INFECTION AND IMMUNITY, Sept. 2011, p. 3677–3682 Vol. 79, No. 9
0019-9567/11/$12.00 doi:10.1128/IAI.00136-11
Copyright © 2011, American Society for Microbiology. All Rights Reserved.

Laboratory Adaptation of *Bordetella pertussis* Is Associated with the Loss of Type Three Secretion System Functionality▽

M. E. Gaillard, D. Bottero, C. E. Castuma, L. A. Basile, and D. Hozbor*

Instituto de Biotecnología y Biología Molecular, Departamento de Ciencias Biológicas, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CCT La Plata CONICET, Calles 47 y 115, La Plata 1900, Argentina

Received 10 February 2011/Returned for modification 15 March 2011/Accepted 21 June 2011

Although *Bordetella pertussis* contains and transcribes loci encoding type III secretion system (TTSS) homologues, expression of TTSS-associated proteins has been reported only for non-laboratory-adapted Irish clinical isolates. Here we confirm such a result for clinical isolates obtained from patients treated in Argentinean hospitals. Moreover, we demonstrate that the expression of TTSS-associated proteins is independent both of the year in which the isolate was obtained and of the types of polymorphic alleles for other virulence factors but is dependent on environmental growth conditions. Interestingly, we observed that TTSS-associated protein expression is lost after successive *in vitro* passages but becomes operative again when bacteria come into contact with the host. This *in vivo* activation of TTSS expression was observed not only for clinical isolates previously adapted to the laboratory after successive *in vitro* passages but also for vaccine strains that did not express the system *in vitro*. The reversibility of TTSS expression, demonstrated by its switching off-on when the bacterium comes into contact with the host, appears to be an adaptive response of this pathogen.

Many bacteria can modulate host cell traits not only by secreting proteins into the extracellular environment but also by translocating them directly into the interiors of host cells. One of the most widespread means for translocating bacterial proteins into host cells is the type III secretion system (TTSS). This system, composed of 20 to 25 proteins, is responsible for the transport of bacterial proteins that act as powerful "effectors" and take control of host cells by hijacking their intracellular machinery (18). The TTSS has been identified in many animal pathogens, such as *Yersinia* spp., *Salmonella* spp., *Shigella* spp., enteropathogenic and enterohemorrhagic *Escherichia coli* (e.g., O157:H7), *Pseudomonas aeruginosa*, *Vibrio parahaemolyticus*, *Bordetella* spp., and *Chlamydia* spp. (6, 16, 21, 38). Certain bacteria, such as *Salmonella enterica* serovar Typhimurium, *Yersinia pestis*, and *Yersinia enterocolitica*, encode more than one TTSS (6, 16). The distribution of the TTSS is not limited exclusively to animal pathogens; it has also been identified in plant pathogens and certain endosymbiotic bacteria (22, 40, 42).

Although the sequences of many components of the TTSS apparatus used to transport effectors are conserved among bacteria, and some of these components are also functionally interchangeable, the target cells respond to this kind of "injection" in different ways depending on the system involved (5, 7, 8, 17).

It is noteworthy that the effector proteins of the TTSS not only can be translocated but also can be secreted into the extracellular medium under appropriate laboratory conditions. For example, the Ysc TTSS, one of the two TTSSs of *Yersinia*, can secrete effector proteins into the extracellular environment when bacteria are grown at 37°C in a medium containing a low concentration of calcium (29). *Shigella* can also secrete effector proteins when it is grown in the presence of the dye Congo red (33). Interestingly, in the case of *Bordetella pertussis*, a pathogen that causes a resurgent respiratory disease called whooping cough or pertussis, the conditions for secretion by the TTSS seem to differ between the circulating clinical bacterial population and laboratory-adapted strains (12). That is, *in vitro* expression of one of the known TTSS effector proteins, Bsp22, was detected in a significant proportion of Irish clinical isolates but not in common laboratory-adapted strains of *B. pertussis*. In parallel, by a comparative proteomic strategy, we had already obtained evidence showing that a TTSS was expressed in one Argentinean clinical isolate but not in a Tohama phase I strain (1). These findings are important not only because they show new aspects of the regulation of TTSS expression but also because they represent the first data on the functionality of the system in *B. pertussis*. Although the TTSS gene cluster has been found, and designated the *bsc* locus (11, 45), in three species of the *Bordetella* genus, *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, the functionality of the system has been described only for *B. bronchiseptica* (27). Through this system *B. bronchiseptica* secretes Bsp22 as the most abundantly expressed TTSS protein during *in vitro* growth. Bsp22 is highly immunogenic and has the ability to confer protection against *B. bronchiseptica* infection (28). This protein also appears to play a role in mediating persistent infection of the lower respiratory tract and is required for TTSS-mediated cytotoxicity toward eukaryotic cells (28, 44).

The recently demonstrated expression of TTSS proteins in *B. pertussis* clinical isolates but not in strains adapted to laboratory culture conditions (12), such as vaccine strains, is an interesting phenomenon, which should be more thoroughly analyzed not only in the context of *B. pertussis* evolution *in vitro* but also in the context of the reemergence of the disease. Thus, we analyze here whether the findings of Fennelly et al. (12) can

* Corresponding author. Mailing address: Instituto de Biotecnología y Biología Molecular, Facultad de Ciencias Exactas, Universidad Nacional de La Plata, CCT La Plata CONICET, Calle 47 y 115, La Plata 1900, Argentina. Phone: 54 221 4229777. Fax: 54 221 4226947. E-mail: hozbor@biol.unlp.edu.ar.

▽ Published ahead of print on 5 July 2011.

Downloaded from http://iai.asm.org/ on October 9, 2019 by guest

TABLE 1. *Bordetella* strains used in this study

Strain/clinical isolate	Yr of isolation	*prn*	*ptx*	Origin	Reference
*Bb*9.73				Collection de l'Institut Pasteur	26a
*Bb*9.73 Δ*bvgA*				Argentine	13
*Bp*18323	1947	*prn6*	*ptxS1E*	United States	33a
Tohama I	1954	*prn1*	*ptxS1C*	Japan	24
*Bp*10536		*prn1*	*ptxS1A*	United States	34
*Bp*509	1950	*prn7*	*ptxS1D*	Netherlands	18a
*Bp*6901	1969	*prn1*	*ptxS1A*	Argentina	1
*Bp*8201	1982	*prn1*	*ptxS1A*	Argentina	1
*Bp*005	2000	*prn2*	*ptxS1A*	Argentina	1
*Bp*006	2000	*prn2*	*ptxS1A*	Argentina	1
*Bp*007	2000	*prn1*	*ptxS1A*	Argentina	1
*Bp*102	2001	*prn1*	*ptxS1A*	Argentina	1
*Bp*106	2001	*prn2*	*ptxS1A*	Argentina	1
*Bp*204	2002	*prn2*	*ptxS1A*	Argentina	1
*Bp*301	2003	*prn2*	*ptxS1A*	Argentina	1
*Bp*305	2003	*prn2*	*ptxS1A*	Argentina	1
*Bp*314	2003	*prn1*	*ptxS1A*	Argentina	1
*Bp*407	2004	*prn2*	*ptxS1A*	Argentina	1
*Bp*506	2005	*prn2*	*ptxS1A*	Argentina	This study
*Bp*610	2006	*prn2*	*ptxS1A*	Argentina	This study
*Bp*708	2007	*prn2*	*ptxS1A*	Argentina	This study

also be extended to other, geographically unrelated clinical isolates and to those with different genotypes. Moreover, we evaluate the hypotheses proposing that the differential expression of the TTSS between vaccine strains and clinical isolates relies on bacterial adaptation to *in vitro* growing conditions. Both aspects were addressed by performing assays comparing reference and vaccine strains with clinical isolates from Argentina exhibiting different allelic variants of pertactin (Prn) and pertussis toxin (Ptx).

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Bordetella* strains and clinical isolates used in this study are listed in Table 1. *B. pertussis* clinical isolates were recovered from the nasopharyngeal aspirates of infants with whooping cough. Clinical samples were collected in Argentina during the years 1969 to 2007. Strains and isolates from glycerol stocks were cultured on Bordet-Gengou agar (BGA; Difco) supplemented with 1% glycerol and 10% (vol/vol) defibrinated sheep blood and were incubated at 36°C for 3 days. Then, from BGA hemolytic colonies (a marker of the virulent state of the bacteria), bacteria were replated in the same medium for 24 h in order to obtain *B. pertussis* biomass retaining virulent characteristics. To obtain supernatant proteins, subcultures were grown in Stainer-Scholte (SS) liquid medium (34). Mutants were selected on BGA or SS medium supplemented with gentamicin (Gm; 10 μg ml^{-1}) and kanamycin (Km; 75 μg ml^{-1}). To modulate virulence factor expression, bacteria were grown in SS medium supplemented with 40 mM $MgSO_4$ at 36°C.

For passage experiments, strains were grown at 36°C on BGA with 10% defibrinated sheep blood until individual colonies were well-defined, and a single colony was streaked onto a fresh plate.

Escherichia coli strains TOP10 and BL21(DE3) (both from Invitrogen) were cultured in Luria-Bertani (LB) medium supplemented, when appropriate, with ampicillin (Ap) and Km, at final concentrations of 100 and 50 μg/ml, respectively.

PCR amplification, cloning, and expression of recombinant His_6-Bsp22. Primers *bsp22*F (5′-CACCATGAGCATTGATCTCG-3′) and *bsp22*R (5′-TTGCTC CTGCATGGAATACT-3′) were used to amplify the open reading frame (ORF) of Bsp22 from *B. pertussis* Tohama I. The PCR assay was conducted in a final volume of 20 μl with 0.5 U of Platinum *Pfx* polymerase (Invitrogen). The amplified product of 727 bp was cloned first into the pENTR/SD/D-TOPO entry vector and then into the Gateway pDEST17 destination vector according to the manufacturer's instructions (Invitrogen). The recombinant clones were confirmed by PCR and were sequenced. The pDEST17:*bsp22* vector was transformed first into TOP10 competent *E. coli* cells and then into BL21 Star (DE3) host expression cells. For Bsp22 expression, a selected clone was grown in LB medium containing ampicillin (100 μg ml^{-1}). When the cell culture reached an optical density at 600 nm (OD_{600}) of ~0.6, protein expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM, and incubation was continued for another 3 h. *E. coli* cells were harvested and resuspended in Laemmli buffer (26), and the total proteins in soluble whole lysates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis was performed at room temperature and constant voltage. Molecular weight was estimated by means of the GE calibration kit, and proteins resolved on gels were visualized by using a Coomassie blue staining method.

Purification of recombinant His_6-Bsp22. For His_6-Bsp22 purification, recombinant *E. coli* DE3 cells obtained from 100 ml of IPTG-induced cultures were resuspended in 4 to 8 ml of denaturing lysis buffer (Invitrogen). The recombinant protein was solubilized, and bacterial cells were disrupted by sonication (30-s pulses with 30-s intervals for 5 min). After centrifugation, the resultant supernatant was loaded onto Ni-nitrilotriacetic acid (NTA) columns (Invitrogen) equilibrated in denaturing lysis buffer (Invitrogen). The recombinant protein was purified under denaturing conditions (buffers used contained 8 M urea at different pH values ranging from 7.9 to 4.0). The purification steps were performed according to the manufacturer's protocols. Fractions containing recombinant His_6-Bsp22 were pooled and concentrated 16-fold using an Amicon Ultra-4 10K centrifugal filter device (Millipore). The final product was judged to be pure by SDS-PAGE. Fractions containing purified protein were dialyzed at 4°C against phosphate-buffered saline (PBS) to remove urea from the preparation and were stored at −20°C until use. The identity of the expressed protein and its molecular weight were confirmed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry. The protein concentration of purified His_6-Bsp22 was determined by the Bradford method, with bovine serum albumin as a standard (3).

Production of a polyclonal serum against His_6-Bsp22. To obtain specific sera against His_6-Bsp22, 3 mice obtained from the Instituto Biológico Argentino (Biol. SAIC, Argentina) were immunized intraperitoneally twice, at intervals of 14 days, with 5 μg of the purified recombinant protein each time. $Al(OH)_3$ was used as an adjuvant. Fourteen days after the last immunization, mice were bled, and the serum was prepared in order to assess the presence of specific circulating antibodies against His_6-Bsp22 by immunoblotting.

***B. pertussis* Δ*bsp22* mutant strain construction.** Gentamicin-resistant Δ*bsp22* derivatives of *B. pertussis Bp*106 (*Bp*106 Δ*bsp*22) were constructed as follows. Two single-stranded oligonucleotide primers were designed on the basis of the *bsp*22 sequence: $bsp22_{RT}$F (5′-AACTCCTCACGGCTCAAATG-3′) and $bsp22_{RT}$R (5′-ACGGTGGTGTAGGCACTTTC-3′). Using these primers and *Taq* DNA polymerase (0.5 U), we accordingly amplified a 346-bp fragment corresponding to an internal sequence of the *bsp22* gene by PCR. This PCR product was cloned into the shuttle plasmid pGEM-T Easy (Ap^r *lacZ*). After ligation, a recombinant fragment from this plasmid was released with EcoRI and was cloned into the EcoRI site of the *Bordetella* suicide plasmid pG18mob (Gm^r) (25). The recombinant suicide plasmid pG18mob:*bsp*22 was transformed into electrocompetent *Bp*106 cells, which were thereafter selected for single genetic crossovers on BGA supplemented with 10 μg/ml gentamicin. A gentamicin-resistant single recombinant of *B. pertussis Bp*106 was screened by PCR using M13 and the bsp_{RT22}F/bsp_{RT22}R primers for the presence of the expected DNA structure and was also analyzed by immunoblotting for Bsp22 expression.

RNA isolation and RT-qPCR. Total RNA from mid-log-phase bacterial cultures was isolated using a commercial RNAspin Mini RNA isolation kit (GE Healthcare) according to the manufacturer's protocols. Reverse transcription-quantitative PCR (RT-qPCR) was performed using 300 ng of total RNA and Moloney murine leukemia virus reverse transcriptase (Promega). The resulting cDNA was amplified in triplicate using a SYBR green PCR assay (Bio-Rad Laboratories, Hercules, CA), and products were detected on an iQ5 cycler (Bio-Rad). PCR samples were incubated for 4 min at 95°C, followed by 40 amplification cycles with 30 s of annealing/extension at 60°C and 15 s of denaturation at 95°C. For analysis of *bscN* and *bscC* gene transcription, the following primers were used: *bscN*F (5′-CATGCCCACAAGTTCTATCC-3′)/*bscN*R (5′-GACCGAGTCCATCAGAAAAA-3′) and *bscC*F (5′-CCTTGATCGTGCAGG ATATT-3′)/*bscC*R (5′-GGGTGCGAATGTAGAAGGTA-3′). For analysis of *bsp*22 gene transcription, the bsp_{RT22}F and bsp_{RT22}R primers, described above, were used. PCR specificity was checked by melting curves. *recA* transcription was used as a normalizer with primers *rec*AF (5′-AAGACCACGCTCACGCTGCA GGTCA-3′) and *rec*AR (5′-ATCAGCAGGTCGGTCAGGTTGACGC-3′). The relative mRNA expression levels of each gene in each sample were calculated using the comparative threshold cycle (C_T) method (35). The categorical variables were compared by using one-way analysis of variance with a Tukey posttest. A P value of <0.05 was considered statistically significant.

Downloaded from http://iai.asm.org/ on October 9, 2019 by guest

Immunoblot analysis. *B. pertussis* grown on BGA or SS liquid medium was used for the different preparations. The whole-cell lysate was prepared as described previously (14). To analyze supernatant fractions of *Bordetella* spp., bacteria were grown in SS medium in 100 ml of culture inoculated at a starting OD_{650} of 0.20. Cultures were harvested for each bacterial strain at the same stage of bacterial growth as determined by OD_{650} measurements. Cell-free supernatant fractions obtained after filtering through a 0.22-μm-pore-size polycarbonate filter were precipitated with 10% (wt/vol) trichloroacetic acid at 4°C for 4 h and were then resuspended in sample buffer. *Bordetella* sp. whole-cell lysates and supernatants were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P; Millipore). For this purpose, SDS-PAGE was performed according to the method of Laemmli (26) using denaturing 15% (wt/vol) SDS-polyacrylamide gels. Proteins separated by SDS-PAGE were transferred to a polyvinylidene difluoride membrane (Immobilon P; Millipore) at a constant voltage (100 V; 1 h) and were then probed either with a polyclonal anti-Bsp22 antibody or with a polyclonal anti-PtxS1 antibody (diluted 1:500), followed by incubation with anti-mouse IgG conjugated with alkaline phosphatase (AP) at a 1:1,000 dilution. Nitroblue tetrazolium (NBT)–5-bromo-4-chloro-3-indolylphosphate (BCIP) was used as the AP substrate according to the manufacturer's protocol (Promega).

i.n. infection of mice with *B. pertussis*. Female BALB/c mice, 3 to 4 weeks of age, obtained from the Instituto Biológico Argentino (Biol. SAIC, Argentina) were infected intranasally (i.n.) with 10^7 CFU of *B. pertussis* strain Tohama I, the *Bp*106 clinical isolate, or its laboratory-adapted derivative strain (*Bp*106 after 35 passages) in 50 μl of a PBS suspension. Bacteria from glycerol stocks were cultured on BGA (Difco) supplemented with 1% glycerol and 10% (vol/vol) defibrinated sheep blood and were incubated at 36°C for 3 days. Then the bacteria were replated in the same medium for 24 h, resuspended, and adjusted to approximately 10^8 CFU ml^{-1} in phosphate-buffered saline. Infections were performed by pipetting the inoculum down into the nostrils. Animals were sacrificed 8 days after infection, and lungs were excised for bacterial recovery as described previously (1). The expression of Bsp22 in recovered bacteria was evaluated by immunoblotting. At least two independent experiments were performed.

RESULTS AND DISCUSSION

The reemergence of the disease called pertussis has been attributed mainly to waning vaccine-induced immunity and pathogen adaptation (31). Pathogen adaptation is supported by the antigenic divergence observed between vaccine strains and clinical isolates with respect to several vaccine components, such as pertussis toxin (Ptx) and pertactin (Prn) (10, 14, 23, 30, 31, 39). In addition to antigenic variation, increased Ptx production has been associated with the resurgence of pertussis. Strains with a novel allele for the *ptx* promoter (*ptxP3*) emerged in the 1990s, replacing the resident *ptxP1* strains (32). Sporadically, isolates obtained from pertussis patients do not produce Prn or Ptx (2).

An interesting recent report demonstrates the secretion of the *Bordetella* TTSS effector, Bsp22, by a significant number of Irish *B. pertussis* clinical isolates but not by common laboratory-adapted vaccine strains, such as Tohama I (12). Despite reports describing the transcription of genes encoding components of the *Bordetella bsc* TTSS machinery in *B. pertussis* Tohama I (9, 20, 27), studies performed before that of Fennelly et al. (12) have failed to demonstrate TTSS effector secretion by *B. pertussis* either *in vitro* or *in vivo* (20, 27). Fennelly et al. suggested, but did not demonstrate, that *B. pertussis* might lose the capacity to express TTSS proteins after prolonged *in vitro* culture (12). In this work, we extended the analysis of TTSS protein expression to Argentinean clinical isolates and evaluated the previous hypothesis by performing successive *in vitro* cultures and *in vivo* infection assays.

As a first step, we corroborated by quantitative PCR that a laboratory-adapted vaccine strain (strain Tohama I) and a clinical isolate (*Bp*106) representative of our bacterial collection transcribe differential levels of mRNA for TTSS-associated genes. In particular, we evaluated *bscN*, *bscC*, and *bsp22* mRNA levels. Although both strains transcribed the three genes analyzed, *Bp*106 showed higher mRNA levels than Tohama I, and these differences were most pronounced for the *bsp22* gene (Fig. 1). Taking these results into account, we decided to measure the expression of the TTSS in clinical isolates by using the effector protein Bsp22 as a marker. To this end, we performed immunoblot assays using a specific serum, obtained from the recombinant His_6-Bsp22 protein constructed here, whose identity was confirmed by MALDI-TOF spectrometry. The specificity of the anti-Bsp22 serum against the characteristic epitopes of wild-type Bsp22 protein and not against the recombinant protein was evaluated through immunoblot assays using antigens derived from *B. bronchiseptica*. In particular, we used the cell-free supernatant proteins from *B. bronchiseptica* CIP 9.73 (*Bb*9.73) and its derivative BvgA-defective mutant (*Bb*9.73 Δ*bvg*A), which is fixed in the well-known phenotypic avirulent state (13). This mutant was employed for the assay because it had already been demonstrated that TTSS is not expressed when the bacterium is in the avirulent phase (27). We also included in the experiments the supernatant proteins from the *Bp*106 clinical isolate and its derivative Bsp22-defective mutant (*Bp*106 Δ*bsp22*), obtained in this work by site-specific insertional mutagenesis (Fig. 2). As shown in Fig. 2, the anti-Bsp22 serum recognized a band with the expected molecular size for Bsp22 in the parental *Bb*9.73 and *Bp*106 strains but not in either defective mutant (*Bb*9.73 Δ*bvg*A or *Bp*106 Δ*bsp*22).

For immunoblotting using the specific anti-Bsp22 polyclonal serum, cell-free supernatant proteins from 15 selected *B. pertussis* clinical isolates in our collection with different genotypic characteristics, obtained during the years 1969 to 2007, were used (Table 1). As shown in Fig. 3, the specific serum reacted against a protein with the expected molecular size of 22 kDa in all clinical isolates assayed, independently of the year of isolation or genotypic characteristics. As expected, this Bsp22-specific antiserum, which recognized the presence of Bsp22 in *B. bronchiseptica*, did not react with samples obtained from ref-

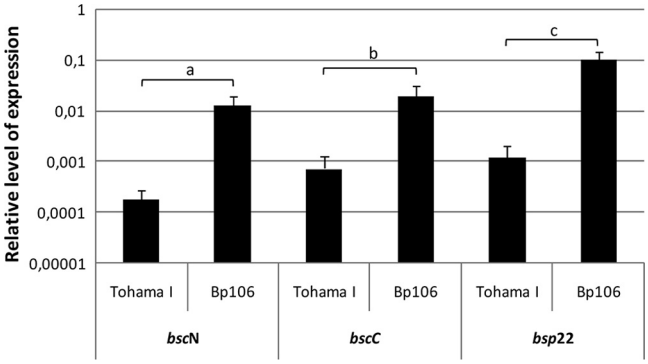

FIG. 1. Quantitative PCR analysis of *bscN*, *bscC*, and *bsp*22 transcript levels in the vaccine strain Tohama I and the clinical isolate *Bp*106. cDNA obtained from total RNA of Tohama I and *Bp*106 was used as a template for PCR. The *recA* transcript was used as a reference. Comparisons of Tohama I with *Bp*106 were performed for each gene evaluated; *P* values were <0.05 (a and b) and <0.01 (c).

Downloaded from http://iai.asm.org/ on October 9, 2019 by guest

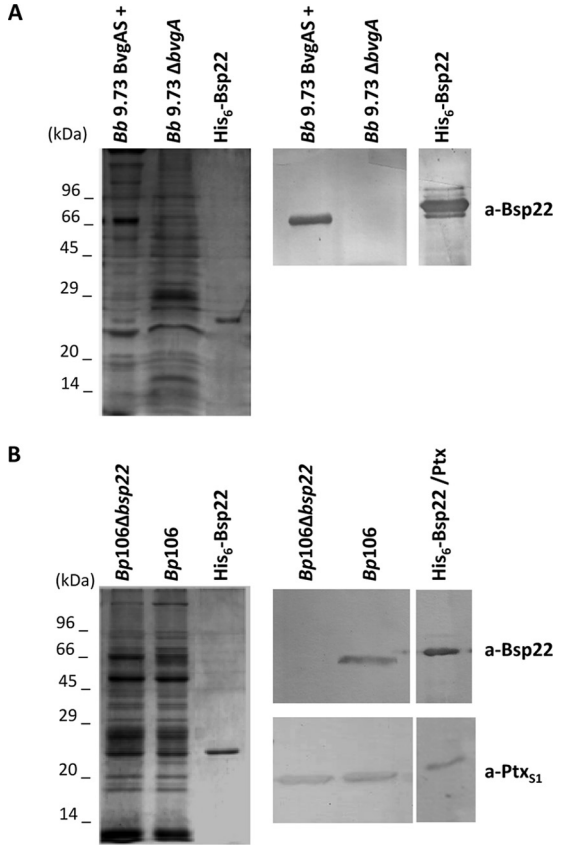

FIG. 2. Reactivities of an anti-Bsp22 serum against supernatant proteins from *B. bronchiseptica* and *B. pertussis* strains. Proteins from *B. bronchiseptica* 9.73 grown under Bvg^+ conditions (*Bb*9.73 $BvgAS^+$) and from its avirulent constitutive derivative mutant (*Bb*9.73 Δ*bvgA*) (A) and from a Bsp22-defective mutant of *B. pertussis* *Bp*106 (*Bp*106 Δ*bsp22*) and its parental strain *Bp*106 (B) were resolved by 15% (wt/vol) SDS-PAGE (left) and were probed with the indicated sera (right). Purified His_6-Bsp22 and Ptx were used as positive controls.

erence strains, such as Tohama I or *Bp*18323 (the WHO reference strain), or vaccine strains, such as *Bp*10536 and *Bp*509 (Fig. 3). To assess whether the expression of the system in these isolates was lost after they were adapted to *in vitro* culture conditions, we selected a subset of isolates (*Bp*106, *Bp*6901, and Tohama I) to be subjected to 35 successive passages *in vitro*. In these bacteria, TTSS expression was again assessed by immunoblot assays for the Bsp22 protein (Fig. 4). Interestingly, while all the bacteria studied expressed Ptx, TTSS expression was observed only in clinical isolates recently obtained from the host, not in long-term laboratory-adapted cultures (Fig. 4A). In fact, TTSS expression was already lost at the 5th *in vitro* passage (Fig. 4B). In contrast, strain Tohama I, which has been extensively grown through laboratory passages since its isolation in Japan in 1954 (24), did not express Bsp22 protein in any of the culture steps assessed. To establish precisely in which passage the expression of Bsp22 in *Bp*106 is lost, we performed a new immunoblot assay for passages 1 to 5 (Fig. 5). As can be seen in Fig. 5, Bsp22 expression stops between passages 3 and 4. As a control for all immunoblot assays performed, we included Bsp22 recombinant protein obtained from an engineered *E. coli* strain constructed by us.

These results demonstrating the inability of *in vitro* culture-adapted bacteria to express the marker Bsp22 raise the question of whether these laboratory species, including the vaccine strain Tohama I, are able to reexpress the TTSS effector Bsp22 when they regain contact with the host. To check this phenomenon, mice were infected with laboratory-adapted or non-adapted bacteria and were sacrificed 8 days after infection in order to test the abilities of the microorganisms recovered from their lungs to express Bsp22. Immunoblot assays using the Bsp22-specific antiserum showed that both laboratory-adapted clinical isolates and the vaccine strain Tohama I were now able to express proteins of the TTSS system (Fig. 6).

Taken together, the results presented here not only confirm that the expression of TTSS is a feature of natural *B. pertussis*

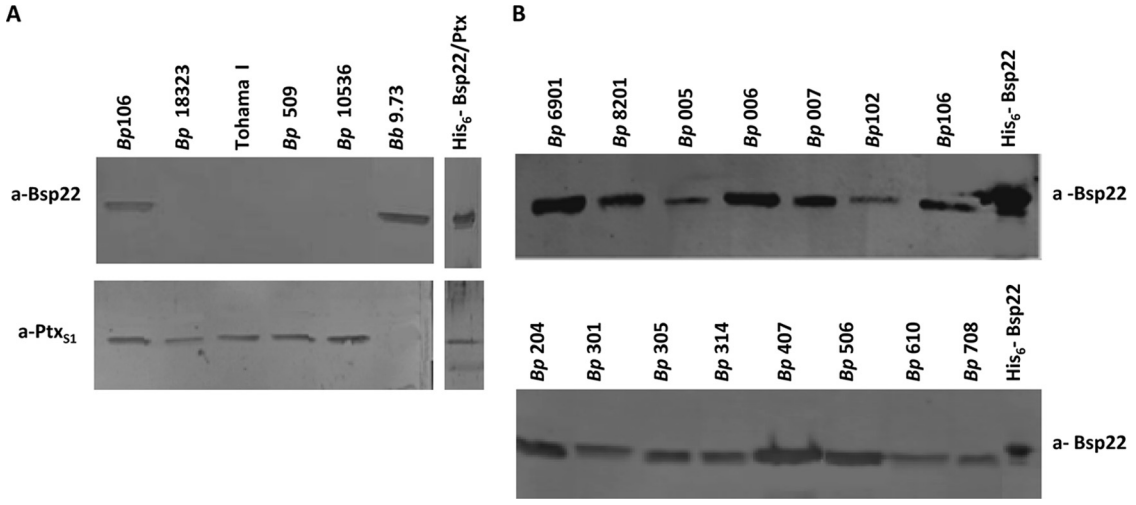

FIG. 3. Immunoblot analysis of supernatant proteins from Argentinean *B. pertussis* clinical isolates and laboratory-adapted strains. Proteins from reference and vaccine strains (A) and from clinical isolates (B) were resolved by 15% (wt/vol) SDS-PAGE, transferred to a PVDF membrane, and probed with a polyclonal serum specific for Bsp22. Samples in panel A were also probed with a polyclonal antiserum specific for subunit S1 of pertussis toxin (Ptx_{S1}). Positive controls were purified recombinant His_6-Bsp22 and Ptx_{S1} proteins, as well as samples obtained from *B. bronchiseptica* strain 9.73 (*Bb*9.73) and the clinical isolate *Bp*106.

Downloaded from http://iai.asm.org/ on October 9, 2019 by guest

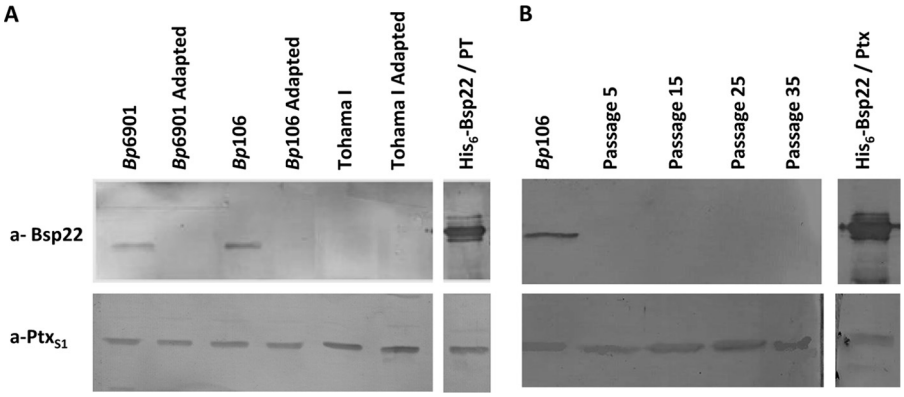

FIG. 4. Immunoblot analysis of laboratory-adapted *B. pertussis* isolates/strains for TTSS expression. (A) Supernatant proteins of *B. pertussis* strains *Bp*6901, *Bp*106, and Tohama I with their derivatives obtained after 35 successive *in vitro* passages (Adapted). (B) TTSS expression through 35 *in vitro* passages of *Bp*106. The sera used in immunoblotting are indicated on the left.

infection but also demonstrate the reversibility of the expression system depending on whether the bacteria do or do not come into contact with the host. Even though this work was not intended to elucidate the molecular mechanism of the phenomenon demonstrated, some evidence indicates that *B. pertussis*, a pathogen with little gene loss or gain, can generate biodiversity by rearranging its chromosome and altering gene expression (4, 36, 37). For example, it has been reported recently (19) that an IS*481* insertion could cause the differential expression of the TTSS cytotoxic effector protein BteA in clinical isolates of *B. pertussis*. This protein is expressed at higher levels in *B. pertussis* non-vaccine-type strains than in vaccine-type strains. This type-dependent expression suggests that the increased BteA protein level might play a key role in the type shift of *B. pertussis* (19). It would be interesting to analyze if this mechanism could be involved in the expression switch of the TTSS between *in vivo* and *in vitro* conditions. Independently of the mechanism involved, the findings of this paper represent concrete evidence for an intuitively expected feature that has been described in other pathogens (15, 41, 43): differential protein expression between bacteria grown under *in vitro* versus *in vivo* conditions. However, the outcome of our experiments is particularly relevant for the pertussis scenario, where the well-documented genomic divergence between vaccine strains (adapted to the laboratory) and bacteria recently isolated from the host (clinical isolates) might render control of the disease difficult. The *in vivo*/*in vitro* differences reported here add a new layer of complexity that should be taken into account in designing novel formulations, since current vaccines, based on strains subcultured extensively *in vitro*, might not reflect the real protein profile of the pathogen under *in vivo* conditions.

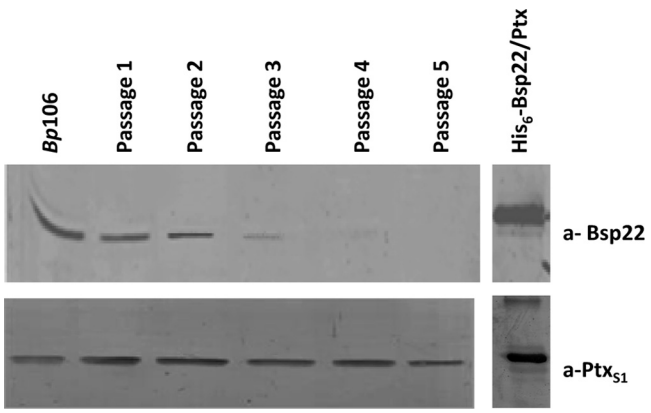

FIG. 5. Immunoblot analysis of TTSS expression through 5 *in vitro* passages of *Bp*106. Supernatant proteins of *B. pertussis* *Bp*106 with their derivatives obtained after 5 successive *in vitro* passages were resolved by 15% (wt/vol) SDS-PAGE, transferred to a PVDF membrane, and probed with a polyclonal serum specific for Bsp22. Samples were also probed with a polyclonal antiserum specific for subunit S1 of pertussis toxin (Ptx_{S1}). Purified recombinant His_6-Bsp22 and Ptx_{S1} proteins were included in this assay as positive controls.

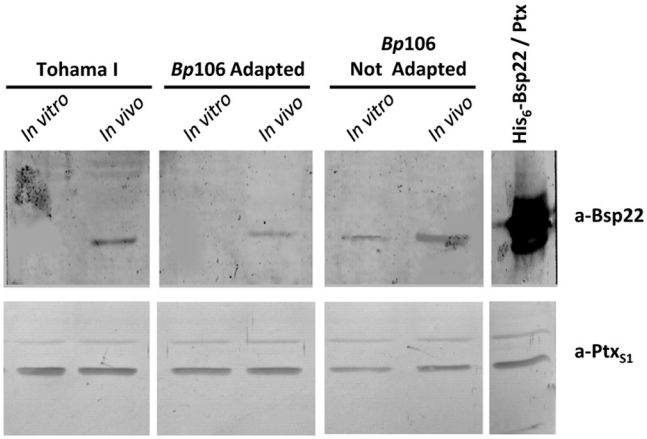

FIG. 6. Analysis of TTSS expression *in vivo*. Protein samples obtained from *B. pertussis* supernatant cultures (*in vitro*) or from whole-cell bacteria recovered from infected mice (*in vivo*) were analyzed for Bsp22 expression by immunoblotting. Samples obtained from Tohama I, *Bp*106 (*Bp*106 Not Adapted), and isolate *Bp*106 subjected to 35 *in vitro* passages (*Bp*106 Adapted) were probed with a polyclonal antiserum specific for Bsp22 or subunit S1 of pertussis toxin (Ptx_{S1}). Purified His_6-Bsp22 and Ptx were used as positive controls.

Downloaded from http://iai.asm.org/ on October 9, 2019 by guest

ACKNOWLEDGMENTS

This work was supported by ANCPyT and CICBA (Argentina) grants to D.H. D.H. is a member of the Scientific Career of CICBA. M.E.G. and D.B. have fellowships from CONICET; C.E.C. is a researcher at the Facultad de Ciencias Exactas (Universidad Nacional de La Plata); and L.A.B. has a fellowship from ANCPyT.

REFERENCES

1. **Bottero, D., et al.** 2007. Pulsed-field gel electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical *Bordetella pertussis* strains. Clin. Vaccine Immunol. **14:**1490–1498.
2. **Bouchez, V., et al.** 2009. First report and detailed characterization of *B. pertussis* isolates not expressing pertussis toxin or pertactin. Vaccine **27:**6034–6041.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
4. **Brinig, M. M., et al.** 2006. Significant gene order and expression differences in *Bordetella pertussis* despite limited gene content variation. J. Bacteriol. **188:**2375–2382.
5. **Büttner, D., and U. Bonas.** 2002. Port of entry—the type III secretion translocon. Trends Microbiol. **10:**186–192.
6. **Coburn, B., I. Sekirov, and B. B. Finlay.** 2007. Type III secretion systems and disease. Clin. Microbiol. Rev. **20:**535–549.
7. **Cornelis, G. R.** 1998. The *Yersinia* deadly kiss. J. Bacteriol. **180:**5495–5504.
8. **Cornelis, G. R., and F. Van Gijsegem.** 2000. Assembly and function of type III secretory systems. Annu. Rev. Microbiol. **54:**735–774.
9. **Cummings, C. A., H. J. Bootsma, D. A. Relman, and J. F. Miller.** 2006. Species- and strain-specific control of a complex, flexible regulon by *Bordetella* BvgAS. J. Bacteriol. **188:**1775–1785.
10. **de Greeff, S. C., et al.** 2010. Seroprevalence of pertussis in The Netherlands: evidence for increased circulation of *Bordetella pertussis*. PLoS One **5:**e14183.
11. **Fauconnier, A., et al.** 2001. Characterization of the type III secretion locus of *Bordetella pertussis*. Int. J. Med. Microbiol. **290:**693–705.
12. **Fennelly, N. K., et al.** 2008. *Bordetella pertussis* expresses a functional type III secretion system that subverts protective innate and adaptive immune responses. Infect. Immun. **76:**1257–1266.
13. **Fernández, J., F. Sisti, D. Bottero, M. E. Gaillard, and D. Hozbor.** 2005. Constitutive expression of *bvgR*-repressed factors is not detrimental to the *Bordetella bronchiseptica*-host interaction. Res. Microbiol. **156:**843–850.
14. **Fingermann, M., et al.** 2006. Differences of circulating *Bordetella pertussis* population in Argentina from the strain used in vaccine production. Vaccine **24:**3513–3521.
15. **Fux, C. A., M. Shirtliff, P. Stoodley, and J. W. Costerton.** 2005. Can laboratory reference strains mirror "real-world" pathogenesis? Trends Microbiol. **13:**58–63.
16. **Galán, J. E.** 2001. *Salmonella* interactions with host cells: type III secretion at work. Annu. Rev. Cell Dev. Biol. **17:**53–86.
17. **Galán, J. E., and A. Collmer.** 1999. Type III secretion machines: bacterial devices for protein delivery into host cells. Science **284:**1322–1328.
18. **Ghosh, P.** 2004. Process of protein transport by the type III secretion system. Microbiol. Mol. Biol. Rev. **68:**771–795.

18a. **Hamstra, H. J., B. Kuipers, D. Schijf-Evers, H. G. Loggen, and J. T. Poolman.** 1995. The purification and protective capacity of Bordetella pertussis outer membrane proteins. Vaccine **13:**747–752.

19. **Han, H. J., A. Kuwae, A. Abe, Y. Arakawa, and K. Kamachi.** 2011. Differential expression of type III effector BteA protein due to IS481 insertion in *Bordetella pertussis*. PLoS One **6:**e17797.
20. **Hot, D., et al.** 2003. Differential modulation of *Bordetella pertussis* virulence genes as evidenced by DNA microarray analysis. Mol. Genet. Genomics **269:**475–486.
21. **Hsia, R. C., Y. Pannekoek, E. Ingerowski, and P. M. Bavoil.** 1997. Type III secretion genes identify a putative virulence locus of *Chlamydia*. Mol. Microbiol. **25:**351–359.
22. **Hueck, C. J.** 1998. Type III protein secretion systems in bacterial pathogens of animals and plants. Microbiol. Mol. Biol. Rev. **62:**379–433.
23. **Kallonen, T., and Q. He.** 2009. *Bordetella pertussis* strain variation and evolution postvaccination. Expert Rev. Vaccines **8:**863–875.
24. **Kasuga, T., Y. Nakase, K. Ukishima, and K. Takatsu.** 1954. Studies on *Haemophilus pertussis*. V. Relation between the phase of bacilli and the progress of the whooping-cough. Kitasato Arch. Exp. Med. **27:**57–62.
25. **Kirchner, O., and A. Tauch.** 2003. Tools for genetic engineering in the amino acid-producing bacterium *Corynebacterium glutamicum*. J. Biotechnol. **104:**287–299.
26. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.

26a. **Le Blay, K., P. Gueirard, N. Guiso, and R. Chaloy.** 1997. Antigenic polymorphism of the lipopolysaccharides from human and animal isolates of Bordetella bronchiseptica. Microbiology **143:**1433–1441.

27. **Mattoo, S., M. H. Yuk, L. L. Huang, and J. F. Miller.** 2004. Regulation of type III secretion in *Bordetella*. Mol. Microbiol. **52:**1201–1214.
28. **Medhekar, B., R. Shrivastava, S. Mattoo, M. Gingery, and J. F. Miller.** 2009. *Bordetella* Bsp22 forms a filamentous type III secretion system tip complex and is immunoprotective in vitro and in vivo. Mol. Microbiol. **71:**492–504.
29. **Michiels, T., P. Wattiau, R. Brasseur, J. M. Ruysschaert, and G. Cornelis.** 1990. Secretion of Yop proteins by Yersiniae. Infect. Immun. **58:**2840–2849.
30. **Mooi, F. R.** 2010. *Bordetella pertussis* and vaccination: the persistence of a genetically monomorphic pathogen. Infect. Genet. Evol. **10:**36–49.
31. **Mooi, F. R., I. H. van Loo, and A. J. King.** 2001. Adaptation of *Bordetella pertussis* to vaccination: a cause for its reemergence? Emerg. Infect. Dis. **7:**526–528.
32. **Mooi, F. R., et al.** 2009. *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg. Infect. Dis. **15:**1206–1213.
33. **Parsot, C., R. Menard, P. Gounon, and P. J. Sansonetti.** 1995. Enhanced secretion through the *Shigella flexneri* Mxi-Spa translocon leads to assembly of extracellular proteins into macromolecular structures. Mol. Microbiol. **16:**291–300.

33a. **Pittman, M.** 1984. Genus *Bordetella*, p. 388–393. *In* N. R. Krief and J. G. Holt (ed.), Bergey's manual of systemic bacteriology, vol. 1. Williams and Wilkins Company, Baltimore, MD.

34. **Stainer, D. W., and M. J. Scholte.** 1970. A simple chemically defined medium for the production of phase I *Bordetella pertussis*. J. Gen. Microbiol. **63:**211–220.
35. **Stefanelli, P., et al.** 2006. Differential in vitro expression of the *brkA* gene in *Bordetella pertussis* and *Bordetella parapertussis* clinical isolates. J. Clin. Microbiol. **44:**3397–3400.
36. **Stibitz, S., and M. S. Yang.** 1997. Genomic fluidity of *Bordetella pertussis* assessed by a new method for chromosomal mapping. J. Bacteriol. **179:**5820–5826.
37. **Stibitz, S., and M. S. Yang.** 1999. Genomic plasticity in natural populations of *Bordetella pertussis*. J. Bacteriol. **181:**5512–5515.
38. **Troisfontaines, P., and G. R. Cornelis.** 2005. Type III secretion: more systems than you think. Physiology (Bethesda) **20:**326–339.
39. **van Boven, M., F. R. Mooi, J. F. Schellekens, H. E. de Melker, and M. Kretzschmar.** 2005. Pathogen adaptation under imperfect vaccination: implications for pertussis. Proc. Biol. Sci. **272:**1617–1624.
40. **Van den Ackerveken, G., and U. Bonas.** 1997. Bacterial avirulence proteins as triggers of plant disease resistance. Trends Microbiol. **5:**394–398.
41. **Viboud, G. I., and J. B. Bliska.** 2005. *Yersinia* outer proteins: role in modulation of host cell signaling responses and pathogenesis. Annu. Rev. Microbiol. **59:**69–89.
42. **Viprey, V., A. Del Greco, W. Golinowski, W. J. Broughton, and X. Perret.** 1998. Symbiotic implications of type III protein secretion machinery in *Rhizobium*. Mol. Microbiol. **28:**1381–1389.
43. **von Götz, F., et al.** 2004. Expression analysis of a highly adherent and cytotoxic small colony variant of *Pseudomonas aeruginosa* isolated from a lung of a patient with cystic fibrosis. J. Bacteriol. **186:**3837–3847.
44. **Yuk, M. H., E. T. Harvill, P. A. Cotter, and J. F. Miller.** 2000. Modulation of host immune responses, induction of apoptosis and inhibition of NF-κB activation by the *Bordetella* type III secretion system. Mol. Microbiol. **35:**991–1004.
45. **Yuk, M. H., E. T. Harvill, and J. F. Miller.** 1998. The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. tMol. Microbiol. **28:**945–959.

Editor: J. B. Bliska

Downloaded from http://iai.asm.org/ on October 9, 2019 by guest