The Bordetella pertussis Type III Secretion System Tip Complex Protein Bsp22 Is Not a Protective Antigen and Fails To Elicit Serum Antibody Responses during Infection of Humans and Mice

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The type III secretion system (T3SS) of pathogenic bordetelae employs a self-associating tip complex protein Bsp22. This protein is immunogenic during infections by Bordetella bronchiseptica and could be used as a protective antigen to immunize mice against B. bronchiseptica challenge. Since low-passage clinical isolates of the human pathogen Bordetella pertussis produce a highly homologous Bsp22 protein (97% homology), we examined its vaccine and diagnostic potential. No Bsp22-specific antibodies were, however, detected in serum samples from 36 patients with clinically and serologically confirmed whooping cough disease (pertussis syndrome). Moreover, although the induction of Bsp22 secretion by the laboratory-adapted 18323 strain in the course of mice lung infection was observed, the B. pertussis 18323-infected mice did not mount any detectable serum antibody response against Bsp22. Furthermore, immunization with recombinant Bsp22 protein yielded induction of high Bsp22-specific serum antibody titers but did not protect mice against an intranasal challenge with B. pertussis 18323. Unlike for B. bronchiseptica, hence, the Bsp22 protein is nonimmunogenic, and/or the serum antibody response to it is suppressed, during B. pertussis infections of humans and mice.

Efficient pertussis vaccines have been available for over 6 decades, and their generalized use resulted in an impressive decline in global whooping cough incidence. Nevertheless, respiratory infections by Bordetella pertussis remain the least controlled vaccine-preventable infectious illness and account for more than 48 million people infected and as many as ~300,000 deaths annually worldwide (1–4). Since the 1980s, pertussis is again on the rise in developed countries, and resurgence of the disease has been observed in a number of vaccinated populations (4–12). Despite high acellular pertussis (aP) vaccine intake, a true whooping cough epidemic started in Australia in 2009, with 38,588 reported cases in 2011 (http://www9.health.gov.au/cda/source/Rpt_3_cfm). In 2010, a serious pertussis outbreak occurred in California, with 9,156 disease cases and 10 deaths http://www.cdph.ca.gov/programs/immunize/Documents/PertussisReport2012-04-24.pdf), and in 2012 the highest annual whooping cough incidence in the United States in 70 years was reached. A total of 16 pertussis-related deaths and more than 32,000 cases of pertussis were reported to the Centers for Disease Control and Prevention (CDC) as of 15 October 2012 (http://www.cdc.gov/pertussis/outbreaks.html). Studies involving persons with prolonged cough suggest that up to 1 million pertussis infections may be occurring in the United States per year, revealing that B. pertussis circulation in highly vaccinated populations is far more widespread than previously assumed (see reference 4 and references therein). Moreover, statistical analysis of medical records of populations living in the California counties hit by the 2010 outbreak revealed an unexpectedly limited duration of protective immunity induced by the aP vaccine (13). The resurgence of pertussis in highly vaccinated populations of the most developed countries thus raises questions about the composition and effectiveness of currently used aP vaccine formulations and their administration practices and schedules (14, 15). A better understanding of the molecular mechanisms accounting for the pathogenesis of pertussis infection, as well as identification and characterization of further protective antigens for the development of next-generation pertussis vaccines, is sorely needed.

Low-passage clinical isolates of B. pertussis have recently been found to express some components of the type III secretion system (T3SS), the role of which in pathophysiology of pertussis syndrome remains entirely unknown (16). The T3SS apparatus is exploited by a wide range of Gram-negative bacteria to deliver numerous effector proteins from bacterial cytosol directly into host cells to which the bacteria adhere, thus hijacking the intracellular machinery of the infected cells (17–19). The T3SS locus (bse) is positively regulated by the BvgAS two-component regulatory system and appears to be highly conserved among members of the Bordetella genus, where it was shown to play a major role in virulence of B. bronchiseptica (20–24). Type III secretion in B. pertussis was recently suggested to play a role in subverting the protective innate and adaptive immunity of the host (16) and, to date, four B. pertussis proteins, BopN, BopD, BteA, and Bsp22, were found to be secreted by this machinery (16, 25). Interestingly, secretion of these proteins appears to be switched-off in laboratory-adapted strains, while it is observed in vitro for a significant portion of clinical strains that had not been repeatedly passaged (16, 25, 26). Moreover, a recent study demonstrated that even laboratory-
adapted B. pertussis strains are able to switch-on the type III secretion following contact with the host, such as during experimental infections of mice (26). The T3SS, hence, is likely to be expressed during natural infections of humans and may be contributing to bacterial virulence by subverting the host immune system, as is the case for B. bronchiseptica infections (23, 24, 27).

During growth in vitro, Bsp22 is the most abundantly secreted T3SS protein of B. bronchiseptica, and it was found to be highly immunogenic during natural infections (23, 24). Bsp22 is also absolutely required for T3SS activity in cocultures of B. bronchiseptica with host cells in vitro, where the Bsp22 protein extends from the bacterial cell surface as a filamentous T3SS tip complex that binds the translocon complex (28). Furthermore, immunization with purified recombinant Bsp22 significantly protected mice from lung colonization by B. bronchiseptica upon intranasal challenge (28). Therefore, we examined here the vaccine and diagnostic potential of a recombinant form of the B. pertussis Bsp22 antigen.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Wild-type B. pertussis 18323 and the Tohama-derived B. pertussis BPRA strain lacking the PTXstructural gene (29) were grown on Bordet-Gengou (BG) agar supplemented with 15% defibrinated sheep blood at 37°C for 72 h. For animal studies, subcultures of B. pertussis 18323 were performed in Stainer-Scholte medium (30) for 20 h at 37°C until the optical density at 650 nm (OD650) reached 1.2. For filamentous hemagglutinin (FHAA) purification, subcultures of B. pertussis BPRA were performed under the same conditions but left growing until the optical density reached 5.

**Production and purification of recombinant Bsp22.** To construct a plasmid for the expression of the recombinant Bsp22, the gene bsp22 was amplified from B. pertussis 18323 genomic DNA by PCR using oligonucleotide primers (5′-GGG CAT ATG AGC ATT GAT CTC GGA G-3′ and 5′-CCC TCG AGT TAG CGC ATG TTG CTG GT-3′) with the Ndel and Xhol restriction sites introduced at each end, respectively. The PCR product was cloned into a PET11c-derived expression vector (PET11cHis) and fused in frame to a sequence encoding a double-6 His tag. The absence of undesired mutations in the PCR-amplified DNA fragment was verified by DNA sequencing.

**E. coli BL21 (ADE3) PET11c-bsp22 cells were grown in MDO medium (yeast extract, 20 g/liter; glycerol, 20 g/liter; KH2PO4, 1 g/liter; K2HPO4, 3 g/liter; NH4Cl, 2 g/liter; Na2SO4, 0.5 g/liter; thiamine hydrochloride, 0.01 g/liter) supplemented with 150 μg of ampicillin/ml at 30°C. Bsp22 production was induced at an OD650 of 0.6 by 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) over 4 h, and the cells were harvested, resuspended in phosphate-buffered saline (PBS; 12 mM Na2HPO4 and 2 mM KH2PO4, 3 mM KCl, 132 mM NaCl [pH 7.4]), and disrupted by sonication. The cell debris was extracted with 8 M urea in PBS (PBSU). Cleared extract (20,000×g, 30 min) was loaded onto Ni-NTA agarose (Qiagen, Hilden, Germany) equilibrated with PBSU. After column washing with 8 M urea; PBSU, Bsp22 protein was eluted with 350 mM imidazole in PBSU, dialyzed against 8 M urea in 50 mM sodium acetate (pH 5.5; AcU), and loaded onto an SP-Sepharose column (GE Healthcare, Little Chalfont, United Kingdom) in AcU. After a washing step with 100 mM NaCl in AcU, the bound Bsp22 was eluted from the column with 760 mM NaCl in AcU and stored at −20°C. The purity of the eluted Bsp22 protein was analyzed by SDS-PAGE, and its identity was confirmed by mass spectrometry.

For use in immunoassays, Bsp22 was purified to homogeneity on a preparative Vydac C4 reversed-phase column (catalog no. 214TP1010; Grace, Deerfield, IL) run in 0.1% TFA with a shallow gradient of 5 to 62% of aqueous acetonitrile.

Transmission electron microscopy. The ability of recombinant Bsp22 to form the previously described filamentous structures (28) was examined by transmission electron microscopy. Bsp22 purified on Ni-NTA agarose and SP-Sepharose columns and diaлизed against PBS was applied onto a glow-discharge activated carbon coated grid (5 μl of Bsp22 at a concentration of 83 μg/ml) and adsorbed for 30 s (32). Excess of solution was blotted with filter paper, and grids were washed with 1% ammonium molybdate in double-distilled H2O (ddH2O) for 30 s. The grids were immediately negatively stained with 2% uranyl acetate in ddH2O for 30 s, before being blotted again and air dried. Samples were examined in a Philips CM100 electron microscope at 80 kV at magnification of ×64,000. Digital images were recorded using MegaViewII slow scan camera at magnification of ×64,000, giving a pixel size of ~1 nm. The recorded images were processed in AnalySis3.2 software suite using embedded modules (Shading correction and Optimize 16-bit image for 8-bit display).

**FHA and CyaA-AC− antigen purification.** FHA was purified from supernatants of B. pertussis BPRA cultures as previously described with minor modifications (33). Briefly, liquid cultures were grown to an optical density at 650 nm of ≥5 and supplemented with protease inhibitors (1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride). Solid ammonium sulfate was added to culture supernatants to a final 30% saturation, and FHA precipitate was allowed to form for 16 h at 4°C. The protein was collected at 29,600×g for 30 min at 4°C and dissolved in 1 M NaCl in 50 mM sodium phosphate (pH 8.0). FHA solution was cleared at 17,300×g for 30 min at 4°C and dialyzed against 50 mM sodium phosphate (pH 6.8) containing 2 M urea (buffer A), and the sample was applied at a flow rate of 2 ml per min onto a C10/10 column (GE Healthcare) filled with SP Sepharose (GE Healthcare). FHA was eluted with a 0 to 500 mM NaCl gradient in buffer A, supplemented with excess of polyvinyl B (370 μg/ml), and dialyzed against buffer A. The obtained FHA preparation contained ~4 endotoxin units per μg of protein, as determined with the Limulus amebocyte lysate assay (QCL-1000 kit; BioWhittaker, Walkersville, MD). Recombinant CyaA-AC− toxoid was produced as previously described (34, 35).

**Serum samples.** Human sera were obtained from 36 individuals having developed characteristic clinical symptoms of pertussis and in which the disease was confirmed serologically by standard agglutination assays run at the National Reference Laboratory for pertussis at the National Institute of Public Health in Prague in the years 2005 to 2010. Their diagnosis was confirmed for the purpose of the present study by a PT-specific enzyme-linked immunosorbent assay (ELISA) according to EU Perti strain group recommendations (36). Sera taken at different time points were available for these individuals and the average of the antibody content values for the individual sera pairs was used. A control set of sera from 20 adult healthy volunteers with no known pertussis infection was used for determination of ELISA cutoff values. The sera of mice colonized repeatedly by the PT-deficient live attenuated vaccine strain B. pertussis BPZE1 were kindly provided by Camille Locht from Institute Pasteur in Lille, France.

**Antibody level determination by ELISA.** Polysorp 96-well ELISA plates (Nunc, Denmark) were coated with purified Bsp22, FHA, or CyaA-AC− at 10 μg/ml in 50 mM sodium carbonate buffer (pH 9.6) for 16 h at 4°C. Plates were blocked for 1 h at 37°C with 0.05% Tween 20 (PBST) and 1% bovine serum albumin (BSA) in PBS (PBST-BSA) and diluted human or mouse serum samples (1:100 and 1:1,000) in PBST-BSA were added for 1 h at 37°C. Upon three washes, the reactions were revealed using horse-radish peroxidase conjugates of swine anti-human IgG (1:5,000; SEVAC, Prague, Czech Republic) or sheep anti-mouse IgG (1:5,000; GE Healthcare) and o-phenylenediamine as a substrate. Cutoff values for the determination of anti-Bsp22, anti-FHA, and anti-CyaA antibody levels were determined as means plus two standard deviations from the test results for healthy, noninfected volunteers or nonimmunized, negative mouse sera at 1:100 or 1:1,000 dilutions. Plasma levels of IgG antibodies directed against pertussis toxin (PT) were determined by using the B. pertussis
IgG-PT ELISA kit validated for clinical diagnosing of pertussis (Statens Serum Institute Diagnostica, Hillerod, Denmark) according to the manufacturer’s instructions.

Intranasal infection with *B. pertussis* 18323. Five female 4-week-old BALB/c mice (Charles Rivers Genetic Models, Inc., Indianapolis, IN) per group were infected intranasally with 2.5 × 10^5 CFU of *B. pertussis* 18323 in Stainer-Scholte medium (50 µl). Two mice received medium only, and all of the mice were bled at 6 weeks postinfection. To confirm that Bsp22 was produced during infection, *B. pertussis* was recovered from lungs of one mouse 27 days after infection, and the secretion of Bsp22 was examined using concentrated supernatants of liquid cultures by Western blotting. The experiment was performed twice, yielding identical results.

All animal experiments were approved by the Animal Welfare Committee of the Institute of Microbiology of the ASCR, Prague, Czech Republic. The handling of animals was performed according to the Guidelines for the Care and Use of Laboratory Animals, the Act of the Czech National Assembly, Collection of Laws no. 149/2004, inclusive of the amendments, on the Protection of Animals against Cruelty, and Public Notice of the Ministry of Agriculture of the Czech Republic, Collection of Laws no. 207/2004, on care and use of experimental animals.

Production and secretion of Bsp22 by *B. pertussis* 18323. To determine Bsp22 levels in whole-cell lysates, 1-ml aliquots were withdrawn, and the pellets cells were boiled in protein sample buffer. To assay for the presence of secreted Bsp22 protein, 20-ml aliquots of cell-free supernatants of *B. pertussis* cultures were filtered and precipitated with 10% (wt/vol) trichloroacetic acid. The precipitates were washed with 80% acetone, dissolved in Laemmli sample buffer and boiled. Samples equivalent to 0.1 OD_600 unit (pellets) and to 1 OD_600 unit (supernatants) were separated on SDS-PAGE gels and either stained with Coomassie brilliant blue or transferred onto nitrocellulose membrane. Membranes were probed with mouse polyclonal antibodies raised against recombinant Bsp22 protein (kindly provided by K. H. G. Mills), followed by incubation with antimouse IgG conjugated with alkaline phosphatase. The antibody-antigen complexes were visualized using NBT/BCIP staining according to standard protocols.

Mouse immunization and *B. pertussis* challenge. A total of 30 µg of Bsp22 or CyaA-AC^- antigen in 180 µl of PBS was mixed with 20 µl of 2% aluminum hydroxide hydrate (A1577; Sigma-Aldrich) and left for 30 min on ice prior to injection. Groups of 17 female 4-week-old BALB/c or CD-1 mice (Charles Rivers Genetic Models, Inc., Indianapolis, IN) were injected intraperitoneally twice at a 2-week interval. Control mice received only adjuvant. Two mice per group were bled 14 days after the last injection for the assessment of anti-Bsp22 or anti-CyaA antibodies.

For the determination of protection against bacterial colonization, BALB/c mice were challenged intranasally with 1.5 × 10^5 CFU of *B. pertussis* 18323 on day 14 after the second immunization. Three mice per group were sacrificed 2 h and 5, 8, 13, and 16 days after challenge, and lungs were aseptically removed and homogenized in saline with tissue grinders. Serial dilutions of homogenates were plated on BG agar, and the CFU were counted after 3 days at 37°C. Reisolated bacteria were subcultured in liquid Stainer-Scholte medium, and Bsp22 secretion was examined in concentrated culture supernatants by Western blotting.

For determination of the 50% lethal dose (LD_{50}) values, groups of five immunized CD-1 mice were challenged intranasally 14 days after the second immunization with series of 2 × 10^5, 4 × 10^5, and 8 × 10^5 CFU of *B. pertussis* 18323, and the LD_{50} was calculated from the day 7 survival values.

Statistical analysis. Statistical differences between the study groups were examined by using the Student t test.

**RESULTS**

*B. pertussis* infection does not elicit a serum antibody response to Bsp22. To determine whether production of Bsp22-specific serum antibodies was induced upon natural infection by *B. pertussis*, we developed an ELISA protocol for the determination of anti-Bsp22 IgG antibody levels in human sera. Toward this aim, a recombinant form of Bsp22 antigen was produced in *E. coli* as a fusion protein with an N-terminal double-6×His purification tag (31). As documented in Fig. 1A, the antigen was purified close to homogeneity by a combination of affinity chromatography on Ni-NTA agarose with cation-exchange chromatography on SP-Sepharose and reversed-phase chromatography on a C4 resin. The purified Bsp22 sample was separated by SDS-PAGE (12.5%) and stained with Coomassie blue. St, molecular mass standards. (B and C) Sera obtained from patients with confirmed whooping cough disease and from healthy volunteers (controls) were diluted 1:1,000 and examined for anti-PT IgG using a validated ELISA kit (B) or by conventional ELISA with purified Bsp22 or FHA used as coating antigens (C). PT IgG values are given as international units (IU) per ml (B), and Bsp22 and FHA IgG values are given as the absorbance at 492 nm (C). Box-whisker plots represent boxes for medians with 25th and 75th percentiles and whiskers for the 10th and 90th percentiles. Closed circles represent outliers. The statistical difference between the pertussis-infected group and the control group was calculated by using the Student t test and was shown to be significant for anti-PT antibody levels (*P* < 0.001) and anti-FHA antibody levels (*P* < 0.001) but not for anti-Bsp22 antibody levels (*P* = 0.118) compared to control sera.
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FIG 2 B. pertussis infection of mice does not elicit a serum antibody response against Bsp22. (A) BALB/c mice were intranasally infected with a sublethal dose of 2.5 × 10^5 CFU of B. pertussis 18323, and the presence of serum antibodies against Bsp22 and FHA was monitored by ELISA at 6 weeks after infection. Average values of four sera ± standard deviations were calculated from two independent experiments, where only the anti-FHA antibodies were significantly increased (P < 0.01). (Inset) B. pertussis 18323 was recovered from lungs 27 days after infection and subcultured in vitro. Concentrated culture supernatants were tested for production of Bsp22 by Western blotting with hyperimmune mouse anti-Bsp22 serum (lane S). Cultures of the laboratory-adapted strain B. pertussis 18323, having the Bsp22 (T3SS) production switched off (lane C), were used as negative control. Purified recombinant Bsp22 protein was used as a positive control (lane rBsp22) and migrated at a higher molecular mass than did natural Bsp22 because of the N-terminal double-6×His tag extension (~5 kDa). (B) Production and secretion of Bsp22 in B. pertussis 18323 cells recovered from infected mice. Whole-cell lysates (P) or concentrated supernatants (S) from cultures of B. pertussis recovered 2 h (D0) or 8 days (D8) after challenge were separated on a 12.5% SDS-polyacrylamide gel and stained with Coomassie brilliant blue (upper panel) or transferred to nitrocellulose and probed with anti-Bsp22 antisera (lower panel, only the relevant part of the membrane is shown). The supernatant fraction of a B. bronchiseptica RB50 (Bb) culture and purified recombinant Bsp22 (rBsp22) were loaded as positive controls. Position of Bsp22 protein is indicated by an asterisk. Prestained molecular mass standards (St) are shown in the left lane of the stained gel and Western blot.

36 human individuals who developed characteristic whooping cough disease symptoms and for which the diagnosis of pertussis was confirmed serologically by agglutination assays and determination of elevated serum anti-PT antibody levels according to EU Pertastrain recommendations (36). As a negative control, a set of sera from 20 healthy adult volunteers without any recent cough episode were used for determination of Bsp22 and FHA antibody cutoff levels. As documented in Fig. 1B, the anti-PT IgG antibody levels were significantly higher in the pertussis-confirmed patient sera than in the control set (P < 0.001). The latter set contained a single serum that was positive for PT antibodies, and this sample was excluded from further assays.

As shown in Fig. 1C, however, no Bsp22-specific antibodies were detected at a 1:1,000 dilution in the group of sera from patients with confirmed whooping cough disease. In contrast, when FHA was coated on plates instead of Bsp22 under otherwise identical ELISA conditions, high FHA-specific IgG antibody levels were unambiguously detected in the sera of pertussis patients. These were significantly higher (P < 0.001) than the levels of FHA-specific antibodies detected in sera from adult healthy volunteers that were vaccinated against whooping cough by a whole-cell pertussis vaccine in their infancy (Fig. 1C). It can therefore be concluded that the B. pertussis-infected individuals failed to develop any detectable serum antibody response to the Bsp22 antigen, while responding with high antibody levels to PT and FHA produced by the infectious agent.

Restoration of Bsp22 production in the course of B. pertussis infection does not elicit a serum antibody response to Bsp22. The lack of a serum antibody response to Bsp22 in B. pertussis-infected patients was rather unexpected, since Bsp22 was found to be highly immunogenic during natural infections of rabbits or rats by B. bronchiseptica (23, 24). Moreover, the highly conserved B. pertussis Bsp22 protein appears to be produced in vitro by low-passage clinical isolates (16), and the loss of Bsp22 production could be reverted upon infectious passage of laboratory-adapted B. pertussis strains on mice (26). Therefore, we tested whether anti-Bsp22 antibody response was induced upon experimental intranasal infection of mice by the B. pertussis 18323 strain that is used as a World Health Organization (WHO) reference strain in intracerebral challenge assays in pertussis vaccine testing. A dose of 2.5 × 10^5 CFU was used, which is ~2 orders of magnitude below LD50 of this strain and results in bacterial colonization of challenged animals in the absence of any obvious morbidity (K. Baslerova et al. [unpublished data]; see also the colonization curve of the control group in Fig. 3C below). As shown in Fig. 2A, whereas significantly increased levels of FHA-specific antibodies were detected in sera of infected mice compared to control mice (P < 0.01), no significant serum antibody response specific for Bsp22 could be detected at 6 weeks after B. pertussis infection. This was clearly not due to failure of the colonizing bacteria to turn on the secretion of Bsp22 while in the animal, since the unambiguous presence and selective enrichment of Bsp22 was observed in supernatants of in vitro subcultures of the bacteria isolated on day 8 from lungs of infected mice (Fig. 2B, asterisk). Moreover, Bsp22 continued to be produced also by the bacteria recovered from lungs of infected animals on day 27 (Fig. 2A, inset).

High preexisting levels of Bsp22-specific antibodies do not protect mice from B. pertussis colonization and virulence. In the light of these results, it was important to assess whether active preimmunization of animals, inducing high levels of anti-Bsp22
antibodies, would modulate the course of*B. pertussis* infection and virulence. For this purpose, we used as Bsp22 antigen a protein preparation that was purified close to homogeneity by a combination of Ni-NTA agarose and SP Sepharose chromatography. As shown in Fig. 3A by transmission electron microscopy, such purified recombinant Bsp22 protein was still able to adopt a native-like conformation and assemble into the previously described Bsp22 filaments (28).

Groups of 17 mice were intraperitoneally immunized twice at a 2-week interval with 30 μg of recombinant Bsp22 or of the recombinant*B. pertussis* adenylate cyclase toxoid (CyaA-AC−) used as a known protective antigen (37, 38) or with the aluminum hydroxide adjuvant alone. Two mice per group were bled 14 days after the second immunization, and the induced levels of antigen-specific antibodies were determined. As documented in Fig. 3B, the recombinant Bsp22 protein was highly immunogenic when used as a vaccine and induced higher serum titers of specific antibodies than CyaA-AC−. To determine whether this high Bsp22-specific immune response would confer protection against*B. pertussis* challenge, mice were intranasally infected with 1.5 × 10^8 CFU of*B. pertussis* 18323 2 weeks after the last immunization. As summarized in Fig. 3C and compared to naive control mice in which the administered bacteria proliferated and their CFU count in lungs of infected mice increased by about 2 orders of magnitude within 8 days after infection, the immunization with CyaA toxoid conferred on mice a clear protection against bacterial proliferation in the lungs. In contrast, no impact of immunization with Bsp22 on mouse lung colonization by*B. pertussis* 18323 was observed. CFU counts in the lungs of Bsp22-immunized mice increased between days 0 and 5, as in the lungs of control mice. Moreover, no significant restriction of lung colonization was observed on days 5, 8, 13, and 16 compared to the course of colonization for naive control mice in which the bacteria reisolated on days 5, 8, 13, and 16 from the lungs of the Bsp22-immunized mice still continued to secrete the antigen-like conformation and assemble into the previously described Bsp22 filaments (28).

To test whether preimmunization with Bsp22 would at least restrict the virulence of*B. pertussis* and delay the appearance of virulence determinants, a challenge strain reisolated from lungs was analyzed for the secretion of Bsp22 by Western blotting. For lane C and the rBsp22 lane, see Fig. 3E, (B) The postvaccination sera were taken from two BALB/c mice from each group immediately before challenge and individually examined for anti-Bsp22 and anti-CyaA-AC− antibody levels using ELISA, yielding identical results. The dashed line indicates the cutoff value calculated as the mean plus two standard deviations of the test results of 1:100 diluted negative sera from mice that received adjuvant alone (cutoff value for Bsp22 = 0.1; cutoff value for CyaA-AC− = 0.1). (C) At 2 weeks after the second immunization, BALB/c mice in all three groups were intranasally infected with 1.5 × 10^6 CFU of*B. pertussis* 18323 and sacrificed on days 0, 5, 8, 13, and 16, respectively. The lungs were removed and homogenized, serial dilutions of lung homogenates were plated on BG agar, and the CFU were counted 3 days later. The plots show average values ± the geometric standard deviations for three mice per time point. The results are representative of two experiments with the exception of the group immunized with CyaA-AC−, an experiment that was performed once. (D) The challenge strain reisolated from lungs was analyzed for the secretion of Bsp22 by Western blotting. For lane C and the rBsp22 lane, see the explanations in legend for Fig. 2. (E) At 2 weeks after the last immunization, CD-1 mice in all three groups were intranasally infected with 2 × 10^5 CFU/mouse of*B. pertussis* 18323, and the survival of mice was monitored for 7 days.
morbidity signs and death, mice were vaccinated as described above and challenged with various doses of \textit{B. pertussis} 18323. As shown in Fig. 3E, however, at a dose of $2 \times 10^6$ CFU/mouse, no difference in survival was observed between mice immunized with Bsp22 and the animals that received only the adjuvant. All animals in both groups succumbed to \textit{B. pertussis} infection by day 3 postinfection, with the LD$_{50}$ value found to be $6 \times 10^7$ CFU for Bsp22-immunized mice compared to $3 \times 10^7$ CFU for control mice. In contrast, immunization with the known protective antigen, CyaA-AC$^-$, resulted in the survival of 80% of the challenged mice (LD$_{50} > 2 \times 10^7$ CFU; Fig. 3E). It can therefore be concluded that, despite its high immunogenicity when used as a vaccine, recombinant Bsp22 antigen did not confer any protection on mice against an intranasal \textit{B. pertussis} challenge.

**DISCUSSION**

We show here that the T3SS tip complex protein Bsp22 does not elicit a specific serum antibody response in the course of \textit{B. pertussis} infections in humans and mice. In line with that, active immunization of mice with recombinant Bsp22 failed to induce any protective immunity against \textit{B. pertussis} challenge. This, however, is in contrast to the reported immunogenicity of Bsp22 in the course of mouse, rat, or rabbit infections by \textit{B. bronchiseptica} and to the protective efficacy of a comparable recombinant Bsp22 vaccine in the \textit{B. bronchiseptica} challenge model in mice.

Intriguingly, no Bsp22-specific antibody response was detected either in a set of sera from patients with confirmed clinical pertussis, or in the sera of mice subjected to controlled infections with the WHO reference strain \textit{B. pertussis} 18323. This is puzzling in the light of previous reports that fresh clinical isolates of \textit{B. pertussis} continue to produce Bsp22 in vitro and that the laboratory-adapted strains regain the capacity to produce Bsp22 upon infectious passage in mice (16, 26), as also shown here.

A whole array of plausible hypotheses can be proposed and would need to be tested, in order to decipher the reasons for the lack of Bsp22 immunogenicity in \textit{B. pertussis} infections in vivo. The simplest explanation would be that in contrast to \textit{B. bronchiseptica} RB50, \textit{B. pertussis} 18323 secretes importantly lower amounts of Bsp22 protein, at least under in vitro subculture conditions, as indicated here by Western blots shown in Fig. 2B. Another appealing hypothesis would be that immunosuppressive action of some coexpressed \textit{B. pertussis}-specific virulence factors, such as the pertussis toxin, might have actively and specifically suppressed the antibody response against \textit{B. pertussis}-produced Bsp22. PT is not produced during \textit{B. bronchiseptica} infections, in the course of which Bsp22 appears to induce a high serum antibody response and Carbonetti et al. have previously shown that PT action can selectively repress serum antibody responses to several \textit{B. pertussis} antigens, including an otherwise immunodominant lipoprotein of bordetelae (39). The absence of serum response to the produced Bsp22 protein, however, most likely was not due to immunosuppressive action of PT, since we did not detect any Bsp22 antibodies in the sera of mice colonized by \textit{B. pertussis} strains producing detoxified PT (R9K/E129G) or not producing PT at all (data not shown).

It remains unknown whether the B cell epitopes of Bsp22 might be shielded from recognition by the immune system by other components of the tip complex during a natural \textit{B. pertussis} infection. Alternatively, low production levels of Bsp22 in vivo may also account for its poor immunogenicity. It cannot, however, be excluded that Bsp22 production by \textit{B. pertussis} resolated from animals is selectively enhanced to detectable levels only under in vitro culture conditions. Possibly, some putative \textit{B. pertussis}-specific posttranslational modifications might also render the tip complex protein Bsp22 poorly immunogenic in vivo. The above-mentioned possibilities of poor visibility of Bsp22 to the immune system under in vivo growth conditions would be compatible with the observation that Bsp22 was still produced by bacteria resolated from lungs of mice in which high titers of Bsp22-specific serum antibodies were induced by active immunization.

A potential limitation of the present study was also the unavailability of matched pairs of sera and bacterial isolates from the same patients, which would be needed for confirmation that Bsp22 was indeed produced by the strains infecting the donors of the sera. In the light of the observation that Bsp22 secretion is switched on even in laboratory-adapted strains during infection of mice, we consider it unlikely that Bsp22 was not produced and secreted at all by the strains infecting the donors of the sera.

It should, however, be mentioned that the notion that \textit{B. bronchiseptica} Bsp22 was highly immunogenic during natural host infections, and not only upon experimental infection of mice, is based on two early studies. There, the immunoblots with antisera from different natural hosts infected with \textit{B. bronchiseptica} RB50 were able to recognize several polypeptides in protein precipitates from culture supernatants of RB50 that were considered to contain also Bsp22 (23, 24). Nevertheless, a clear band corresponding to Bsp22 detection could only be observed when sera of infected rabbits were used. There is, therefore, still no definitive evidence showing that the sera of \textit{B. bronchiseptica}-infected rats and mice truly recognize Bsp22.

In conclusion, the results reported here do not support the extrapolation of the previous report on \textit{B. bronchiseptica} Bsp22 immunogenicity and protective antigen efficacy in mice (28) to the human infections with \textit{B. pertussis}. In contrast to expectations, therefore, the Bsp22 protein does not appear to be a suitable antigen for the development of next-generation pertussis vaccines and diagnostics.

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The *Bordetella pertussis* Type III Secretion System Tip Complex Protein Bsp22 Is Not a Protective Antigen and Fails To Elicit Serum Antibody Responses during Infection of Humans and Mice

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Volume 81, no. 8, p. 2761–2767, 2013. “*Bordetella pertussis* 18323” should be replaced with “*Bordetella pertussis* Tohama I” throughout.

We recently became aware that since 2008, our laboratory has used a *Bordetella pertussis* strain that was erroneously labeled and referred to in this publication as *Bordetella pertussis* 18323. Single nucleotide polymorphism analysis and determination of the draft genome sequence of the strain performed by Dr. Harris at the Sanger Institute revealed that we have been working with the *B. pertussis* Tohama I strain. The genome sequence data for this strain (assembled sequence contigs) from the study named “Sequencing Czech *Bordetella pertussis* wP vaccine strains” can be downloaded from the ENA under study accession number ERP003836, using the following link: http://www.ebi.ac.uk/ena/data/view/SAMEA2177726.

In all future experiments and publications, we will refer to this strain as “*B. pertussis* Tohama I.” We sincerely apologize to all colleagues for any confusion that may have arisen from the unfortunate error that, however, has no effect whatsoever on the validity of the published data obtained with a reference *B. pertussis* strain and mutants derived thereof.