BopC Is a Novel Type III Effector Secreted by Bordetella bronchiseptica and Has a Critical Role in Type III-dependent Necrotic Cell Death*5

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In Bordetella bronchiseptica, the functional type III secretion system (TTSS) is required for the induction of necrotic cell death in infected mammalian cells. To identify the factor(s) involved in necrotic cell death, type III-secreted proteins from B. bronchiseptica were analyzed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and electrospray ionization tandem mass spectrometry. We identified a 69-kDa secreted protein designated BopC. The gene encoding BopC is located outside of the TTSS locus and is also highly conserved in both Bordetella parapertussis and Bordetella pertussis. The results of a lactate dehydrogenase release assay and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling assay demonstrated that BopC is required for necrotic cell death. It has been reported that tyrosine-phosphorylated proteins (PY) of host cells are dephosphorylated during B. bronchiseptica infection in a TTSS-dependent manner. We found that BopC is also involved in PY dephosphorylation in infected host cells. It appears that the necrotic cell death triggered by BopC occurs prior to the PY reduction in host cells, because Bordetella-induced cell death was not affected even in the presence of a dephosphorylation inhibitor. Furthermore, a translocation assay showed that the signal sequence for both secretion into culture supernatant and translocation into the host cell is located in 48 amino acid residues of the BopC N terminus. This report reveals for the first time that a novel type III effector, BopC, is required for the induction of necrotic cell death during Bordetella infection.

Bordetella pertussis is a causative agent of whooping cough (pertussis) in humans (1, 2). Bordetella parapertussis is also a cause of whooping cough, and its pathology is generally milder than that of B. pertussis (2). Bordetella bronchiseptica is known to be the evolutionary progenitor of B. pertussis and B. parapertussis (3). B. bronchiseptica has been isolated from a large number of four-legged animals (4) and causes kennel cough in dogs (5) and atrophic rhinitis in swine (6), although most cases of B. bronchiseptica infection are asymptomatic (7). These three Bordetella species colonize the host’s respiratory tract.

In Bordetella, most virulence factors, such as adhesion factors and toxins, are expressed under the control of a two-component regulatory system composed of BvgA and BvgS (BvgAS system) (8). BvgS is a sensory histidine kinase and is localized in the bacterial membrane (9). When the bacteria are situated in conditions resembling the extracellular environment of the host tissue during colonization, BvgS is auto-phosphorylated and transfers its phosphate group to BvgA (10). The phosphorylated BvgA binds to the upstream of the promoters of specific genes that encode mainly virulence factors, and acts as the transcriptional activator (11–13). In this way, the activation of the BvgAS system leads the bacteria to the virulent phase. The BvgAS system also positively regulates the expression of a virulence factor secretion system called the type III secretion system (TTSS) (14).

The TTSS is highly conserved in a number of Gram-negative bacteria and functions as the injector of virulence proteins, so-called effectors, to the host cell (reviewed in Ref. 15). For example, functional TTSS is required for the formation of the pedestal-like structure beneath the bacterial adhesion site in enteropathogenic Escherichia coli (EPEC) (16) and for the bacterial invasion of epithelial cells in Shigella (17, 18). If these pathogens are deficient in the TTSS function, their virulence is greatly reduced, indicating that TTSS is one of the major mechanisms for exerting their pathogenicity. In general, the bacteria possessing TTSS exploit several effectors to achieve infection. In EPEC, seven effectors, Tir, EspG, Orf3, EspF, EspH, Map, and Cif, have been identified (19, 20), and Shigella secrete VirA, IcsB, IpaH3.5, and IpgD (21–24) via the TTSS as effectors. Thus, effector variations provide different pathological features among bacterial infection.

In B. bronchiseptica, the functional TTSS is required for the induction of cytotoxicity, including necrotic cell death, in cultured mammalian cells during infection (25). Moreover, B. bronchiseptica with TTSS defi-

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2 The abbreviations used are: TTSS, type III secretion system; EPEC, enteropathogenic E. coli; SS, Stainer and Scholte; BG, Bordet and Gengou; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; ESI-MS/MS, electrospray ionization tandem mass spectrometry; CBB, Coomassie Brilliant Blue; ORF, open reading frame; m.o.i., multiplicity of infection; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling; PBS, phosphate-buffered saline; LDH, lactate dehydrogenase; PY, tyrosine-phosphorylated protein; F-actin, filamentous actin; aa, amino acid(s); PBS, phosphate-buffered saline; PTPase, protein-tyrosine phosphatase.
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Bacteriological, Cell Culture, and Media—The wild-type strain used in this study was B. bronchiseptica S798 (27). The type III secretion mutant (type III−) and BopB mutant (ΔbopB) were derived from S798 (27). Bordetella strains were cultured in Stainer and Scholte (SS) liquid medium with a starting A600 of 0.2, and the inoculum was prepared from fresh colonies grown on Bordet and Gengou (BG) agar as described previously (29–31). For the infection assay, B. bronchiseptica strains cultured for 18 h at 37 °C with vigorous shaking were used. E. coli DH10B, MC1061, and SM10Apri were used as hosts for the construction of various plasmids. L2 cells (ATCC CCL-149) were maintained in Dulbecco’s modified Eagle’s medium (Sigma) and were maintained in Dulbecco’s modified Eagle’s medium (Sigma).

MALDI-TOF MS and ESI-MS/MS Analyses—The protocols for preparing samples for MALDI-TOF MS or ESI-MS/MS analyses were described elsewhere (33). Briefly, after SDS-PAGE, protein bands were stained with Coomassie Brilliant Blue (CBB) and excised from the gels. The resulting gel pieces were treated with a reducing agent such as dithiothreitol. After alkylation by iodoacetamide, the proteins were digested with trypsin (sequencing grade, Roche Molecular Biochemicals), and the resulting samples were analyzed by the Voyager-DE PRO MALDI mass spectrometer (Applied Biosystems) or Q-Tof 2 (Micromass). To retrieve the amino acid sequence of proteins analyzed by mass spectrometry, all speculative open reading frames (ORFs) were picked from the genomic sequence obtained from the B. bronchiseptica S798 genomic DNA as the template. This PCR product was cloned into the vector by means of adaptor PCR and site-specific recombination techniques using the Gateway cloning system. The resulting plasmid was designated pDONR-bflA. Likewise, to insert the rrnB terminator region into pDONR P2R-P3 (Invitrogen), a 0.4-kbp fragment was amplified by PCR with the primers B2f-flbA (5′-TGGTGGCAA-3′) and B2r-flbA (5′-TAATAAAGTTGAAACAAAAAGTGTGGTGAAGAACC-3′) using pTrc99A (Amersham Biosciences) as the template. This PCR product was cloned into the vector, and the resulting plasmid was designated pDONR-rrnB.

For the complementation of the bopC defect in ΔbopC, pBopC was constructed as follows. A 2.3-kbp fragment encoding BopC was amplified by PCR with the primers B1-bopC (5′-AAAAAGCGAGGGTTGGTACATATGCTGACCT-3′) and B2-bopC (5′-AGGGGTGTCGTTCTCGGATCATATGCGCGG-3′) using B. bronchiseptica S798 genomic DNA as the template. The resulting PCR product was cloned into pDONR201, designated pDONR-bopC. To control the transcription of the bopC gene by the flbA promoter and rrnB terminator in Bordetella, pDONR-flbA, pDONR-bopC-comp, pDONR-rrnB, and pRK415 R4-R3-F were mixed and treated with LR Clonase Plus (Invitrogen) to clone the flbA promoter, bopC gene, and rrnB terminator into pRK415 R4-R3-F using the MultiSite Gateway system (Invitrogen), and the resulting plasmid was designated pDONR-rrnB.

To construct the plasmids encoding the TEM-1-fused protein, we cloned bopC, bcrH2, and map genes into pCX340 (38). Then, 2.2- and 0.7-kbp fragments encoding BopC, BcrH2, and Map, respectively, were amplified by PCR with the following primer sets. For bopC: pBopC-pCX340-Ndel (5′-GAATTCCATATGCGTACATATGCGGACCT-3′) and B2-bopC (5′-AGGGGTGTCGTTCTCGGATCATATGCGCGG-3′) using B. bronchiseptica S798 genomic DNA as the template. The resulting PCR product was cloned into pDONR201 to obtain pDONR-bopC using the adaptor PCR method in the Gateway cloning system (Invitrogen). Then, inverse PCR was carried out with the primers R1-bopC (5′-CGGGATCCCTTACCGGGGCTGTCCGGGCTTCT-3′) and R2-bopC (5′-GGGATCCCTTACCGGGCTGTCCGGGCTTCT-3′) using circular pDONR-bopC as the template. The underlined portions indicate the BamH I sites.

The resulting plasmids were digested with BamH I and self-ligated to obtain pDONR-ΔbopC, which contained a 178-bp deletion around the 5′ region of the bopC. pDONR-ΔbopC was mixed with pABB-CRS2 to obtain pABB-ΔbopC using the Gateway cloning system. pABB-ΔbopC was then introduced into E. coli SM10Apri and was transconjugated into the B. bronchiseptica S798 (streptomycin-resistant), as described previously (36). The resulting mutant strain was designated ΔbopC.

Construction of Plasmid for Gene Expression in Bordetella and Mammalian Cells—A vector used for the gene expression in Bordetella, pRK415 R4-R3-F, was constructed as follows. A 1.8-kbp fragment containing attR4, ccdB, the chloramphenicol acetyl transferase gene, and attR3 was amplified by PCR with the primers HindIII-R4-F (5′-CCCAAGCTTACAGGGGAGCTATGACAAGTGC-3′) and HindIII-R4-R3 (5′-CCCAAGCTTACAGGGGAGCTATGACAAGTGC-3′) using pDEST R4-R3 (Invitrogen) as the template. The underlined portions indicate the HindIII sites. This fragment was digested with HindIII and then inserted into the HindIII site in pRK415 (37). The resulting plasmid, in which the ccdB is oriented in the opposite direction from the lac promoter in pRK415, was designated pRK415 R4-R3-F. To insert the flbA promoter region into pDONR P4-P1R (Invitrogen), a 0.5-kbp fragment was amplified by PCR with the primers B4f-flbA (5′-ATAAGAAAGGTTTTGTGTTTCTCTCACAGTT-3′) and B1R-flbA (5′-TGTAATACGACATCACTTTTTATTTAGGTCG-3′) using B. bronchiseptica S798 genomic DNA as the template. This PCR product was cloned into the vector by means of adaptor PCR and site-specific recombination techniques using the Gateway cloning system. The resulting plasmid was designated pDONR-flbA. Likewise, to insert the rrnB terminator region into pDONR P2R-P3 (Invitrogen), a 0.4-kbp fragment was amplified by PCR with the primers B2f-rrnB (5′-TGTAATACGACATCACTTTTTATTTAGGTCG-3′) and B3R-rrnB (5′-ATAATAGTTGAAACAAAAAGTGTGGTGAAGAACC-3′) using pTrc99A (Amersham Biosciences) as the template. This PCR product was cloned into the vector, and the resulting plasmid was designated pDONR-rrnB.

Sequential cloning of the bopC gene into ΔbopC, pBopC was constructed as follows. A 2.3-kbp fragment encoding BopC was amplified by PCR with the primers B1-bopC (5′-AAAAAGCGAGGGTTGGTACATATGCTGACCT-3′) and B2-bopC (5′-AGGGGTGTCGTTCTCGGATCATATGCGCGG-3′) using B. bronchiseptica S798 genomic DNA as the template. The resulting PCR product was cloned into pDONR201, designated pDONR-bopC. To control the transcription of the bopC gene by the flbA promoter and rrnB terminator in Bordetella, pDONR-flbA, pDONR-bopC-comp, pDONR-rrnB, and pRK415 R4-R3-F were mixed and treated with LR Clonase Plus (Invitrogen) to clone the flbA promoter, bopC gene, and rrnB terminator into pRK415 R4-R3-F using the MultiSite Gateway system (Invitrogen), and the resulting plasmid was designated pDONR-rrnB.

To construct the plasmids encoding the TEM-1-fused protein, we cloned bopC, bcrH2, and map genes into pCX340 (38). Then, 2.2-, 0.5-, and 0.7-kbp fragments encoding BopC, BcrH2, and Map, respectively, were amplified by PCR with the following primer sets. For bopC: pBopC-pCX340-Ndel (5′-GAATTCCATATGCGTACATATGCGGACCT-3′) and B2-bopC (5′-AGGGGTGTCGTTCTCGGATCATATGCGCGG-3′) using B. bronchiseptica S798 genomic DNA as the template. Then, inverse PCR was carried out with the primers R1-bopC (5′-CGGGATCCCTTACCGGGGCTGTCCGGGCTTCT-3′) and R2-bopC (5′-GGGATCCCTTACCGGGGCTGTCCGGGCTTCT-3′) using circular pDONR-bopC as the template. The underlined portions indicate the BamH I sites.

The resulting plasmids were digested with BamH I and self-ligated to obtain pDONR-ΔbopC, which contained a 178-bp deletion around the 5′ region of the bopC. pDONR-ΔbopC was mixed with pABB-CRS2 to obtain pABB-ΔbopC using the Gateway cloning system. pABB-ΔbopC was then introduced into E. coli SM10Apri and was transconjugated into the B. bronchiseptica S798 (streptomycin-resistant), as described previously (36). The resulting mutant strain was designated ΔbopC.
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pCX-Map, respectively. To obtain a fragment containing a different gene fused with bla (encoding β-lactamase; TEM-1) and bla alone, 3.1-, 1.4-, 1.5-, and 0.9-kbp fragments were amplified by PCR with the following primer sets: B1-bopC-comp (see above) and B2-bla-pCX340 (5'-AGAAGATCTGGAATATCCATGTTAATCACTG-3') or B2-bla-pCX340 (5'-AAAAACGAGCTCCTAGAGAGGTTTCTG-3') and B2-bla-pCX340; B1-map-bla (5'-AAAAACGAGCTGCTTTTATTATATGTTAATG-3') and B2-bla-pCX340; and B1-bla-pCX340 (5'-AAAAACGAGCTCCTATATTAGAGAGGTTTCTG-3') and B2-bla-pCX340 using pCX-BopC, pCX-BcrH2, pCX-Map, and pCX340 circular DNA as the template, respectively. The resulting fragments were cloned once into pDONR201 and designated as pDONR-bopC-bla, pDONR-bcrH2-bla, pDONR-map-bla, and pDONR-bla, respectively. To obtain the plasmid encoding truncated versions of BopC fused with TEM-1, inverse PCR was performed with the primer sets bopC-N1-bla-KpnI (5'-GGGGCTACCGCCGAGACC-TGGGGCAGCAG-3') for 448 aa residues of the BopC N terminus fused with TEM-1, or bopC-N2-bla-KpnI (5'-GGGGCTACCGCGAGACC-TGGGGCAGCAG-3') for 248 aa residues of the BopC N terminus fused with TEM-1; or bopC-N3-bla-KpnI (5'-GGGGCTACCGCGAGACC-TGGGGCAGCAG-3') for 48 aa residues of the BopC N terminus fused with TEM-1; and bla-KpnI (5'-GGGGCTACCGCGAGACC-TGGGGCAGCAG-3') for 3.1-kbp fragments encoding BopC were amplified by PCR with the primers pCX-bopC-U (5'-CCCAAGCTTTCATGCGCGTAGATTCAGCG-3') and pCX-bopC-L (5'-CCCAAGCTTTCATGCGCGTAGATTCAGCG-3'). These cross-linked peptides were used to immunize rabbits, and the resulting antisera were incubated with each peptide immobilized on epoxy-activated Sepharose 6B (Amersham Biosciences) to obtain specific immunoglobulin fractions. The mixture of these purified anti-BopC antibodies was used for immunoblot assays.

**Infection Assays**—L2 cells seeded on coverslips were infected with bacteria at a multiplicity of infection (m.o.i.) of 200 and then were centrifuged for 5 min and incubated for 20 min at 37 °C under an atmosphere of 5% CO2. The cells were then washed with phosphate-buffered saline (PBS) and fixed in methanol. Fixed cells were stained with Giemsa solution. In the case of the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay, 4% paraformaldehyde was used as the reagent for fixation. The TUNEL assay was performed by using an In Situ Cell Death Detection Kit (Roche Applied Science). To examine the release of lactate dehydrogenase (LDH) from *B. bronchiseptica*-infected cells, 7.5 × 105 HeLa cells seeded on 24-well plates were infected at an m.o.i. of 100. They were then centrifuged for 5 min and incubated at 37 °C under an atmosphere of 5% CO2. The amounts of LDH were measured spectrophotometrically using a CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega). To calculate the percentage of non-viable cells, 3 × 105 HeLa cells seeded on 6-well plates were infected as in the LDH assay described above. After 60 min of infection, cells were washed with cold PBS twice, and then PBS containing 1 mM EDTA was added to the wells. After 15 min of incubation at 4 °C, the cells were detached from substrata using a Pasteur pipette, and then the cell suspensions were centrifuged. The cells were resuspended with PBS containing 0.4% trypan blue and the resulting stained cells were counted under a microscope using a hemocytometer. Staurosporine (Sigma) was used for inducing apoptosis at a final concentration of 2.5 mM.

**Hemolytic Assay**—The measurement of type III-dependent hemolytic activity was carried out as described previously (35). Briefly, bacterial pellets from overnight cultures and rabbit red blood cells were washed with PBS and adjusted to 5 × 10^7 bacteria/ml and 3 × 10^8 cells/ml with PBS, respectively. The suspensions were mixed together (50-μl aliquots per suspension) on a 96-well plate and centrifuged for 5 min for close contact; the combined suspension was then incubated at 37 °C for 30 min in a CO2 incubator. The bacteria-red blood cell suspensions were gently resuspended with an additional 100 μl of PBS, and then the plate was centrifuged. The supernatants were transferred to a fresh plate, where the optical density at 492 nm was measured.

**Detection of Tyrosine-phosphorylated Proteins (PY) in Host Cells**—The protocol of immunofluorescence staining has been described elsewhere (40). HeLa cells were infected with bacteria as mentioned above. When sodium orthovanadate (Na2VO3) was used to inhibit of phosphatase activity, this reagent was added to the extracellular medium at a final concentration of 1 mM during infection. After the fixation, PY were stained with anti-PY monoclonal antibody 4G10 (Upstate Biotechnology). In a secondary antibody, Alexa Fluor 488 or 594 goat anti-mouse IgG (Molecular Probes) was used. Filamentous actin (F-actin) was stained with rhodamine phalloidin (Molecular Probes). Bacteria were stained with anti-*B. bronchiseptica* antiserum (Denka Seiken). To detect PY in HeLa cells infected with bacteria by immunoblot assay, infected cells were washed with ice-cold PBS three times, and then the cells were treated with lysis buffer (41). The cell lysates were sonicated for 30 s and clarified by centrifugation at 15,000 × g for 15 min. The supernatants were then separated by SDS-PAGE with 10% gel and immunoblotted with anti-PY antibody RC20 (BD Biosciences) or anti-actin monoclonal antibody (Chemicon).
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**Effector Translocation Assay**—HeLa cells were seeded at $5 \times 10^4$ cells/well in a 6-well plate, and then the plate was incubated at 37 °C overnight in a CO$_2$ incubator. HeLa cells were exposed to *B. bronchiseptica* for 60 min at an m.o.i. of 1000. The infected cells were washed with Hanks’ balanced salt solution (Sigma), and then cells were stained with CCF2-AM solution according to the manufacturer’s protocol for the GeneBLAzer In Vivo Detection Kit (Invitrogen). The stained cells were analyzed under a fluorescence microscope using a filter set for 4’,6-diamidino-2-phenylindole (Zeiss).

**Transfection Assay**—HeLa, COS-7, or 293T cells were seeded at $2.5 \times 10^4$ cells/well in a 24-well plate, and then the plate was incubated at 37 °C overnight in a CO$_2$ incubator. Mock vector or pcDNA-BopC was introduced into the cells using FuGENE6 (Roche Applied Science) according to the manufacturer’s protocol. After incubation for 18 h in a CO$_2$ incubator, the amounts of LDH released into the extracellular media were measured as described above.

**RESULTS**

*BopC Is a Novel Secreted Protein via the Bordetella TTSS*—The *B. bronchiseptica* wild-type and type III- strains were cultured in SS medium for 18 h, and the supernatant proteins were recovered by trichloroacetic acid precipitation. These samples were separated by SDS-PAGE with 6% or 12% gel, then stained with silver or CBB, respectively. As expected (26, 27), BopB, BopN, BopD, and Bsp22 were detected specifically in the lanes where the wild-type supernatants were loaded. Additionally, ten bands were observed specifically in the lanes where the wild-type supernatants were loaded (Fig. 1A, arrows). Each band was excised from CBB-stained gels and treated with trypsin. The resulting peptides were analyzed by MALDI-TOF MS or ESI-MS/MS. Three bands were assigned as degraded products of Bsp22 or BopD (Fig. 1A, small arrows in right panel). The ESI-MS/MS analysis indicated that the deduced amino acid sequence of a trypsinized peptide derived from band a (Fig. 1A) was LLEPNNDFEVFR (Fig. 1B). This sequence corresponds to amino acid residues 34–44 of a 69-kDa hypothetical protein that is encoded in an ORF located at nucleotides 4,502,054–4,504,030 of the *B. bronchiseptica* genome that Parkhill et al. published (34). By the MALDI-TOF MS analysis of trypsinized peptides from band b, five molecular masses, 961.4953, 1345.7384, 1541.8305, 2089.0730, and 2342.3590, were obtained. These masses corresponded to the following amino acid residues of the 69-kDa protein described above, respectively, 161–168, 34–44, 149–160, 169–186, and 214–235 (Table 1). Although the other five protein bands indicated by small arrows in Fig. 1A (left panel) were also analyzed by both MALDI-TOF MS and ESI-MS/MS, clear results were not obtained. These results strongly suggest that bands a and b were derived from the presumed 69-kDa protein, designated BopC in this study.

**Construction and Secretion Profile of the bopC-deficient Mutant**—To examine whether or not bands a and b shown in Fig. 1 are really derived from 69-kDa BopC, a mutant of BopC was constructed, as documented under “Experimental Procedures.” The culture supernatant samples prepared from the wild-type strain, BopC mutant (ΔBopC), BopC mutant harboring pBopC (ΔBopC/pBopC), and type III- were subjected to SDS-PAGE, and the resulting gels were stained with CBB or silver (Fig. 2, A and B). In the ΔBopC culture supernatant, both bands corresponding to a and b shown in Fig. 1A disappeared. In contrast, both bands were detected in the ΔBopC/pBopC-complemented strain (Fig. 2, A and B). The band intensities of other type III-secreted proteins, such as BopB, BopN, BopD, and Bsp22, were slightly higher in the supernatant sample prepared from ΔBopC than in those from the wild-type strain (Fig. 2A), and the band intensities of those proteins from the ΔBopC/pBopC strain were similar to those from the wild-type strain (Fig. 2A). Interestingly, five bands also disappeared in the ΔBopC supernatant sample (Fig. 2B, small arrows). The results of immunoblot analysis using anti-BopC antibodies indicated that the specific signals were detected in band a (Fig. 2C, large arrow), band b (data not shown), and in the other five bands (Fig. 2C, small arrows). Four of these bands were each larger than 200 kDa. These results clearly demon-

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**TABLE 1**

Tryptic peptides obtained from band b in Fig. 1A by MALDI-TOF MS analyses

The identities of the peptides were assigned by matching the measured mono isotopic mass values of single-charged ions ([M+H]+) to those calculated for the predicted p69 tryptic fragments.

| No. | Mass | Position |
|-----|------|----------|
|     | Observed | Calculated | Start | End |
| 1   | 961.4953 | 961.4850 | 161 | 168 |
| 2   | 1345.7384 | 1345.6746 | 34 | 44 |
| 3   | 1541.8305 | 1541.7529 | 149 | 160 |
| 4   | 2089.0730 | 2089.0349 | 169 | 186 |
| 5   | 2342.3590 | 2342.2714 | 214 | 235 |

**FIGURE 1. Identification of unknown type III-secreted protein in *B. bronchiseptica*. A**, supernatant samples were prepared from the wild-type strain and TTSS mutant (type III-) and were separated by SDS-PAGE with 6% (left panel) or 12% (right panel) gel. The gels were then stained with silver (left panel) or CBB (right panel). Bands indicated by arrows were unidentified proteins whose secretions were dependent on the TTSS. After the band indicated by arrows was excised from the gels, those samples were analyzed by TOF MS. B, the result of analysis of band a in panel A by using ESI-MS/MS. The obtained amino acid sequence corresponds to residues 34–44 in a 69-kDa hypothetical protein (p69).
strated that bands a and b were derived from BopC. This protein seemed to form multimeric complexes during SDS-PAGE.

**BopC Is Required to Induce Cytotoxicity in Mammalian Cells**—Infection of cultured mammalian cells with wild-type *B. bronchiseptica* induces morphological changes and injury to the host cell membrane, and these events depend on the TTSS (14, 25, 27). To examine whether or not the type III-secreted protein BopC is involved in these cytotoxic phenotypes, L2 rat lung epithelial cells were exposed to *B. bronchiseptica*. The cells infected with bacteria for 20 min were stained with Giemsa solution and examined under a light microscope (Fig. 3A). Almost 95% of the L2 cells infected with the wild-type or ΔBopC/pBopC strain were detached from substrata, and the remainder of the adherent cells had shrunken cytoplasm and condensed nuclei. In contrast, the cells infected with ΔBopC or type III− had the same normal morphology (data not shown). Next, HeLa cells were infected with *B. bronchiseptica*. The release of LDH into the extracellular medium was measured, and the released amount was compared with that of a positive control (Triton X-100 treatment) (Fig. 3B). When the cells were infected with the wild-type strain or ΔBopC/pBopC for 120 min, the amount of LDH release reached ~75% or 50%, respectively. In contrast, neither ΔBopC nor type III− showed any ability to elicit LDH release in the infected cells, even when the infection time was prolonged to 360 min (Fig. 3B). The amount of LDH release from HeLa cells treated with staurosporine for 5 h was very similar to that of uninfected cells (data not shown). To calculate the percentage of non-viable cells, HeLa cells were infected with the various strains of *B. bronchiseptica* for 60 min, and the number of dead HeLa cells was counted by trypan blue staining (Fig. 3, C and D). About 57% or 44% of cells infected with the wild-type strain or ΔBopC/pBopC, respectively, were stained by trypan blue (Fig. 3D). In contrast, cells treated with staurosporine were scarcely stained at this time point (Fig. 3D).

As reported previously (25), although *B. bronchiseptica* induces necrotic cell death, in which caspases are not activated, there is a population of infected cells having TUNEL-positive signals in their nuclei (26–28). In this population, the nuclei were stained diffusely by TUNEL reagent (26, 27). In contrast, the nuclei of typical apoptotic cells, including chromatin condensation, were not observed. No TUNEL-positive nuclei were observed in cells infected with ΔBopC or type III− (Fig. 3, E and F). These results indicate that BopC is required for the induction of mammalian cell death.

**BopC Is Not a Pore-forming Factor for Effector Translocation**—It is well known that TTSS-possessing bacteria, including *B. bronchiseptica*, induce hemolysis in which hemoglobin leaks through the pores formed by type III-secreted proteins, so-called translocators. Although *B. bronchiseptica* has a cyclic adenylylase toxin/hemolysin, we have measured the TTSS-dependent hemolytic activity and demonstrated that type III-secreted proteins BopB and BopD are translocators in *B. bronchiseptica* (27, 28). To determine whether BopC is included in the translocator or not, ΔBopC was exposed to rabbit red blood cells for 30 min, and hemolysis was measured as described under “Experimental Procedures” (Fig. 3G). The hemolytic activity of ΔBopC (29.3% against positive control in which red blood cells were treated with 1% Triton X-100) was at almost the same level as that of the wild-type strain (33.4%), indicating that BopC is not a translocator.

**BopC Induces Dephosphorylation of PY in Host Cells**—A previous study showed that the wild-type *B. bronchiseptica* induces dephosphorylation of infected L2 cells and that this phenotype is dependent on the TTSS (14). To examine whether or not BopC is involved in the dephosphorylation of PY in host cells, immunofluorescence and immunoblot assays were performed using anti-PY antibodies (Fig. 4A). HeLa cells infected with the wild-type strain, ΔBopC, ΔBopC/pBopC, and type III− were stained with not only anti-PY antibodies but also rhodamine-phalloidin (Fig. 4A) or anti- *B. bronchiseptica* antisera (supplemental Fig. S1) to visualize F-actin or bacterial adherence to host cells, respectively. The results of fluorescence microscopy showed that fluorescent signals from adherent bacteria had almost the same intensity regardless of whether the cells were infected for 60 min with strains used here (supplemental Fig. S1). This suggests that BopC is not involved in bacterial adherence...
FIGURE 3. Cytotoxic activity of BopC mutant. A, L2 cells were infected with indicated B. bronchiseptica strains for 20 min. Those infected cells were stained with Giemsa solution. B, the graph shows time courses of amounts of LDH released into the extracellular medium from HeLa cells infected with indicated strains. The values are the percentages of Triton X-100-lysed HeLa cells after subtraction of the value measured in the uninfected cells. The data points for ΔBopC and type III superimpose on the graph. C, HeLa cells were infected with indicated strains for 60 min at an m.o.i. of 100. Those infected cells were stained with trypan blue. The cells treated with staurosporine for 1 h was shown as stsp. D, the histogram shows the percentage of cells stained with trypan blue in panel C. At least 500 cells were counted in each assay. E, HeLa cells were infected with indicated strains for 60 min at an m.o.i. of 100. Staurosporine treatment was performed for 5 h. After fixation, those infected cells were stained with fluorescent TUNEL reagent. The cells were analyzed with Nomarski (upper panels) and fluorescent microscope (lower panels). F, the graph shows time courses of the number of cells with the fluorescent TUNEL signal. At least 500 cells were randomly chosen to calculate the rate of fluorescence in each assay. G, the histogram shows the hemolytic activity of indicated strains. The values are the percentages of Triton X-100-lysed RBC after subtraction of the value measured in the uninfected cells. The error bars in B, D, F, and G represent the means ± S.E. from triplicate experiments.
Bordetella BopC Is a Novel Type III Effector

Dephosphorylation of PY Occurs after the BopC-dependent Cell Death—Upon infection with wild-type *B. bronchiseptica*, BopC induces in both cell death (estimated by LDH release and TUNEL assays) and dephosphorylation of PY in host cells. To determine which phenomenon, cell death or dephosphorylation, is induced by BopC more directly, HeLa cells were infected with the wild-type *B. bronchiseptica* in the presence or absence of a dephosphorylation inhibitor, sodium orthovanadate (Na$_3$VO$_4$). First, the degree of inhibition was examined. Cells exposed for 60 min were immunostained as described above, and then the PY signal-lacking cells were counted under fluorescent microscope. Although the bacterial adherence was not affected by addition of sodium orthovanadate (supplemental Fig. S2), this inhibitor reduced the proportion of PY signal-lacking cells from 19.3% to 9.8% (Fig. 5, A and B). Lysates were prepared from these infected cells and were subjected to the immunoblot assay (Fig. 5C). As reported previously (14), signal intensities of PY proteins were increased in the presence of the inhibitor (Fig. 5C). These results indicate that treatment with Na$_3$VO$_4$ partially attenuates the extent of PY dephosphorylation induced by *B. bronchiseptica* infection. Next, to examine whether or not the *B. bronchiseptica*-induced dephosphorylation is associated with DNA damage to the host cells or injury to the host membrane, TUNEL and LDH release assays were performed on HeLa cells infected with the wild-type strain in the presence of an inhibitor. The rate of TUNEL-positive cells and the amount of released LDH were almost the same in the presence or absence of the inhibitor (Fig. 5, D–F). In fluorescent microscopy, TUNEL-positive cells were detected in both PY-positive (Fig. 5D, arrow in top row) and PY-lacking (Fig. 5D, arrowhead in top row) cells. In contrast, the PY signal-lacking phenotype was not detected in the TUNEL-negative cells. These findings suggest that the decrease in PY proteins in host cells infected with *B. bronchiseptica* occurred after BopC-dependent cell death.

BopC Seems to Be Translocated into Mammalian Cells by TTSS—It has been well known that type III effectors are delivered into host cells directly from bacterial cells. The TEM-1 reporter system (38) was adopted to examine whether or not BopC is translocated into the host cell during wild-type *B. bronchiseptica* infection. Plasmid encoding TEM-1 (β-lactamase) fused at its N terminus with each of the following: the full-length of BopC (658 aa), BopC truncated versions (448, 248, or 48 aa of the BopC N terminus); BcrH2 (cytoplasmic protein of *B. bronchiseptica*); or Map (one of the EPEC effectors) was constructed. The resulting plasmids and pTEM-1 (encoding TEM-1 alone) were

![FIGURE 4. Detection of tyrosine-phosphorylated proteins (PY) in cells infected with *B. bronchiseptica* strains. A, HeLa cells were infected with indicated strains for the indicated time at an m.o.i. of 100. After fixation, the cells were stained with anti-PY antibodies to infected cells. In the case of HeLa cell infection with the wild-type strain, all cells infected for 0 min still had specific PY signals that seemed to localize mainly in focal adhesions. However, the cells infected for 20 or 60 min had a certain proportion of cells with no PY signals (Fig. 4A, arrowheads). In contrast, all cells infected with ΔBopC or type III′ for 60 min still had PY signals to the same extent as did uninfected cells (Fig. 4A). Next, lysates were prepared from HeLa cells infected with *B. bronchiseptica* and were subjected to immunoblot assay using anti-PY or anti-actin antibodies. The amount of each loaded sample was confirmed and were subjected to immunoblot assay using anti-PY (Fig. 4B). As reported previously (14), the PY signal profiles were changed and the signal intensity of signals was significantly lower in HeLa cells infected with the wild-type strain or ΔBopC/pBopC for 60 min than that with type III′ (Fig. 4B). In contrast, HeLa cells infected with ΔBopC had almost the same profiles as those from the cells infected with type III′ or from uninfected cells (Fig. 4B). These results indicate that BopC induces dephosphorylation of PY in infected mammalian cells.](http://www.jbc.org/content/281/10/6595/F4)

(PY, green) and rhodamine phallolidin (F-actin, red). Merge (PY and F-actin) and Nomarski pictures are also shown. Arrowheads indicate the PY signal-lacking cells. B, lysates prepared from HeLa cells infected with indicated strains for 0, 30, and 60 min were analyzed by immunoblot assay using anti-PY (upper panel) or anti-actin (lower panel) antibodies.

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FIGURE 5. Cytotoxic activity of *B. bronchiseptica* in the presence of the phosphatase inhibitor. **A**, HeLa cells were infected with the wild-type strain in the presence (upper panels) or absence (lower panels) of a phosphatase inhibitor, sodium orthovanadate. Those infected cells were stained with anti-PY antibodies (PY, green) and rhodamine phalloidin (F-actin, red). Merge (PY and F-actin) and Nomarski pictures are also shown. Arrowheads indicate PY signal-lacking cells. **B**, HeLa cells were infected with indicated strains for 60 min in the presence (+) or absence (−) of the phosphatase inhibitor. After cells were stained with anti-PY antibodies, at least 200 cells were randomly chosen and PY signal-lacking cells were counted under fluorescent microscope. The histogram shows the percentages of PY signal-lacking cells against all examined cells. **C**, the lysates prepared from HeLa cells infected with indicated strains as described in **B** were analyzed by immunoblot analysis using anti-PY (upper panel) or anti-actin (lower panel) antibodies. **D**, HeLa cells were infected with indicated strains for 60 min at an m.o.i. of 200 in the presence or absence of the phosphatase inhibitor. After fixation, the cells were stained with anti-PY (red) antibodies and fluorescent TUNEL reagent (green). Merge (PY and TUNEL) and Nomarski pictures were also shown. **E**, after TUNEL assay of HeLa cells infected with indicated strains in the presence or absence of the phosphatase inhibitor for 60 min at an m.o.i. of 200, at least 500 cells were randomly chosen to calculate the rate of TUNEL-positive cells in each assay. The histogram shows the percentages of cells with a fluorescent TUNEL signal against all examined cells. **F**, the histogram shows relative amounts of LDH released from HeLa cells infected with indicated strains for 60 min in the presence or absence of the phosphatase inhibitor. The values are the percentages of Triton X-100-lysed HeLa cells after subtraction of the value measured in the uninfected cells. The error bars in **B**, **E**, and **F** represent the means ± S.E. from triplicate experiments.
Bordetella BopC Is a Novel Type III Effector

To investigate whether cytotoxicity is induced by BopC alone, the bopC gene was introduced into mammalian cell lines. The mock vector or pcDNA-BopC was transfected to COS-7, HeLa, or 293T cells. After 18 h of incubation, the amounts of LDH released into the extracellular media were measured (Fig. 7). No significant LDH release was detected in the media of mock vector-introduced cells. When the bopC gene was introduced into COS-7, 293T, and HeLa cells, the amount of LDH release reached 22.0%, 13.4%, and 4.6%, respectively (Fig. 7). These results strongly suggest that BopC has the ability to induce cytotoxicity and that other factors were not involved in this series of events.

DISCUSSION

Thus far, no protein has been characterized as a definite type III effector in Bordetella. In this study, we identified a novel effector that introduced into respective ΔBopC, ΔBopB, and type III - strains. These strains were cultured in SS broth to the stationary phase, and then whole cell lysates and supernatant fractions were prepared. These samples were subjected to immunoblot analysis with anti-TEM-1 antibodies (Fig. 6A). All signals of the TEM-1-fused proteins or TEM-1 alone were detected in whole cell lysates as their deduced molecular masses (Fig. 6A, asterisks). The band intensities were similar among ΔBopC, ΔBopB, and type III - . As expected, although the signal was detected for the full-length of BopC fused with TEM-1 (FL-TEM-1) in the supernatant sample prepared from ΔBopC or ΔBopB, it was not detected in that from type III - (Fig. 6A). Signals of BcrH2 fused with TEM-1 (BcrH2-TEM-1) or TEM-1 alone were not detected in the supernatant fractions prepared from ΔBopC and ΔBopB (Fig. 6A). Signals of the BopC truncated versions fused with TEM-1 (N1-TEM-1, N2-TEM-1, N3-TEM-1, and N3-TEM-1) were also detected in supernatant fractions prepared from ΔBopC and ΔBopB (Fig. 6A) but not from type III - (data not shown). Interestingly, signals of EPEC Map fused with TEM-1 (Map-TEM-1) were detected in the supernatant fractions prepared from ΔBopC and ΔBopB. However, the signal intensity of the supernatant fraction prepared from ΔBopB was much weaker than that from ΔBopC (Fig. 6A). These results indicate that BopC fused with TEM-1 was secreted, as was native BopC, and 48 aa residues of N terminus were enough for BopC secretion via the TTSS. Next, HeLa cells were infected with B. bronchiseptica expressing TEM-1 fused protein, and then infected cells were stained with CCF2-AM solution. When TEM-1-fused proteins were translocated into host cell cytosol, the fluorescence emitted by CCF2-AM-stained cells turned from green to blue in fluorescence microscopy as observed by means of a 4’,6-diamidino-2-phenylindole filter set. Although the cells infected with ΔBopC expressing FL-, N1-, N2-, BcrH2-, Map-TEM1, or TEM-1 alone still emitted green fluorescence (data not shown), ~70% of cells infected with ΔBopC expressing N3-TEM-1 emitted blue fluorescence (Fig. 6B). As expected, the cells infected with ΔBopB or type III - expressing TEM-1-fused proteins emitted green fluorescence (data not shown). These results suggest that BopC is translocated into the host cell via the TTSS, and the signal sequence of both secretion and translocation is located within 48 aa residues of the BopC N terminus.

BopC Has the Ability to Induce Cytotoxicity—To investigate whether cytotoxicity is induced by BopC alone, the bopC gene was introduced into mammalian cell lines. The mock vector or pcDNA-BopC was transfected to COS-7, HeLa, or 293T cells. After 18 h of incubation, the amounts of LDH released into the extracellular media were measured (Fig. 7). No significant LDH release was detected in the media of mock vector-introduced cells. When the bopC gene was introduced into COS-7, 293T, and HeLa cells, the amount of LDH release reached 22.0%, 13.4%, and 4.6%, respectively (Fig. 7). These results strongly suggest that BopC has the ability to induce cytotoxicity and that other factors were not involved in this series of events.

DISCUSSION

Thus far, no protein has been characterized as a definite type III effector in Bordetella. In this study, we identified a novel effector that

FIGURE 6. Translocation assay of BopC from the bacterial cytosol into the host cell cytoplasm. A, whole cell lysates (upper panel) and supernatant samples (lower panel) prepared from indicated strains harboring pBopC-FL-TEM-1, pBopC-N1-TEM-1, pBopC-N2-TEM-1, pBopC-N3-TEM-1, pBcrH2-TEM-1, pMap-TEM-1, or pTEM-1 were separated by SDS-PAGE with 5–20% gradient gels, and the gels were then analyzed by immunoblot with anti-TEM-1 antibodies. Asterisks indicate the position of the intact length of each protein. B, HeLa cells were infected with the indicated strains for 60 min and then stained with CCF2-AM solution. These stained cells were analyzed under fluorescent microscope using a 4’,6-diamidino-2-phenylindole filter set. Two representative images (left and right panels) obtained from each experiment were shown. C, results of both secretion (A) and translocation (B) assays are shown with schematic diagrams of TEM-1 fusion proteins used in those assays. Amino acid numbers of full-length BopC, truncated BopC, BcrH2, Map, and TEM-1 are shown. ND, indicates not detected.
Bordetella BopC Is a Novel Type III Effector

BopC in *B. bronchiseptica* RB50 has been registered in National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) as a 69-kDa hypothetical protein with accession number is BX640449 (nucleotide, supplemental Fig. S3) and NP_890763 (amino acid; supplemental Fig. S4). BopC in *B. pertussis* Tohama I and *B. parapertussis* 12822 were also registered in NCBI as NP_879352 and NP_885936, respectively. Although the expression of BopC is unknown in both species, BopC has high identity (>95%) among the three species (supplemental Fig. S4), suggesting that the function of BopC is probably equivalent among the Bordetella species. In *B. bronchiseptica*, the genes encoding BopB, BopD, BopN, and Bsp22 are located within the TTSS locus (26, 27). In contrast, the *bopC* gene is located outside the locus, at 2.5 Mbp (in *B. bronchiseptica*), 1.7 Mbp (in *B. parapertussis*), and 1.8 Mbp (in *B. pertussis*) from their respective TTSS loci. It is unclear why the BopC locus on the genome is separated from the TTSS locus. However, similar observations have been made in other effectors, such as SopE (55) and SigD (56) in *Salmonella*, OrbB in *EPEC* (57), and NleA in enterohemorrhagic *E. coli* (58). The 10-kbp upstream and downstream flanking regions of *bopC* in both *B. bronchiseptica* and *B. parapertussis* encode nearly identical proteins, which are mainly housekeeping proteins. One exception to this similarity is a gene encoding a transposase homolog located 5-kbp upstream from *bopC* in *B. parapertussis* but not found in *B. bronchiseptica*. In contrast, in *B. pertussis*, genes encoding transposable and integrase homologs are located 2.0 kbp upstream and 1.6 kbp downstream from *bopC*, respectively. Neither insertion is found in those regions of *B. bronchiseptica* or *B. parapertussis*, whereby the outside region of *bopC* in *B. pertussis* encodes different proteins from those of *B. bronchiseptica* or *B. parapertussis*. These findings suggest that the extensive genome arrangements, including recombinations, gene conversions, and transpositions, occurred during the divergence of *B. pertussis* from the progenitor, *B. bronchiseptica* (3).

The BvgAS system was shown to be down-regulated when bacteria were cultured in the presence of MgSO4 (12). To analyze whether or not BopC is regulated by the BvgAS system, the whole cell lysate was prepared from the wild-type *B. bronchiseptica* cultured in SS broth containing MgSO4 and was subjected to an immunoblot assay using anti-BopC antibodies. No BopC signal was detected in the whole cell lysate (data not shown), suggesting that the expression of BopC is regulated positively by the BvgAS system.

Although the deduced molecular mass of BopC is 69 kDa, the secreted BopC bands appeared at ~150 kDa and at over 200 kDa as multimers on SDS-PAGE (Figs. 1A and 2B). The monomer band of BopC was detected only as a faint band on overexposed x-ray film (data not shown). It has been reported that polytopic membrane proteins, which have been defined generally as proteins having multiple transmembrane regions, form an SDS-resistant multimer (60). For example, DotA, which is secreted via the type IV secretion machinery in *Legionella pneumophila*, has eight transmembrane domains (61) and forms SDS-resistant multimers that were detected in the immunoblot assay, although the signal of the DotA monomer was stronger than that of the DotA multimer (62). The DotA multimer disappeared when the sample was loaded on the gel without boiling (62). In contrast, the BopC multimer was still detected and the monomer band intensity was not increased, even when the whole cell lysate or the supernatant sample was prepared without boiling (data not shown). Prediction of the transmembrane domain in BopC by the TMPred program (www.ch.embnet.org/software/TMPRED_form.html) showed that one probable transmembrane domain exists in the C terminus (amino acid residues 619–643). This suggests that BopC is unlikely to be a typical polytopic protein. When TEM-1 was fused with the C terminus of the full-length

![FIGURE 7. LDH release from mammalian cells by introduction of the bopC gene. The histogram shows the relative amounts of LDH release from the indicated cells by introduction of mock vector or pcDNA-BopC. The values are the percentages of Triton X-100-lysed cells after subtraction of the value measured in cells with the transfection manipulation without DNA. The error bars represent the means ± S.E. from triplicate experiments.](image-url)
BopC, the monomer band was detected clearly, although the multimer bands of the fused protein were still detected (Fig. 6A, Lane FL-TEM-1). In contrast, multimer bands were not detected in the C-terminal truncated versions of BopC (BopC-N1-TEM-1), suggesting that the C-terminal region (amino acid residues 449–658) is needed to form multimers. These findings suggest that BopC would interact with itself tightly like polytopic proteins, such as DotA.

The Xanthomonas TTSS can secrete the Yersinia effector YopE (63), and the Yersinia TTSS recognizes Pseudomonas effectors AvrB and AvrPto as substrates (64). In this study, we also confirmed that the EPEC effector Map fused with TEM-1 (Map-TEM-1) was secreted via the type III-secretion pathway (64). However, in TEM-1 assays, translocation into host cells was not detected in the TEM-1 assay (data not shown). One possibility is that Map may be unstable in B. bronchiseptica. Indeed, degraded Map-TEM-1 products were detected, and a small amount of the intact length of Map-TEM-1 was detected in the supernatant sample (Fig. 6A). Presumably, CesT, a chaperone for Map (65), is required to stabilize Map in B. bronchiseptica. The amount of secreted Map-TEM-1 from ΔBopB is lower than that from ΔBopC, probably due to the competition between the endogenous BopC and Map-TEM-1 in the secretion capacity of the TTSS. The amounts of type III-secreted proteins, such as BopB, BopD, BopN, and Bsp22, in ΔBopC are higher than they are in the wild-type strain and were decreased by the introduction of pBopC into ΔBopB (Fig. 2A). These findings suggest that BopC has a regulatory effect on other type III-secreted proteins.

We showed that the BopC-N3-TEM-1 protein was translocated into HeLa cells during infection (Fig. 6B). However, in TEM-1 assays, translocation into host cells was not detected in three BopC derivatives, including full-length BopC. Conceivably, these BopC-TEM-1 derivatives are unstable in B. bronchiseptica proteins, because the degraded products were detected in the supernatant samples (Fig. 6A); or because the amount of delivered BopC-TEM-1 proteins is insufficient for detection by TEM-1 assay. The precise reason why only BopC-N3-TEM-1 was detected in the TEM1 translocation assay is unknown. However, the signal intensities of BopC-N3-TEM-1 in whole cell lysates and supernatant samples seemed to be stronger than those of any other fusion protein used (Fig. 6A, asterisks). In this context, we speculate that detectable amounts of TEM-1 fusion protein were translocated to host cells in the case of BopC-N3-TEM-1. Indeed, even in the case of HeLa cells infected with ΔBopC expressing BopC-N3-TEM-1, the infected cells turned greenish-blue, whereas HeLa cells infected with EPEC expressing Map-TEM-1 turned from green to rich blue (data not shown).

Collectively, BopC is the first type III effector to be identified in Bordetella, and this effector is crucial for inducing cytoxicity in cultured mammalian cells. The mechanisms of necrotic cell death induced by B. bronchiseptica infection in mammalian cells will be clarified by analyses of the detailed function(s) and localization of translocated BopC.

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REFERENCES
1. Yeh, S. H. (2003) Expert Rev. Vaccines 2, 113–127
2. Heininger, U., Stehr, K., Schmitt-Grobe, S., Lorenz, C., Rost, R., Christenson, P. D., Uberall, M., and Cherry, J. D. (1994) Pediatr. Infect. Dis. J. 13, 306–309
3. van der Zee, A., Mooi, F., Van Embden, J., and Musser, J. (1997) J. Bacteriol. 179, 6609–6617
4. Goodnow, R. A. (1980) Microbiol. Rev. 44, 722–738
5. Foley, I. E., Rand, C., Bannasch, M. J., Norris, C. R., and Milan, J. (2002) Prev. Vet. Med. 54, 141–156
6. Magyar, T., King, V. L., and Kovacs, F. (2002) Vaccine 20, 1797–1802
7. Yoda, H., Nakayama, K., and Nakagawa, M. (1982) Jikken Dobutsu 11, 113–118
8. Sibitz, S., Aaronson, W., Monack, D., and Falkow, S. (1989) Nature 338, 266–269
9. Uhl, M. A., and Miller, J. F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 1163–1167
10. Hoch, J. A. (2000) Curr. Opin. Microbiol. 3, 165–170
11. Boucher, P. E., and Sibitz, S. (1995) J. Bacteriol. 177, 6486–6491
12. Boucher, P. E., Murakami, K., Ishihara, A., and Sibitz, S. (1997) J. Bacteriol. 179, 1753–1763
13. Karinova, G., Bellalou, J., and Ullmann, A. (1996) Mol. Microbiol. 20, 489–496
14. Yuki, M. H., Harville, E. T., and Miller, J. F. (1998) Mol. Microbiol. 30, 911–921
15. Sasakawa, C., Kamata, K., Sakai, T., Makino, S., Yamada, M., Okada, N., and Yoshikawa, M. (1988) J. Bacteriol. 170, 2480–2484
16. Niebuhr, K., Giuriato, S., Pedron, T., Philip, J. D., Guiot, F., Sable, J., Sheetz, M. P., Parsot, C., Sansonetti, P. J., and Payrastre, B. (2002) EMBO J. 21, 5069–5078
17. Sasakawa, S., Kuwae, A., Yoshida, S., Sasakawa, C., and Abe, A. (2004) J. Bacteriol. 186, 3800–3813
18. Kajimura, S., Kakuta, H., Itoh, K., Hori, M., and Sasakawa, C. (2000) J. Bacteriol. 182, 3111–3117
19. Kajimura, S., Kakuta, H., Itoh, K., and Sasakawa, C. (2001) J. Bacteriol. 183, 3363–3367
20. Kawakami, T., Sasakawa, C., Kamata, K., Sakai, T., Makino, S., Yamada, M., Okada, N., and Yoshikawa, M. (1988) J. Bacteriol. 170, 2480–2484
21. Gu, X., and Sansonetti, P. J. (2004) J. Bacteriol. 186, 2293–2307
22. Guan, K. L., and Dixon, J. E. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 3384–3388
23. Charpentier, X., and Oswald, E. (2004) J. Bacteriol. 186, 165–170
24. Izawa, Y., and Nakamura, K. (2003) J. Bacteriol. 185, 5200–5202
25. Izawa, Y., and Nakamura, K. (2004) J. Bacteriol. 186, 165–170
26. Izawa, Y., and Nakamura, K. (2005) J. Bacteriol. 187, 6707–6714
27. Izawa, Y., and Nakamura, K. (2006) J. Bacteriol. 188, 6607–6617
28. Izawa, Y., and Nakamura, K. (2007) J. Bacteriol. 189, 2071–2078
Bordetella BopC Is a Novel Type III Effector

1625–1630
48. Henikoff, J. G., Greene, E. A., Pietrokovski, S., and Henikoff, S. (2000) *Nucleic Acids Res.* **28**, 228–230
49. Henikoff, S., Henikoff, J. G., and Pietrokovski, S. (1999) *Bioinformatics* **15**, 471–479
50. Corpet, F., Gouzy, J., and Kahn, D. (1999) *Nucleic Acids Res.* **27**, 263–267
51. Corpet, F., Gouzy, J., and Kahn, D. (1998) *Nucleic Acids Res.* **26**, 323–326
52. Attwood, T. K., Blythe, M. J., Flower, D. R., Gaulton, A., Mabey, J. E., Maudling, N., McGregor, L., Mitchell, A. L., Moulton, G., Paine, K., and Scordis, P. (2002) *Nucleic Acids Res.* **30**, 239–241
53. Attwood, T. K., and Beck, M. E. (1994) *Protein Eng.* **7**, 841–848
54. Bateman, A., Birney, E., Cerruti, L., Durbin, R., Etwiller, L., Eddy, S. R., Griffiths-Jones, S., Howe, K. L., Marshall, M., and Sonnhammer, E. L. (2002) *Nucleic Acids Res.* **30**, 276–280
55. Hardt, W. D., U rsaub, H., and Galan, J. E. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2574–2579
56. Pleifer, C. G., Marcus, S. L., Steele-Mortimer, O., Knodler, L. A., and Finlay, B. B. (1999) *Infect. Immun.* **67**, 5690–5698
57. Elliott, S. J., Krejany, E. O., Mellies, J. L., Robins-Browne, R. M., Sasakawa, C., and Kaper, J. B. (2001) *Infect. Immun.* **69**, 4027–4033
58. Gruenheid, S., Sekirov, I., Thomas, N. A., Deng, W., O’Donnell, P., Goode, D., Li, Y., Frey, E. A., Brown, N. F., Metalnikov, P., Pawson, T., Ashman, K., and Finlay, B. B. (2004) *Mol. Microbiol.* **51**, 1223–1249
59. Lord, J. M., and High, S. (2005) *Curr. Biol.* **15**, R169–R171
60. Roy, C. R., and Isberg, R. R. (1997) *Infect. Immun.* **65**, 571–578
61. Nagai, H., and Roy, C. R. (2001) *EMBO J.* **20**, 5962–5970
62. Rosier, O., Wengelnik, K., Hahn, K., and Bonas, U. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9368–9373
63. Anderson, D. M., Fouts, D. E., Collmer, A., and Schneewind, O. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12839–12843
64. Creasey, E. A., Delahay, R. M., Bishop, A. A., Shaw, R. K., Kenny, B., Knutton, S., and Frankel, G. (2003) *Mol. Microbiol.* **47**, 209–221

57. Elliott, S. J., Krejany, E. O., Mellies, J. L., Robins-Browne, R. M., Sasakawa, C., and Kaper, J. B. (2001) *Infect. Immun.* **69**, 4027–4033
58. Gruenheid, S., Sekirov, I., Thomas, N. A., Deng, W., O’Donnell, P., Goode, D., Li, Y., Frey, E. A., Brown, N. F., Metalnikov, P., Pawson, T., Ashman, K., and Finlay, B. B. (2004) *Mol. Microbiol.* **51**, 1223–1249
59. Lord, J. M., and High, S. (2005) *Curr. Biol.* **15**, R169–R171
60. Roy, C. R., and Isberg, R. R. (1997) *Infect. Immun.* **65**, 571–578
61. Nagai, H., and Roy, C. R. (2001) *EMBO J.* **20**, 5962–5970
62. Rosier, O., Wengelnik, K., Hahn, K., and Bonas, U. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9368–9373
63. Anderson, D. M., Fouts, D. E., Collmer, A., and Schneewind, O. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 12839–12843
64. Creasey, E. A., Delahay, R. M., Bishop, A. A., Shaw, R. K., Kenny, B., Knutton, S., and Frankel, G. (2003) *Mol. Microbiol.* **47**, 209–221