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## *Bordetella pertussis* Expresses a Functional Type III Secretion System That Subverts Protective Innate and Adaptive Immune Responses<sup>∇</sup>

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Certain bacteria use a type III secretion system (TTSS) to deliver effector proteins that interfere with cell function into host cells. While transcription of genes encoding TTSS components has been demonstrated, studies to date have failed to identify TTSS effector proteins in *Bordetella pertussis*. Here we present the first evidence of a functionally active TTSS in *B. pertussis*. Three known TTSS effectors, Bsp22, BopN, and BopD, were identified as TTSS substrates in *B. pertussis* 12743. We found expression of Bsp22 in a significant proportion of clinical isolates but not in common laboratory-adapted strains of *B. pertussis*. We generated a TTSS mutant of *B. pertussis* 12743 and showed that it induced significantly lower respiratory tract colonization in mice than the wild-type bacteria. Respiratory infection of mice with the mutant bacteria induced significantly greater innate proinflammatory cytokine production in the lungs soon after challenge, and this correlated with significantly higher antigen-specific interleukin-17, gamma interferon, and immunoglobulin G responses later in infection. Our findings suggest that the TTSS subverts innate and adaptive immune responses during infection of the lungs and may be a functionally important virulence factor for *B. pertussis* infection of humans.

*Bordetella pertussis* is the causative agent of whooping cough or pertussis, a respiratory disease that is most severe in infants and young children. Although vaccination has significantly reduced morbidity and mortality, pertussis remains an endemic disease and is one of the major causes of vaccine-preventable deaths today, with WHO estimates of 45 million cases and 409,000 deaths each year. In recent years a resurgence of pertussis was observed in a number of vaccinated populations (6, 29). Furthermore, it has become increasingly clear that pertussis is not only a childhood disease but also is highly prevalent among adults (21). This has called into question the level of protection provided by current pertussis vaccines and highlighted the need for a better understanding of the molecular mechanisms underlying the pathogenesis of *B. pertussis* infection.

Bacteria produce a complex array of virulence factors, including toxins and adhesins, which facilitate colonization and/or suppress immune responses and allow the bacteria to establish infection in the host. One of these virulence factors, the type III secretion system (TTSS), is a specialized secretory apparatus that allows gram-negative bacteria to inject proteins, known as effectors, directly into the eukaryotic cell cytosol. In laboratory conditions bacteria can be induced to secrete TTSS substrates, which include effectors and proteins involved in the delivery process, into the extracellular milieu in the absence of eukaryotic cells. TTSSs have been shown to be important me-

diators of virulence of a range of animal pathogens, including *Yersinia* spp., *Salmonella* spp., *Shigella* spp., *Escherichia coli*, and *Bordetella bronchiseptica* (15, 39). Yuk and colleagues have reported that the TTSS of *B. bronchiseptica* modulates dendritic cell (DC) maturation (31, 33), enhancing production of the anti-inflammatory cytokine interleukin-10 (IL-10) and promoting bacterial persistence (32).

Despite reports describing transcription of genes encoding components of the *Bordetella* *bsc* TTSS machinery in *B. pertussis* Tohama I (14, 22), the *B. pertussis* isolate chosen for genome sequencing, studies to date have failed to demonstrate TTSS effector secretion by *B. pertussis* in vitro or in vivo (9, 22). The sequencing of the *B. pertussis* Tohama I genome has revealed an extraordinary high level of genetic flexibility (28), and this raises concerns about the adequacy of laboratory-adapted strains for the study of natural clinical pathogenesis. Differences in gene expression have been shown to affect virulence characteristics of laboratory-adapted versus corresponding low-passage clinical isolates of *E. coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (11, 34, 37).

Here we demonstrate secretion of the *Bordetella* TTSS effector, Bsp22, by a significant portion of low-passage clinical isolates of *B. pertussis*, but not by common laboratory-adapted strains, such as Tohama I and Wellcome 28. Mutation of *bscN*, which encodes an essential component for TTSS secretion across bacterial membranes, abolished in vitro secretion of TTSS substrates by a clinical isolate of *B. pertussis*, resulting in a reduced ability of the bacteria to colonize the respiratory tracts of mice, and this was associated with enhanced local inflammatory and antigen-specific cellular and humoral immune responses. Our data suggest that expression of a functional TTSS is a feature of natural infection of humans with *B.*

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*pertussis* and that this may confer virulence to the bacteria by subverting the protective innate and adaptive immunity of the host.

## MATERIALS AND METHODS

**Bacterial strains and growth media.** Low-passage isolates *B. pertussis* ATCC 12743 (5375 [3865]), ATCC 12742 (5374 [3747]), and ATCC 9340 (5 [17921]), hereafter referred to as *B. pertussis* 12743, 12742, and 9340, respectively, were obtained from the ATCC. *B. pertussis* 12743 and 12742 were from cultures made by E. K. Anderson and deposited in the ATCC by G. Eldering (8), and *B. pertussis* 9340 was from a culture made by P. Kenrick and deposited in the ATCC by M. Pittman in the 1950s. Sixteen clinical isolates were cultivated from the sputum, noses, nasopharynxes, or throats of infants or adults with whooping cough in The Netherlands between 1949 and 2005. Wild-type (WT) *B. pertussis* and *B. bronchiseptica* were grown at 37°C on Bordet-Gengou (BG) agar and in Stainer-Scholte (SS) broth. Gentamicin-resistant  $\Delta bscN$  derivatives of *B. pertussis* 12743 and *B. bronchiseptica* RB50 were grown on BG agar or SS broth supplemented with 10 µg/ml gentamicin (Gibco, United Kingdom). For allelic exchange WT *B. pertussis* 12743 was first rendered streptomycin resistant by subculture in increasing sublethal concentrations of streptomycin (final concentration, 100 µg/ml). For routine cloning and conjugation, *E. coli* XLI-Blue and SM10λpir were grown at 37°C on Luria-Bertani (LB) agar or LB broth (BD Difco) supplemented with the appropriate antibiotics (ampicillin, 150 µg/ml; gentamicin, 10 µg/ml; kanamycin, 25 µg/ml).

**Generation of  $\Delta bscN$  bacteria.** Gentamicin-resistant  $\Delta bscN$  derivatives of *B. pertussis* 12743 and *B. bronchiseptica* RB50, in which a 0.5-kb internal portion of the *bscN* gene was removed and replaced with a 0.7-kb fragment containing a gentamicin resistance cassette, were constructed as follows. Primers PAB20 (5'-GCCCTGCGGATCCCGCG-3') and NF5 (5'-TACTGACGCATGCCCT ATCC-3'), annealing to bp 65 to 81 of *bscL* (5' flanking gene to *bscN*) and bp 1 to 12 of *bscN*, respectively, were used to amplify a 0.5-kb fragment from Tohama I. The resulting PCR product was digested with BamHI and SphI (recognition sequences underlined in primer sequences) and inserted into the corresponding sites of the cloning vector pQE-80 (Qiagen, United Kingdom) to produce pNF2. Primers NF4 (5'-GCTGGGCATGCTGGTCAAGGGC-3') and PAB21 (5'-GC CGGCTCGCGATGCATCG-3'), annealing to bp 560 to 582 of *bscN* and bp 470 to 489 of *bscO* (3' flanking gene to *bscN*), respectively, were used to amplify a 1.3-kb fragment from Tohama I. The resulting PCR product was digested with SphI and AgeI (recognition sequence underlined in primer sequence) to yield a 0.5-kb fragment and inserted into the corresponding SphI and XmaI sites on pNF2 to create pNF7, containing a mutant *bscN* allele. The gentamicin resistance cassette was amplified from pSS1129 using primers Gmr\_for\_2 (5'-ATA GCATGCTGACGCACACCG-3') and Gmr\_rev (5'-GCATGCTTAGGTGGC GGTAC-3') with SphI sites engineered at the 5' and 3' ends, respectively, and inserted into the unique SphI site at the center of the mutant *bscN* allele on pNF7 to create pNF12. BamHI (bp 1 to 6 of the *bscN::Gm<sup>r</sup>* mutant allele) and NheI (138 bases downstream) sites flanking the *bscN::Gm<sup>r</sup>* mutant allele on pNF12 were then used to subclone the *bscN::Gm<sup>r</sup>* mutant allele into the allelic exchange vector pEGBR (1) to create pNF13. pNF13 was then introduced into streptomycin-resistant *B. pertussis* 12743 and *B. bronchiseptica* RB50 by conjugation, and gentamicin-resistant double recombinants of *B. pertussis* 12743 and *B. bronchiseptica* RB50 generated by allelic exchange were screened by PCR for the presence of the *bscN::Gm<sup>r</sup>* mutant allele.

**Recombinant His-Bsp22 and Ab production.** Primers PAB37 (5'-CGGAAG CTTTATAGCGCATGTTGCTGGTG-3'), which binds bp 596 to 615 of the Bsp22 gene, and PAB38 (5'-AGCGGATCCAGCATTGATCTCGGAGTTCA C-3'), which binds bp 4 to 25 of the Bsp22 gene, were used to amplify the Bsp22 gene from *B. pertussis* 18323. The resulting PCR fragment was cloned in frame with the six-His tag located on pQE-80 (Qiagen, United Kingdom), creating pAPB15, from which recombinant His-tagged Bsp22 was expressed in *E. coli* XLI-Blue. His-Bsp22 was purified by affinity chromatography on Ni<sup>2+</sup> columns. For antibody (Ab) production, a rabbit was immunized subcutaneously with recombinant His-Bsp22 (230 µg) emulsified in complete Freund's adjuvant (Difco) and boosted on days 14 and 28 with His-Bsp22 (115 µg) in incomplete Freund's adjuvant. The rabbit was exsanguinated 12 days after the last immunization, and serum was prepared and shown by Western blotting to contain an Ab that specifically recognized Bsp22.

**Protein identification by MALDI-TOF mass spectrometry and immunoblotting.** For matrix-assisted laser desorption-ionization-time of flight (MALDI-TOF) mass spectrometry analysis of protein samples, culture supernatants were harvested from *Bordetella* spp. following 24 h of growth in liquid culture. Filtered

supernatants were precipitated with 30% ammonium chloride. Protein pellets were analyzed by MALDI-TOF mass spectrometry, and identified peptide fragments were searched against the predicted proteins from the complete *B. bronchiseptica* RB50 and *B. pertussis* Tohama I genomes (www.sanger.ac.uk/Projects/B\_pertussis/). For Western blots, cultures were harvested for each bacterial strain at the same stage of bacterial growth, as determined by measurements of optical density at 600 nm (OD<sub>600</sub>), and total protein from a 1-ml supernatant fraction was precipitated with 10% (wt/vol) trichloroacetic acid and resuspended in sample buffer. Proteins were separated on 10% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose membranes (Sigma) prior to Western blotting with a polyclonal Ab raised against *B. pertussis* Bsp22, pertussis toxin (PT), or filamentous hemagglutinin (FHA), followed by the appropriate secondary Ab.

**In vitro growth curves.** *B. pertussis* 12743 and  $\Delta bscN$  *B. pertussis* 12743 were grown in SS medium in 100-ml cultures inoculated at a starting OD<sub>600</sub> of 0.2. Samples (1 ml) were removed at 4, 8, 12, 23, 27, 37, and 49 h, OD<sub>600</sub> was determined, and CFU counts were performed by plating neat and diluted samples on BG agar plates.

**In vitro cytotoxicity and adherence assays.** DC were expanded in vitro from bone marrow of BALB/c mice by culture for 10 days with granulocyte-macrophage colony-stimulating factor as described previously (13). Murine DC, J774 macrophages, and human HEK-293T epithelial cells (1 × 10<sup>6</sup>/ml) were incubated in Dulbecco's modified Eagle's medium supplemented with 8% fetal calf serum in 24-well plates. Stationary-phase cultures of *Bordetella* spp. were harvested, resuspended in 1% casein, and added to triplicate wells at a multiplicity of infection (MOI) of 20, 100, or 500. Cytotoxicity was determined after 4 h using the Cytotox 96 nonradioactive cytotoxicity assay (Promega), which measures lactate dehydrogenase release as a measure of cell lysis. For detection of bacterial adherence to macrophages and of internalization, J774 cells were incubated with *Bordetella* spp. at an MOI of 100. Following incubation at 37°C for 2 h, the contents of each well were harvested, washed to remove unbound bacteria, and resuspended in either 1 ml of medium supplemented with kanamycin (100 µg/ml) to kill extracellular bacteria or 1 ml medium alone. The cell suspensions were incubated at 37°C for a further 1 h and then centrifuged at 1,200 rpm for 5 min. The cells were washed and resuspended in 1 ml phosphate-buffered saline with 1% (wt/vol) Triton X-100 and left at room temperature for 5 min. One hundred-microliter samples of the appropriate dilutions of the lysed cells were then plated in triplicate onto BG agar plates and incubated at 37°C for 2 days for *B. bronchiseptica* and 3 to 5 days for *B. pertussis*.

***B. pertussis* infection of mice.** Mice were infected with WT or  $\Delta bscN$  *B. pertussis* 12743 by exposure to an aerosol of bacteria for 15 min as described previously (20). The standard inoculum was 2 × 10<sup>10</sup> bacteria/ml (based on OD<sub>600</sub>), and this was reduced or increased by two- to threefold to achieve a lower or higher initial colonization. Four mice from each experimental group were sacrificed 3 h and 3, 7, 14, 21, and 28 days after challenge to assess the number of viable bacteria in the lungs. The numbers of CFU in lung homogenates were estimated as described previously (20). Results are given as the mean numbers of *B. pertussis* CFU for individual lungs from four mice per experimental group. The sensitivity of the assay was 0.56 log<sub>10</sub> CFU per lung.

**Quantification of cytokine concentrations and neutrophil infiltration into the lungs.** Lung homogenates were centrifuged at 13,000 rpm for 5 min, and the concentrations of IL-10, IL-12p70, IL-1β, tumor necrosis factor alpha (TNF-α), macrophage inflammatory protein 1 (MIP-1) and MIP-2 (R&D Systems), IL-12p40, IL-6, and transforming growth factor β (BD Pharmingen) were determined by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Neutrophil infiltration into the lungs was quantified by performing differential counts on hematoxylin-and-eosin-stained cytospin preparations of cells recovered by bronchoalveolar lavage as described previously (23).

**Detection of antigen-specific immune responses.** Spleens and serum samples were recovered from mice 14, 21, and 28 days after bacterial challenge. Spleen cells (2 × 10<sup>6</sup> cells/ml) were stimulated with sonicated *B. pertussis*, PT, FHA, or medium alone and incubated at 37°C for 72 h. Supernatants were removed, and gamma interferon (IFN-γ) and IL-17 concentrations were determined by ELISA. Lung T cells were purified on T-cell isolation columns as described previously (23). Lung T cells were stimulated with heat-inactivated *B. pertussis* antigen and splenic antigen-presenting cells (2 × 10<sup>6</sup> irradiated spleen cells). After 5 days the surviving T cells were stimulated with phorbol myristate acetate and ionomycin for 1 h, and then brefeldin A was added for 4 h at 37°C. Cells were stained with fluorescently labeled Abs specific for CD4 (eBiosciences). Cells were then fixed, permeabilized, and incubated with fluorescently labeled anti-IFN-γ or anti-IL-17 Abs (BD Pharmingen) according to the manufacturer's instructions (Fix & Perm cell permeabilization kit; Invitrogen). Immunofluorescence was

analyzed using Summit software on a Cyan ADP flow cytometer (Dako). Anti-*B. pertussis* immunoglobulin G (IgG), IgG2a, and IgG1 Ab titers were determined by ELISA as described previously (13). Results are expressed as  $\log_{10}$  end point Ab titers, determined by extrapolation of the straight part of the dilution curve, versus the OD<sub>492</sub> value of the control serum for naive mice.

**Statistical analysis.** Statistical analysis was performed using Graphpad Prism. Student's *t* test was used to compare the mean values between two groups. Statistical differences in mean values between more than two experimental groups were determined by analysis of variance. Linear regression was used to examine the correlation between bacterial growth estimated by CFU counts and OD<sub>600</sub>. A nonlinear regression analysis was performed on the growth curves for *B. pertussis* 12743 and  $\Delta$ *bscN* *B. pertussis* 12743. An Akaike's information criterion test with a confidence interval of 95% was applied to the data, with a supplemental *t* test, to determine if the growth curves of bacterial strains are different.

## RESULTS

**Secretion of TTSS effector proteins by clinical but not laboratory-adapted isolates of *B. pertussis*.** *B. pertussis* Tohama I is well characterized and was chosen for genome sequencing (28) but has been through extensive laboratory passage since its isolation in Japan in 1954 (16). Since the TTSS expression and secretory function of *Yersinia* is regulated and activated in vivo by eukaryotic cell contact and can be altered by prolonged laboratory passage (4, 36), we examined low-passage *B. pertussis* isolates from the ATCC (*B. pertussis* 12743, 12742, and 9340) and compared these with the laboratory-adapted strains (Tohama I and Wellcome 28) for expression of Bsp22. Bsp22, whose gene is located in the TTSS operon in *B. pertussis* and *B. bronchiseptica* (www.sanger.ac.uk/Projects/B\_pertussis/), is secreted by *B. bronchiseptica* (39) and is a useful marker of TTSS secretory function. In the present study, His-tagged Bsp22 was cloned and expressed in *E. coli* and the purified protein was used to generate polyclonal anti-Bsp22 Abs. Western blotting with anti-Bsp22 revealed significant quantities of secreted Bsp22 protein in culture supernatants of *B. pertussis* 12743 and 9340, but not in 12742, Tohama I, or Wellcome 28 (Fig. 1A). These data demonstrate that two of the three early isolates from the ATCC that were examined expressed TTSS proteins in vitro, but this may have been lost in the common laboratory-adapted strains Tohama I and Wellcome 28.

We next examined supernatants from stationary-phase cultures of 16 clinical isolates, cultivated from infants or adults with whooping cough in The Netherlands between 1949 and 2005. Western blotting with anti-Bsp22 Abs revealed secretion of Bsp22 in 15 of 16 isolates (Fig. 1; results are shown for 8 clinical isolates; an additional 8 [not shown] all expressed Bsp22). Bsp22 was also detected in *B. bronchiseptica* RB50 but not *B. pertussis* Tohama I or Wellcome 28. PT was detected in all strains except *B. bronchiseptica* RB50, confirming that the clinical isolates were *B. pertussis* and not *B. bronchiseptica*. These results demonstrate that a large proportion of the low-passage clinical isolates of *B. pertussis* examined have a functional TTSS.

In order to confirm these findings and to facilitate identification of additional *Bordetella* TTSS substrates, we carried out mass spectroscopy analysis on total secreted proteins from culture supernatants. MALDI-TOF analysis revealed a number of known *Bordetella* virulence factors, including FHA, adenylate cyclase toxin, and pertactin in *B. pertussis* Tohama I, *B. pertussis* 12743, and *B. bronchiseptica* RB50 (Table 1), as well as other proteins not yet implicated in virulence (data not

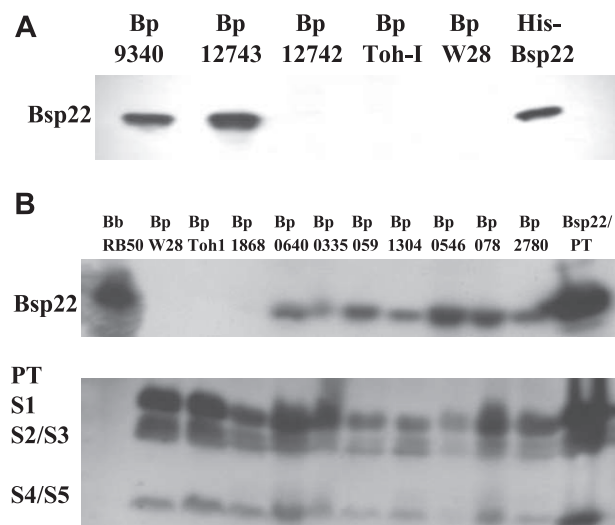


FIG. 1. Clinical isolates but not common laboratory-adapted strains of *B. pertussis* express Bsp22. Protein samples from supernatants of stationary-phase cultures of *B. pertussis* (Bp) 9340, 12743, 12742, Tohama I (Toh-I), and Wellcome 28 (W28) (A) or *B. bronchiseptica* (Bb) RB50, *B. pertussis* W28 and Toh-I, and eight clinical isolates of *B. pertussis* recovered from respiratory tracts of patients with whooping cough (B), prepared by precipitation with 10% trichloroacetic acid or purified His-Bsp22 or PT (2  $\mu$ g), were resolved on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and probed with Abs specific for Bsp22 or PT (S1 to S5 subunits) by Western blotting.

shown). Tracheal colonization factor, a virulence factor of *B. pertussis* but not *B. bronchiseptica* (10), was detected in *B. pertussis* Tohama I and *B. pertussis* 12743 but not *B. bronchiseptica* RB50. Serum resistance protein (BrkA) was detected in *B. pertussis* Tohama I and *B. pertussis* 12743 but not *B. bronchiseptica* RB50; BrkA expression has been demonstrated for some strains of *B. bronchiseptica*, but not for strain RB50 (30). PT, a *B. pertussis*-specific virulence factor, was also detected in *B. pertussis* 12743; this required a separate analysis from that shown in Table 1, using more concentrated culture supernatant (not shown). As well as Bsp22, the TTSS translocator protein, BopD, and the sensor/plug protein, BopN, were detected in supernatants from *B. pertussis* 12743 but not *B. pertussis* Tohama I. The *Bordetella* TTSS effector protein, BteA/BopC, was detected in *B. bronchiseptica* RB50 but not in *B. pertussis* 12743. These results provide further evidence that *B. pertussis* 12743 has a functional TTSS in vitro and demonstrate for the first time that BopD and BopN, in addition to Bsp22, are substrates of the *B. pertussis* TTSS.

**Mutation of *bscN* abolishes expression of TTSS effector proteins by *B. pertussis* 12743 but does not affect bacterial growth in vitro.** In order to assess the contribution of the TTSS to the virulence of BP 12743, we constructed a strain carrying an insertional mutation of the *bscN* gene. Mutation of *bscN* in *B. bronchiseptica* or its homologs, *yscN* in *Yersinia* and *invC* in *Salmonella*, has been shown to abolish in vitro TTSS secretion and in vivo TTSS-mediated effects on host cells (7, 38–40). For comparative purposes, we also mutated *bscN* in *B. bronchiseptica* RB50. Bsp22 protein was detected by Western blotting in the culture supernatants of WT *B. pertussis* 12743 and *B. bronchiseptica* RB50 but not in  $\Delta$ *bscN* derivatives (Fig. 2). Further-

TABLE 1. Identification of secreted TTSS substrates and other virulence factors from *Bordetella* spp. by MALDI-TOF mass spectrometry<sup>a</sup>

Mascot accession no.	Identified protein	Result for:				
		<i>B. pertussis</i> Toh I	<i>B. pertussis</i> 12743	$\Delta bscN$ <i>B. pertussis</i> 12743	<i>B. bronchiseptica</i> RB50	$\Delta bscN$ <i>B. bronchiseptica</i> RB50
BP1879/BB2993	FHA	+ (141)	+ (8,869)	+ (3,776)	+ (567)	+ (434)
BP0760/BB0324	Bifunctional hemolysin-adenylate cyclase toxin (CyaA)	+ (116)	+ (2,423)	+ (1,074)	+ (2,551)	+ (2,571)
BP1054/BB1366	Pertactin (Prn)	+ (111)	+ (2,066)	+ (759)	+ (450)	+ (350)
BP3494/BB0961	Serum resistance protein (BrkA)	+ (234)	+ (2,261)	+ (1,893)	–	–
BP1201 BB3291	Tracheal colonization factor (TcfA)	+ (412)	+ (1,530)	+ (3,117)	–	–
BP2256/BB1617	Putative secreted protein (Bsp22)	–	+ (470)	–	+ (500)	–
BP2253/BB1620	Putative outer protein D (BopD)	–	+ (781)	–	+ (193)	–
BP2257/BB1616	Putative outer protein N (BopN)	–	+ (204)	–	–	–
BP0500/BB4228	Hypothetical protein (BteA/BopC)	–	–	–	+ (67)	–

<sup>a</sup> Quantitative MALDI-TOF tandem mass spectrometry analysis was carried out on extracted proteins from culture supernatant of *B. pertussis* Tohama I (Toh I), *B. pertussis* 12743,  $\Delta bscN$  *B. pertussis* 12743, *B. bronchiseptica* RB50, and  $\Delta bscN$  *B. bronchiseptica* RB50. Identified peptides were searched against the complete *B. bronchiseptica* and *B. pertussis* genome sequences ([www.sanger.ac.uk/Projects/B\\_pertussis/](http://www.sanger.ac.uk/Projects/B_pertussis/)) using the Mascot search engine. Numbers in parentheses represent the individual Mascot ion scores. Individual ion scores >33 indicate identity or extensive homology ( $P < 0.05$ ).

more, Bsp22, BopN, and BopD were detected by mass spectrometry analyses of secreted proteins of WT but not  $\Delta bscN$  derivatives (Table 1). Mutation of *bscN* did not affect secretion of other virulence factors, including FHA, adenylylase toxin, and pertactin (Table 1 and Fig. 2). These results demonstrate that mutation of the *bscN* gene abolishes secretion of TTSS substrates by *B. pertussis* 12743 and *B. bronchiseptica* RB50 and demonstrate that BscN is an essential component of the *B. pertussis* TTSS. Furthermore, mutation of *bscN* did not significantly affect bacterial growth rate. The in vitro growth curves based on OD<sub>600</sub> (Fig. 3A) and CFU (Fig. 3B) were almost superimposable for *B. pertussis* 12743 and  $\Delta bscN$  *B. pertussis* 12743. Nonlinear regression analysis with Akaike's information criterion test determined that there was a 93% probability that the curves for the two bacterial strains were identical. Furthermore linear regression analysis demonstrated a highly significant ( $P < 0.001$ ) correlation between OD<sub>600</sub> and CFU for *B. pertussis* 12743 and  $\Delta bscN$  *B. pertussis* 12743. These findings demonstrate that OD<sub>600</sub> can be used as an accurate estimate of the number of viable bacteria and suggest that equivalent numbers of viable WT and TTSS mutant bacteria were employed for experiments to compare the virulence levels of and immune responses to *B. pertussis* 12743 and  $\Delta bscN$  *B. pertussis* 12743 in vitro and in vivo.

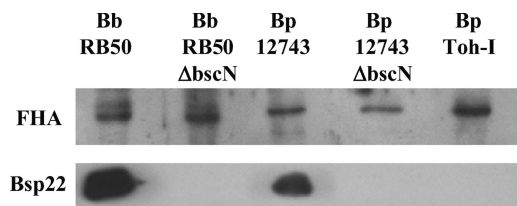


FIG. 2. Mutation of *bscN* in *Bordetella* spp. abolishes Bsp22 production but does not affect secretion of the virulence factor FHA. Proteins from culture supernatants of WT and  $\Delta bscN$  *B. pertussis* (Bp) 12743 and *B. bronchiseptica* (Bb) RB50 or *B. pertussis* Tohama I (Toh-I) were examined for the presence of Bsp22 and FHA by Western blotting.

**The TTSS of *B. pertussis* 12743 promotes adherence to macrophages but does not mediate cytotoxicity in vitro.** Consistent with previous reports (39, 40), *B. bronchiseptica* RB50 was significantly cytotoxic to cultured epithelial cells, macrophages, and DC (Fig. 4A) and this activity was lost in the  $\Delta bscN$  mutant. In contrast, no significant cell lysis was detected in any cell type incubated with WT or  $\Delta bscN$  *B. pertussis* 12743. The TTSS of *Yersinia* spp. plays a crucial role in preventing bacterial uptake during infection (4), while the TTSS of *Salmonella* spp. mediates bacterial uptake to facilitate tissue invasion and intracellular replication (12). Here we investigated the role of the *Bordetella* TTSS in bacterial uptake. Significantly higher CFU were detected in kanamycin-treated macrophages cultured with  $\Delta bscN$  *B. bronchiseptica* than in those cultured with WT *B. bronchiseptica*, indicative of a higher number of internalized viable mutant bacteria (Fig. 4B). After correcting for cell death induced by *B. bronchiseptica*, there was still significantly greater uptake of the mutant than the WT *B. bronchiseptica* (data not shown). In contrast, the numbers of CFU recovered following treatment with medium only were similar following culture with WT or  $\Delta bscN$  *B. bronchiseptica*, suggesting that the TTSS of *B. bronchiseptica* did not facilitate binding to the cell surface. The numbers of viable WT and  $\Delta bscN$  *B. pertussis* 12743 CFU recovered from kanamycin-treated macrophages were very similar, indicative of similar numbers of internalized *B. pertussis* CFU. In contrast, the number of viable *B. pertussis* CFU recovered following treatment with medium alone from cells cultured with  $\Delta bscN$  *B. pertussis* 12743 was significantly lower than the number of WT bacteria, indicating a lower number of cell-associated bacteria following incubation with the mutant bacteria (Fig. 4B). These results demonstrate that the TTSS of *B. bronchiseptica* RB50 inhibits bacterial uptake by murine macrophages, whereas the TTSS of *B. pertussis* 12743 promotes adherence of the bacteria to macrophages in vitro.

**Enhanced innate and adaptive immune responses in mice infected with  $\Delta bscN$  *B. pertussis* 12743.** Protective immunity to *B. pertussis* involves a combination of local innate inflamma-

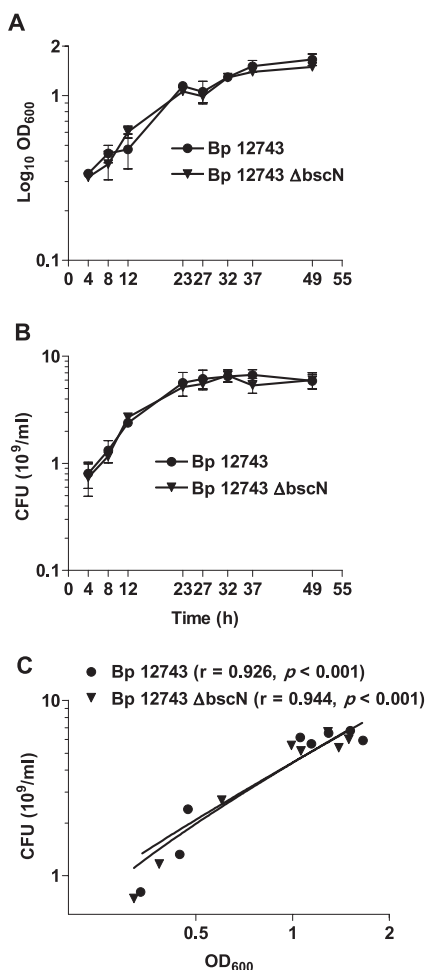


FIG. 3. Correlation between in vitro growth curves for *B. pertussis* (Bp) 12743 and  $\Delta bscN$  *B. pertussis* 12743. *B. pertussis* 12743 and  $\Delta bscN$  *B. pertussis* 12743 were cultured for 2 days in SS medium, and samples were removed at the indicated time points. (A) OD<sub>600</sub> was determined. (B) The number of viable bacteria was determined by performing CFU counts after plating on BG agar. (C) CFU counts were plotted against OD<sub>600</sub> for each strain separately, and linear regression analysis was performed. The correlation ( $r$  value) and level of significance ( $P$  value) are shown.

tory responses in the lungs (13, 24) and adaptive immune responses, mediated by Th1 and Th17 cells, which help to clear bacteria from the respiratory tract (13, 20). Here, we examined local innate inflammatory cytokine production in the lungs of mice infected with WT and  $\Delta bscN$  *B. pertussis* 12743. We detected significantly higher concentrations of the proinflammatory cytokines, TNF- $\alpha$  ( $P < 0.01$ ), IL-1 $\beta$  ( $P < 0.01$ ) and IL-6 ( $P < 0.05$ ), and proinflammatory chemokines MIP-1 $\alpha$  ( $P < 0.01$ ) and MIP-2 ( $P < 0.05$ ) in the lungs 3 h after challenge with the  $\Delta bscN$  bacteria compared with WT *B. pertussis* 12743 (Fig. 5). Furthermore, the concentrations of IL-1 $\beta$ , MIP-1 $\alpha$ , and MIP-2 remained significantly higher in the lungs of mice infected with the  $\Delta bscN$  *B. pertussis* 12743 up to 7 to 14 days postchallenge. We also detected significantly higher concentrations of IL-12p40 and IL-6 in the lungs of mice 7 days after challenge with  $\Delta bscN$  *B. pertussis* 12743. In contrast, the concentrations of the immunosuppressive cytokine IL-10 were

consistently lower in mice infected with  $\Delta bscN$  *B. pertussis* than in those infected with WT bacteria. The concentration of transforming growth factor  $\beta$  in the lungs was not significantly changed over the course of infection with *B. pertussis* 12743 or  $\Delta bscN$  *B. pertussis* 12743 (Fig. 5). These results suggest a role for the TTSS of *B. pertussis* 12743 in suppressing innate proinflammatory cytokine and chemokine responses in the lungs, especially early in infection.

Analysis of the cellular content of bronchoalveolar lavage samples 7 days postchallenge revealed a nonsignificant increase in the number of neutrophils in the lungs of mice infected with  $\Delta bscN$  *B. pertussis* 12743 ( $4.6 \times 10^4 \pm 1.5 \times 10^4$ /lung) compared with that in lungs of mice infected with *B. pertussis* 12743 ( $2.6 \times 10^4 \pm 1.5 \times 10^4$ /lung). However, histological analysis of lungs did not reveal evidence of increased pathology in the lungs of mice infected with the mutant versus WT bacteria (data not shown).

We examined antigen-specific cytokine production in mice infected with WT or  $\Delta bscN$  *B. pertussis* 12743 by stimulation of spleen cells with *B. pertussis* sonicate, purified PT, or FHA. We detected *B. pertussis*-specific IL-17 and IFN- $\gamma$  production by spleen cells recovered 14 or 21 days after infection of mice with WT *B. pertussis* 12743 (Fig. 6). However, antigen-specific IL-17 and IFN- $\gamma$  production was significantly stronger in mice infected with  $\Delta bscN$  *B. pertussis* 12743. The enhanced IL-17 production in mice infected with the mutant bacteria in response to PT and also to *B. pertussis* sonicate was particularly striking, and this was a consistent finding at days 14, 21 (Fig. 6), and 28 (data not shown) postchallenge. Preliminary experiments to determine the frequency of antigen-specific T cells in the lungs of infected mice (based on a single time point, day 14 postchallenge) revealed a higher frequency of *B. pertussis*-specific Th1 and Th17 cells in mice infected with  $\Delta bscN$  *B. pertussis* 12743 (IFN- $\gamma$  secreting, 2.86%; IL-17 secreting, 1.98%) than in mice infected with the WT bacteria (IFN- $\gamma$  secreting, 1.92%; IL-17 secreting, 0.88%). Collectively, these results suggest that the TTSS may suppress antigen-specific IFN- $\gamma$  and IL-17 production during infection of mice with *B. pertussis* 12743. Significantly higher *B. pertussis*-specific serum IgG levels in mice infected with  $\Delta bscN$  *B. pertussis* 12743 than in mice infected with WT bacteria were also observed (Fig. 7). Consistent with the stronger Th1 responses, we detected significantly higher titers of serum IgG2a in mice infected with  $\Delta bscN$  *B. pertussis* 12743 (Fig. 7). In contrast, there were no differences in serum IgG1 between the mutant and WT bacteria. These results suggest that the *Bordetella* TTSS may suppress Ab responses, especially IgG2a, as a consequence of suppressing the induction of Th1 cells.

**The TTSS of *B. pertussis* 12743 enhances bacterial persistence in the lungs of mice.** In order to examine the role of a functional TTSS in virulence of *Bordetella* in vivo, BALB/c mice were infected by exposure to an aerosol containing equivalent concentrations ( $2 \times 10^{10}$ /ml) of either WT or  $\Delta bscN$  *B. pertussis* 12743. After an initial rise in bacterial numbers, peaking at day 7, WT *B. pertussis* 12743 began to be steadily cleared from the lungs between days 7 and 21, with complete clearance of the bacteria 28 days postinfection (Fig. 8A). In contrast,  $\Delta bscN$  *B. pertussis* 12743 colonized the lungs to a significantly lower degree at each time point examined and began to be cleared from the lung earlier in infection and was completely

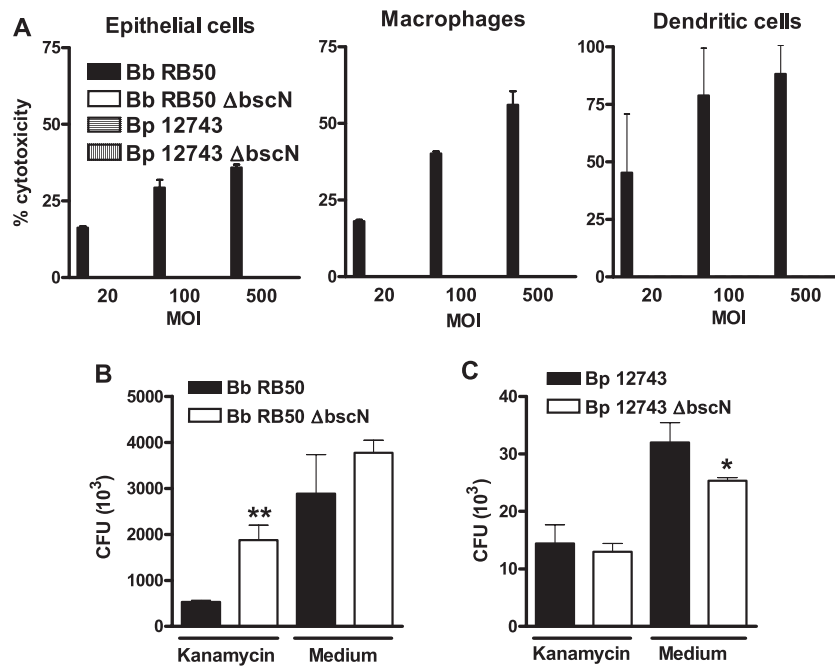


FIG. 4. The TTSS of *B. pertussis* 12743 promotes bacterial adherence to macrophages but does not mediate cytotoxicity. (A) Epithelial cells, J774 macrophages, or bone marrow-derived DC were cultured with WT or  $\Delta$ bscN *B. pertussis* (Bp) 12743 or *B. bronchiseptica* (Bb) RB50 at MOIs of 20, 100, and 500 for 4 h. Cytotoxicity was measured by a lactate dehydrogenase release assay. (B and C) J774 macrophages were cultured with WT or  $\Delta$ bscN *B. bronchiseptica* RB50 (B) or *B. pertussis* 12743 (C) at an MOI of 100:1. Adherence was assessed by assessing CFU counts following 2 h of incubation followed by 1 h of treatment with kanamycin or medium only. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  ( $\Delta$ bscN versus WT). Results are representative of three experiments.

cleared by day 21. The lower number of mutant bacteria recovered 3 h after challenge was a consistent finding in three experiments using our standard challenge inoculum. Evidence from over 100 challenge experiments with *B. pertussis* has shown that this method of infection results in highly reproducible lung colonization of mice within an experiment. Furthermore, we have also shown a highly significant correlation between protection in this model and vaccine efficacy in children (26). We used  $OD_{600}$  to estimate the number of bacteria in the challenge inoculum, and, since there was a highly significant correlation between  $OD_{600}$  and CFU counts (Fig. 3), it is unlikely that the difference in colonization at 3 h reflects exposure to a lower number of viable bacteria. Furthermore, the in vitro growth curves, as determined by measuring either  $OD_{600}$  or CFU, for the TTSS mutant and WT bacteria were almost identical (Fig. 3). Nevertheless, in order to provide further evidence of the impact of the TTSS on virulence in vivo, we examined the course of infection when differing concentrations of the challenge inocula resulted in higher or lower initial lung colonization. In a challenge experiment where the initial colonization was  $3.8 \log_{10}$  CFU per lung for the WT *B. pertussis*,  $\Delta$ bscN *B. pertussis* 12743 was cleared within 7 days and there were significantly greater numbers of CFU recovered at 3, 7, and 14 days postchallenge from the lungs of mice infected with the WT bacteria than with mutant bacteria (Fig. 8B). In contrast, when the initial colonization was  $4.8 \log_{10}$  CFU per lung for the WT *B. pertussis*, the effect of the mutation was less obvious. Although CFU recovered from the mice infected with the TTSS mutant were 4- to 10-fold ( $0.6$  to  $1 \log_{10}$ ) lower than CFU of WT *B. pertussis* on days 3 and 7,

these differences were not statistically significant (Fig. 8C). The absence of the TTSS and the corresponding loss of its cell binding and immunosuppressive effects may be less obvious when the lungs are overwhelmed with a high number of bacteria in the aerosol. Nevertheless the dramatically reduced CFU recovered from mice infected with the TTSS mutant in mice with lower initial colonization (which may be more relevant to the mode of exposure in humans) suggests that the TTSS does play an important role both in colonization and persistence of *B. pertussis* 12743 in the lungs following aerosol challenge of mice. Despite the lower bacterial load, we detected significantly higher concentrations of innate proinflammatory cytokine (Fig. 5) and antigen-specific Th1 and Th17 cells (Fig. 6) in mice infected with the mutant than in mice infected with WT *B. pertussis* (the experiments shown in Fig. 5 to 7 correspond to a level of bacterial colonization shown in Fig. 8A). This provides indirect evidence that the lower lung colonization observed with the mutant bacteria is unlikely to be due to exposure to a lower dose of viable bacteria but may reflect reduced adherence and a stronger host immune response due to the absence of the immune-subversive properties of the TTSS.

## DISCUSSION

This study has demonstrated for the first time that *B. pertussis* expresses a functionally active TTSS, which may be lost following prolonged in vitro culture of the bacteria. We have identified several substrates of the *B. pertussis* TTSS and shown that the TTSS of *B. pertussis* is an important virulence factor



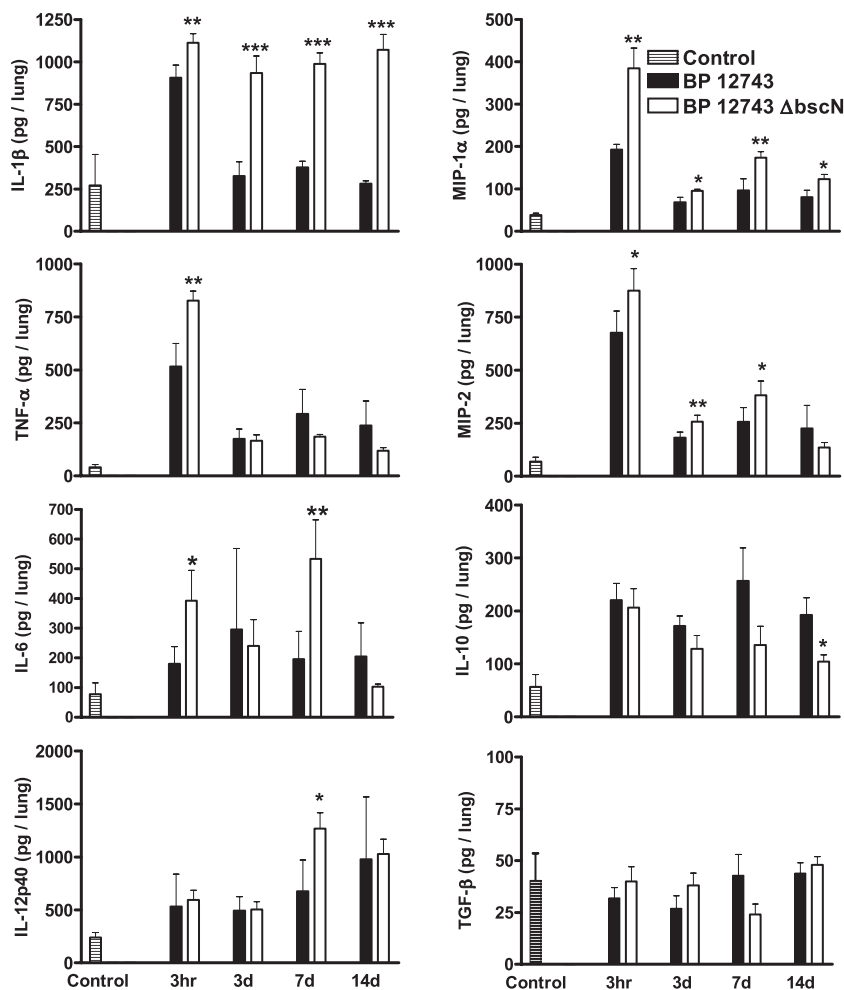


FIG. 5. Enhanced inflammatory cytokine and chemokine induction in the lungs of mice infected with  $\Delta bscN$  *B. pertussis* (BP) 12743. BALB/c mice were challenged with an aerosol of WT or  $\Delta bscN$  *B. pertussis* 12743. Cytokine and chemokine concentrations were determined by ELISA on lung homogenates from mice 3 h and 3, 7, and 14 days (d) after aerosol challenge and in uninfected control mice. Results are means  $\pm$  standard deviations for four mice per group at each time point. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  ( $\Delta bscN$  versus WT).

and immunomodulatory apparatus for subverting protective immune responses and prolonging survival in the host. It appears that the TTSS may facilitate colonization and survival of *B. pertussis* by promoting bacterial adherence and by inhibiting local innate inflammatory responses and the consequent induction of antigen-specific Th1, Th17, and Ab responses that function to clear the bacteria from the respiratory tract.

The closely related pathogens *B. pertussis* and *B. bronchiseptica* produce a range of common virulence factors, including adhesins and toxins, which function to establish and maintain the infection, but have also evolved distinct strategies for survival in the host. The relatively short-lived and acute severity of *B. pertussis* infection of humans, which favors a high rate of host-to-host transmission, contrasts with the relatively asymptomatic and often lifelong persistence of *B. bronchiseptica* infection in animals. This was thought to be consistent with the functional activity of the TTSS in *B. bronchiseptica*, which suppresses DC maturation and migration (32, 33), and the lack of TTSS function in *B. pertussis* Tohama I. However, the present study has revealed that clinical isolates of *B. pertussis* do ex-

press a functionally active TTSS, which plays a role in promoting more efficient bacterial colonization and persistence through immunosuppression following respiratory infection of mice. We demonstrated significant secretion of Bsp22, a reliable marker of a functional TTSS in *Bordetella* spp. (39), by 15 of 16 clinical isolates of *B. pertussis*, in addition to 2 isolates obtained from the ATCC, *B. pertussis* 12743 and 9340, which had not undergone extensive laboratory passage. We also demonstrated secretion of two additional *Bordetella* TTSS substrates, BopN and BopD, by *B. pertussis* 12743. In contrast, secretion of TTSS substrates could not be detected in two well-studied laboratory-adapted strains of *B. pertussis*, Tohama I and Wellcome 28. While the TTSS loci are conserved and have been shown to be transcribed in *B. pertussis* Tohama I during in vitro growth, it has been suggested that protein translation is prevented by a posttranscriptional block (22). The sequencing of the *B. pertussis* Tohama I genome revealed extensive expansion of the insertion sequence element IS481, indicative of large-scale genome rearrangements and thus a high level of genome plasticity. Long-term laboratory passage

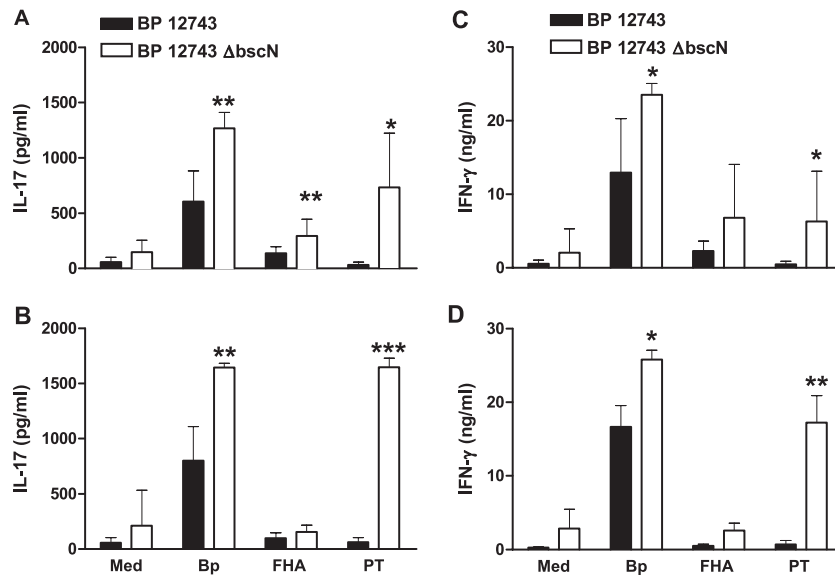


FIG. 6. Enhanced antigen-specific IL-17 and IFN- $\gamma$  production in mice infected with  $\Delta bscN$  *B. pertussis* (BP) 12743. BALB/c mice were challenged with an aerosol of WT or  $\Delta bscN$  *B. pertussis* 12743. Spleen cells recovered 14 (A and C) and 21 (B and D) days postchallenge were stimulated with sonicated *B. pertussis* 12743 (Bp), FHA, PT, or medium (Med) only as a control. Supernatants were removed after 3 days, and IL-17 (A and B) and IFN- $\gamma$  (C and D) concentrations were determined by ELISA. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  ( $\Delta bscN$  versus WT).

can lead to significant changes in gene expression and virulence factor production in *E. coli* and *Pseudomonas* and *Staphylococcus* spp. (11). Compared with that in laboratory-cultured strains, transcription of genes encoding the TTSS and its effector proteins was upregulated in a highly adherent *P. aeruginosa* strain isolated from the lung of a cystic fibrosis patient, and this correlated with increased cytotoxicity in vitro and enhanced virulence in the respiratory tracts of mice (37). Furthermore, it has been reported that the *Yersinia* YopT TTSS effector protein is not expressed by serotype O3 strains of *Y. pseudotuberculosis* that have been extensively passaged in vitro (36). Significant changes in gene transcription by clinical isolates of *B. pertussis* were reported after as few as 12 laboratory passages (2), indicating that *B. pertussis*, like other bacterial species, can alter gene expression when introduced into a new environment. It is therefore possible that the absence of a functional TTSS in *B. pertussis* Tohama I and Wellcome 28 is

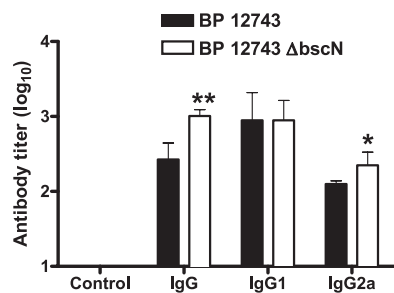


FIG. 7. Enhanced *B. pertussis*-specific IgG and IgG2a responses in mice infected with  $\Delta bscN$  *B. pertussis* (BP) 12743. BALB/c mice were challenged with an aerosol of WT or  $\Delta bscN$  *B. pertussis* 12743. Serum was recovered from infected mice 28 days postchallenge or from uninfected control mice and assayed for anti-*B. pertussis* IgG, IgG1, and IgG2a by ELISA. Results are means  $\pm$  standard deviations for four mice per group. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  ( $\Delta bscN$  versus WT).

a consequence of long-term laboratory culture in the absence of eukaryotic cell contact.

The present study demonstrated that the TTSS of *B. bronchiseptica* may facilitate bacterial persistence through subversion of bacterial uptake by macrophages, a strategy in the pathogenesis of *Yersinia* spp. (5). We found that *B. pertussis* 12743 did not induce cytotoxicity in macrophages, DC, and epithelial cells. However, consistent with previous reports (39, 40), *B. bronchiseptica* was cytotoxic to a range of cultured cells. Although other TTSS effector proteins may also contribute to cytotoxicity, it has been demonstrated that BteA, also called BopC, is required for the induction of necrotic cell death (19, 27). Interestingly, BteA/BopC was not detected in secreted proteins from *B. pertussis* 12743. In contrast, we found evidence that the TTSS of *B. pertussis* facilitates bacterial binding to macrophages in vitro. In addition, compared with the WT bacteria, the TTSS mutant of *B. pertussis* 12743 had significantly reduced colonization in the lungs 3 h after respiratory challenge of mice. Taken together, these data suggest that the TTSS of *B. pertussis* may function as a host adherence factor, as has been demonstrated for enteropathogenic *E. coli* (17).

Significantly, we found evidence that the TTSS facilitates persistence of the bacteria in the respiratory tract by subverting innate and adaptive immune responses. Protective immunity to *B. pertussis* is mediated through recruitment of neutrophils and macrophages to the lungs, local secretion of inflammatory cytokines, and the induction of *B. pertussis*-specific Th1 cells, Th17 cells, and Ab responses (18, 20, 24). IL-12-induced IFN- $\gamma$  production by NK cells and Th1 cells prevents bacterial dissemination from the respiratory tract and activates production of opsonizing and complement-fixing IgG2a Abs in the mouse (3, 20). IL-23, IL-1, TNF- $\alpha$ , and IL-6 promote the differentiation and expansion of Th17 cells, and these cells have been implicated in inflammatory responses that mediate autoimmu-

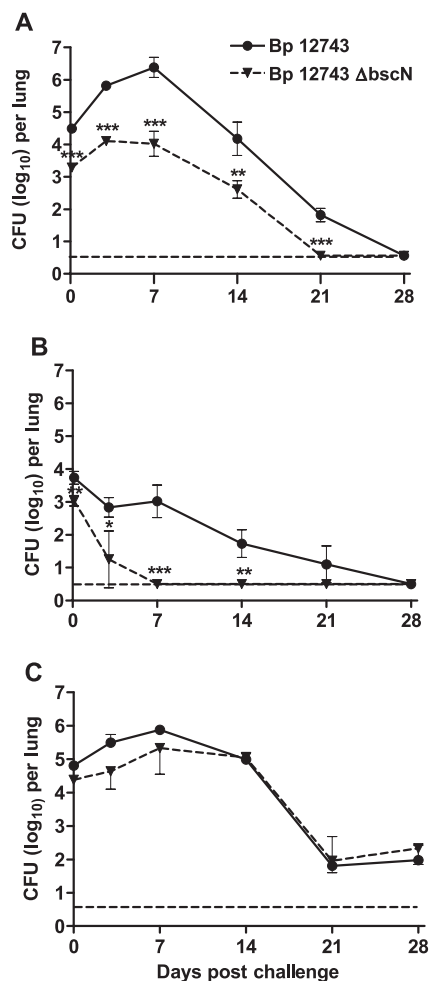


FIG. 8.  $\Delta bscN$  *B. pertussis* 12743 has reduced ability to colonize lungs of mice. BALB/c mice were challenged with an aerosol of WT or  $\Delta bscN$  *B. pertussis* (Bp) 12743, where the challenge inoculum resulted in intermediate (A), low (B), or high (C) initial colonization with the WT *B. pertussis*. Groups of four mice were sacrificed 3 h and 3, 7, 14, 21, and 28 days later, and CFU counts were performed on lung homogenates. The dashed line represents the limit of detection. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  ( $\Delta bscN$  versus WT). Results are representative of three experiments for panel A and one experiment each for panels B and C.

nity and also function in the recruitment of neutrophils to the site of infection, where they may help contain the pathogen until a subsequent clearing IFN- $\gamma$ -producing Th1 response can be generated (25, 35). We have recently reported that Th17 cells have a protective role in vaccine-induced immunity to *B. pertussis* by activating bacterial killing by macrophages (13). In the present study we found evidence that deletion of a functional TTSS from *B. pertussis* 12743 resulted in enhancement of local inflammatory responses in the lungs of infected mice. Despite the lower bacterial burden in the lungs, mice infected with the TTSS mutant had significantly greater local IL-1 $\beta$ , IL-12p40, MIP-1 $\alpha$ , and MIP-2 production. We also observed a modest reduction in IL-10 in mice infected with the TTSS mutant, and this was significant at day 14. This is consistent with the report on *B. bronchiseptica* where antigen-specific IL-10 production in mice infected with the TTSS mutant is

lower than that in mice infected with WT bacteria (32). In contrast, we demonstrated significantly greater *B. pertussis*-specific IFN- $\gamma$ , IL-17, and IgG2a responses of mice infected with the TTSS mutant than with WT *B. pertussis* 14 to 28 days after *B. pertussis* challenge. The augmentation of the IL-17 response to PT in mice infected with the mutant bacteria was particularly striking. Although we do not know the precise mechanisms, this may reflect enhancement of innate IL-1, which together with IL-23 is known to promote the differentiation of Th17 cells (13). The enhanced cellular immune response in mice infected with the TTSS mutant correlated with earlier respiratory clearance of TTSS-defective bacteria than of WT bacteria. Thus, it appears that the TTSS contributes to persistence of *B. pertussis* by suppressing innate inflammatory responses, which not only allows greater bacterial colonization, but also delays clearance due to significant suppression of Th1, Th17, and Ab responses.

Our study provides the first evidence that *B. pertussis* uses the TTSS as a means of colonization and survival in the host and may in particular target the innate immune system. Furthermore, we have demonstrated that one of the substrates, Bsp22, is secreted in significant quantities by clinical isolates of *B. pertussis* and is immunogenic in animals.

#### ACKNOWLEDGMENT

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