGE gel to avoid obtaining an excess signal intensity. NS, nonspecific signals. Loaded WCL and Sup samples were prepared from equal volumes of bacterial culture. Experiments were performed at least three times, and representative data are shown.



**FIG 9** Effects of reducing agents on the production and secretion of type III secreted proteins in *B. pertussis*. Tohama I was cultured in SS<sub>1</sub>AsA<sup>-</sup> medium or SS<sub>1</sub>AsA<sup>-</sup> medium containing ascorbic acid (AsA), 2-mercaptoethanol (2-ME), or dithioerythritol (DTE) at a final concentration of 2.3, 2.1, or 3.0 mM, respectively. WCL and Sup samples were separated by SDS-PAGE and then analyzed by Western blotting with antibodies against BteA, BopB, or FHA. Loaded WCL and Sup samples were prepared from equal volumes of bacterial culture. NS, nonspecific signals. Experiments were performed at least three times, and representative data are shown.

compared to that in the wild type by transcriptome sequencing (RNA-seq) (15). The *bopB* gene is localized in the T3SS apparatus locus (*bsc* locus) adjacent to the *btrS* gene. Since the *bteA* gene is separated from the *bsc* locus by >2.5 Mb (9), BtrS would preferentially regulate transcription of the *bopB* gene over that of the *bteA* gene.

The cytotoxicity against L2 cells was observed by the infection of Tohama I cultured in SS<sub>1</sub>/20AsA<sub>1</sub>/2CaA medium (Fig. 6). Before infection, we exchanged Ham's F-12K medium for the SS<sub>1</sub>/20AsA<sub>1</sub>/2CaA medium. When L2 cells were infected with Tohama I in Ham's F-12K medium, the cytotoxicity against L2 cells was not observed (see Fig. S1 in the supplemental material). To establish oxidizing conditions in the tissue culture media, we used several oxidizing agents, i.e., hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), potassium ferricyanide(III) {K<sub>3</sub>[Fe(CN)<sub>6</sub>]}, methyl 3-nitro-2-pyridinesulfonate (Npys-OMe), and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). We also used another tissue culture medium, i.e., Eagle minimum essential medium (MEM), because MEM contains no reducing agents, while F-12K, which is the medium for the L2 cell culture, contains a reducing agent, i.e., cysteine. Even when L2 cells were infected in F-12K medium containing the oxidizing agent or MEM, *B. pertussis* showed no cytotoxicity (Fig. S1). We therefore speculate that a substance which inhibits the secretion of type III secreted proteins from Tohama I was present in the Ham's F-12K medium or a substance that is necessary for the secretion of type III secreted proteins from Tohama I was absent in the Ham's F-12K medium. We found that infection of the human alveolar epithelium cell line A549 with wild-type *B. pertussis* cultured in SS<sub>1</sub>/20AsA<sub>1</sub>/2CaA medium did not result in cytotoxicity (data not shown). For the observation of cytotoxicity against A549 cells, it is necessary to optimize the conditions for the production and secretion of type III secreted proteins in *B. pertussis*.

A previous study showed that cytotoxicity against the cell line J774A.1 was achieved by infection with wild-type *B. pertussis* 18323 but not by infection with a CyaA-deficient strain (26). This finding suggested that CyaA has the potential to provoke cytotoxicity. However, we confirmed herein that the production and secretion of CyaA in a BteA-deficient strain (MGKU3) were not significantly different from those of the wild type (data not shown). In our results, the amounts of LDH released from L2 cells infected with MGKU3 were greater than those of the T3SS-inactive strain (MGKU2), and no LDH release was detected from L2 cells infected with MGKU2 (Fig. 8). These results raised

the possibility that an unknown type III effector other than BteA is involved in the cytotoxicity of *B. pertussis*.

Although we observed no significant difference in the growth rate of Tohama I between SS medium and SS<sub>AsA</sub><sup>-</sup> medium (data not shown), the production and secretion of type III secreted proteins of Tohama I cultured in the SS<sub>AsA</sub><sup>-</sup> medium were increased compared to those in the SS medium (Fig. 2). It had not been determined whether the expression of type III secreted proteins is regulated by reducing agents at the molecular level, and in this study, the production and secretion of CyaA and FHA in Tohama I cultured in SS medium were not significantly different from those in SS<sub>AsA</sub><sup>-</sup> medium (Fig. 2). It was reported that there is no difference in Prn production by Tohama I cultured in modified SS medium compared to that in a culture in modified SS medium containing DTT, a reducing agent (27). These results suggest that BvgAS-regulated virulence factors other than type III secreted proteins are not affected by reducing agents.

Ascorbic acid, urea, and glutathione (GSH) function as reducing agents in the epithelial lining fluid (ELF) of the human airway. The concentrations of ascorbic acid and urea in ELF in the upper respiratory tract are not significantly different from those in the lower respiratory tract. However, the concentration of GSH in the ELF in the upper respiratory tract is 100-fold lower than that in the lower respiratory tract. One of the possibilities is that GSH forms GS-SG (an oxidized form of GSH) in the ELF in the upper respiratory tract (28). It may thus be considered that the upper respiratory tract is more oxidized than the lower respiratory tract. It was reported that the transcription of genes encoding type III secreted proteins of *B. pertussis* recovered from mouse nasal lavage samples was higher than that from bronchoalveolar lavage samples (29). We thus speculate that the T3SS is important for *B. pertussis* to colonize the upper respiratory tract of the host.

In this study, the production and secretion of BopB by Tohama I cultured in the SS<sub>AsA</sub><sup>-</sup> medium were significantly increased compared to those in SS medium (Fig. 2). Bacteria use redox-sensing proteins to resist oxidative stress (30). For example, OxyR (a member of the LysR family of transcriptional regulators) of *Escherichia coli* is converted into an active form by the oxidation of intramolecular cysteine residues, which then increases the expression of antioxidant genes, e.g., hydroperoxidase I (*katG* [31]). Vfr (a member of the CRP/FNR superfamily) of *Pseudomonas* becomes active by retaining intramolecular cysteine residues as reducing states and then increases the expression of type III secreted proteins (32). Btr is a regulator protein produced by *B. pertussis* and is homologous to Vfr (33). A cysteine cluster is located in the N-terminal domain of Btr, and a helix-turn-helix DNA binding domain is located in the C-terminal domain of Btr. Therefore, when *B. pertussis* is cultured in reducing agent-deprived SS medium, Btr might be oxidized and change its conformation, thereby increasing the expression of type III secreted proteins. Although the signal pathway that *B. pertussis* uses to increase the production of type III secreted proteins is unclear, it is possible that Btr regulates gene expressions for type III secreted proteins.

## MATERIALS AND METHODS

**Bacterial strains.** The strains used in this study are listed in Table 2. *B. pertussis* Tohama I (34) was used as the wild-type strain. BP344, BP348, BP350, and BP351 were used as clinical isolates and were a kind gift from K. Kamachi (National Institute of Infectious Diseases) (35). *Escherichia coli* DH10B and Sm10λpir were used for DNA cloning and conjugation, respectively (Table 2). *B. pertussis* was grown on Bordet-Gengou agar plates at 37°C for 5 days. Bordet-Gengou agar plates were prepared as follows. We mixed 15 g of Bordet-Gengou agar base (BD number 248200), 5 ml of glycerol, 100 ml of defibrinated horse blood (Nippon Bio-Supp. Center), and 400 ml of distilled water. The mixture was autoclaved and poured onto plastic plates. Fresh colonies of *B. pertussis* on each Bordet-Gengou agar plate were suspended in Stainer-Scholte (SS) medium (Table 1) (36). In this study, we used a liquid medium described as a cyclodextrin-containing medium as the SS medium (37). Low-Casamino Acids (LCA) medium was prepared by removing the agar from cyclodextrin solid medium (CSM) contents (Table 1) (22). *B. pertussis* was cultured in SS medium with a starting  $A_{600}$  of 0.23 under static conditions at 37°C. To measure the bacterial density, a spectrophotometer (Ultrospec 2100 pro; Amersham) was used. The final  $A_{600}$  of *B. pertussis* Tohama I culture after 22 h was ca. 0.67, and the culture contained ca.  $1.0 \times 10^9$

**TABLE 3** Primers used in the study

Name	Sequence (5'→3') <sup>a</sup>
B1-bspR	AAAAAGCAGGCTCGCCGTTTCCACCACGTAA
B2-bspR	AGAAAGCTGGGTGGCCAATATCCTGTCTCAGC
R1-bspR	CGCGGATCCCATACGGCGCAAGAATGGCT
R2-bspR	CGCGGATCCAAGACATCAAGTCCAAAG
5-recA	ATGAAGATCGGCGTGATGT
3-recA	TAGAAGTGGAGCGGTTGC
5-bteA	AATGGCCTTGGTGGGAAC
3-bteA	ATTCAGCGCCGTGATCTT
5-bopB	GTCTGCTGACAGCGTTGG
3-bopB	GGGGTTCGATCAGCAAGAC

<sup>a</sup>Restriction enzyme recognitions sites are underlined.

CFU/ml. The liquid cultivation period was 22 h for the protein preparation, mRNA preparation, and infection.

**Generation of a Tohama I nalidixic acid-resistant mutant.** For the generation of a Tohama I nalidixic acid-resistant mutant, Tohama I cultured in SS medium for 24 h as described above was spread on 20 sets of Bordet-Gengou agar plates containing nalidixic acid at the final concentration of 30  $\mu$ g/ml. After 5 days, surviving colonies were designated MGKU1. The growth rate, colony size, and hemolysis on the BG plates of the resulting nalidixic acid-resistant strain were almost equivalent to those of the wild-type strain, and we observed that the amounts of the production and secretion of type III secreted proteins in MGKU1 were not significantly different from those of Tohama I wild type (data not shown).

**Cell culture.** L2 cells, a rat lung epithelial cell line (ACTT CCL-149), were maintained in F-12K (Invitrogen, Tokyo, Japan). The cell culture medium was supplemented with 10% fetal calf serum (FCS). L2 cells were grown at 37°C under a 5% CO<sub>2</sub> atmosphere.

**Construction of gene-disrupted mutants.** The primers used in this study are listed in Table 3. To construct the *bspR* mutant, the 4.0-kb DNA fragment harboring *bspR* and its flanking regions was amplified by PCR with primers B1-bspR and B2-bspR using *B. pertussis* Tohama I genomic DNA as the template. The resulting PCR product was cloned into pDONR201 to obtain pMGKU401 by means of adapter PCR and site-specific recombination techniques using a Gateway cloning system (Invitrogen). An inverse PCR was carried out with the set of primers R1-bspR and R2-bspR using circular pMGKU401 as the template.

The resulting PCR product was digested with BamHI and self-ligated to obtain pMGKU402. This plasmid contained the BamHI site in addition to a 474-bp in-frame deletion from 175 bp downstream of the 5' end of the *bspR* gene to 52 bp upstream of the 3' end of the gene. This plasmid, pMGKU402, was mixed with a positive suicide vector, pABB-CRS2 (38), to obtain pMGKU403 using the Gateway cloning system. This plasmid, pMGKU403, was introduced into *E. coli* Sm10 $\lambda$ pir (18) and transconjugated into MGKU1 as described previously (39). The resulting mutant strain was designated MGKU4 (BspR-deficient strain). Similarly, pABB- $\Delta$ *bscN* (18) and pABB- $\Delta$ *bopC* (10) were separately introduced into *E. coli* Sm10 $\lambda$ pir and were transconjugated into MGKU1. The resulting mutant strains were designated MGKU2 (a T3SS-inactive strain) and MGKU3 (a BteA-deficient strain), respectively.

**Preparation of proteins from culture supernatants and whole bacterial cell lysates.** Secreted proteins released into bacterial culture supernatants and whole bacterial cell lysates were prepared by trichloroacetic acid precipitation. In the case of the supernatant fraction (Sup) samples, the culture supernatants were filtered with a 0.22- $\mu$ m filter (Millipore number SLGVR33RB). Next, 2  $\mu$ l of 5% deoxycholic acid and 100  $\mu$ l of 100% trichloroacetic acid were added to the 1 ml of filtered supernatants. After being incubated on ice for 15 min, the samples were centrifuged at 21,130  $\times$  *g* for 5 min. The resulting precipitated proteins were neutralized with 2  $\mu$ l of 2 M Tris base and dissolved in 13  $\mu$ l of 2 $\times$  SDS-PAGE sample buffer. In the case of the whole-cell lysate (WCL) samples, bacterial pellets collected from 1 ml of bacterial cultures were resuspended in distilled water. One hundred microliters of 100% trichloroacetic acid was added to 1 ml of the bacterial suspension. After being incubated on ice for 15 min, the samples were centrifuged at 21,130  $\times$  *g* for 5 min. The resulting precipitated proteins were neutralized with 5  $\mu$ l of 2 M Tris base and dissolved in 95  $\mu$ l of 2 $\times$  SDS-PAGE sample buffer. When the A<sub>600</sub> of bacterial culture was 0.67, 0.5  $\mu$ l of WCL and 5  $\mu$ l of Sup samples were loaded on the SDS-PAGE gels. The loaded sample amounts were adjusted by the A<sub>600</sub> of each bacterial culture in order to load samples prepared from the same number of bacteria. The protein samples were separated by SDS-PAGE and analyzed by Western blotting.

**Antibodies.** Anti-BteA, anti-BopB, and anti-Bsp22 antibodies were purified from rabbit serum in our previous study (10, 18, 40). To detect filamentous hemagglutinin (FHA) signals, we used mouse anti-FHA serum (41). Mouse anti-CyaA monoclonal antibody was purchased from Santa Cruz Biotechnology (Dallas, TX). To prepare the anti-BtrS antibody, the peptide corresponding to the C-terminal region of BtrS (CALREALRERYDSVP) was conjugated to hemocyanin from keyhole limpets (Sigma) by using 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Sigma). These cross-linked peptides were used to immunize rabbits, and resulting antisera were incubated with peptide immobilized on epoxy-activated Sepharose 6B (Amersham) to obtain specific Ig fractions.

**Quantitative reverse transcription-PCR.** The amounts of mRNA were measured by qRT-PCR. Bacterial total RNA was prepared using a Trizol Max Bacterial RNA isolation kit (Invitrogen), an RNeasy

minikit (Qiagen, Hilden, Germany), and an RNase-free DNase-free kit (Qiagen). The RNA sample was reverse transcribed with Transcriptor Universal cDNA master (Roche Diagnostics, Indianapolis, IN) and a T100 thermal cycler (Bio-Rad, Hercules, CA). The resulting cDNA was amplified by FastStart Essential DNA Probes Master (Roche) using the following primer pairs 5-recA and 3-recA for *recA*, 5-bteA and 3-bteA for *bteA*, and 5-bopB and 3-bopB for *bopB* (Table 3). The results were calculated as described in the Roche manual. The amount of *recA* mRNA was used as an internal control. The mRNA amounts are presented here as relative to the amount in the Tohama I cultured in SS medium, which was defined as 1.

**LDH assays.** To examine the release of lactate dehydrogenase (LDH) from *B. pertussis*-infected cells,  $5.0 \times 10^4$  cells/well of L2 cells seeded in 24-well plates were infected with bacteria at the multiplicity of infection (MOI) of 125, 250, or 500. The plates were centrifuged at  $900 \times g$  for 5 min and incubated for 3 h at 37°C under a 5% CO<sub>2</sub> atmosphere. The amounts of LDH were measured spectrophotometrically using a Cyto-Tox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI). We added 50 μl of 10% Triton X-100 to 950 μl of extracellular medium of L2 cells in a 24-well plate. We then mixed the extracellular medium by pipetting. We subtracted the LDH value obtained from extracellular medium of uninfected cells from the value obtained from the Triton X-100-treated cells and used the resulting value as 100%.

**Immunofluorescent staining.** For the immunofluorescent staining assay,  $2.5 \times 10^5$  cells/well of L2 cells were seeded on coverslips in six-well plates, incubated overnight, and then infected with *B. pertussis* at an MOI of 125. The plates were centrifuged at  $900 \times g$  for 5 min and incubated for 3 h at 37°C under a 5% CO<sub>2</sub> atmosphere. The infected L2 cells were then immunostained as described previously (12). Briefly, the cells were treated with 4% paraformaldehyde, 50 mM NH<sub>4</sub>Cl, 0.2% Triton X-100, and 4% bovine serum albumin (BSA). After blocking, the cells were incubated with an anti-Bsp22 antibody (40). As a secondary antibody, Alexa Fluor 488–goat anti-rabbit IgG (Invitrogen) was used. F-actin was stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR). Average numbers of Bsp22 signal dots on single L2 cells were measured by fluorescence microscopy.

**Statistical analyses.** The statistical analyses were performed using the nonparametric unpaired *t* test with Prism ver. 5.0f software (GraphPad, La Jolla, CA). One-tailed *P* values of <0.05 were considered significant.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, PDF file, 2 MB.

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