

## Pulsed-Field Gel Electrophoresis, Pertactin, Pertussis Toxin S1 Subunit Polymorphisms, and Surfaceome Analysis of Vaccine and Clinical *Bordetella pertussis* Strains<sup>∇</sup>

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To add new insight to our previous work on the molecular epidemiology of *Bordetella pertussis* in Argentina, the *prn* and *ptxS1* gene sequences and pulsed-field gel electrophoresis (PFGE) profiles of 57 clinical isolates obtained during two periods, 1969 to 1989 and 1997 to 2006, were analyzed. Non-vaccine-type *ptxS1A* was detected in isolates obtained since 1969. From 1989 on, a shift of predominance from the vaccine *prn1* type to the nonvaccine *prn2* type was observed. This was also reflected in a transition of PFGE group IV to group VI. These results show that nonvaccine *B. pertussis* strains are currently circulating. To analyze whether the observed genomic divergences between vaccine strains and clinical isolates have functional implications, protection assays using the intranasal mouse challenge model were performed. For such experiments, the clinical isolate *B. pertussis* 106 was selected as representative of circulating bacteria, since it came from the major group of the PFGE dendrogram (PFGE group VI). Groups of mice were immunized either with diphtheria–tetanus–whole-cell pertussis vaccine (*ptxS1B prn1*) or a vaccine prepared by us containing *B. pertussis* 106. Immunized mice were then challenged with a *B. pertussis* vaccine strain (Tohama, harboring *ptxS1B* and *prn1*) or the clinical isolate *B. pertussis* 106 (*ptxS1A prn2*). An adequate bacterial-elimination rate was observed only when mice were immunized and challenged with the same kind of strain. For further characterization, comparative proteomic profiling of enriched membrane proteins was done using three vaccine strains and the selected *B. pertussis* 106 clinical isolate. By matrix-assisted laser desorption/ionization–time of flight mass spectrometry analysis, a total of 54 proteins were identified. This methodology allowed us to detect differing proteins among the four strains studied and, in particular, to distinguish the three vaccine strains from each other, as well as the vaccine strains from the clinical isolate. The differing proteins observed have cellular roles associated with amino acid and carbohydrate transport and metabolism. Some of them have been proposed as novel vaccine candidate proteins for other pathogens. Overall, the global strategy described here is presented as a good tool for the development of next-generation acellular vaccines.

Pertussis is an infectious respiratory disease caused by the bacterium *Bordetella pertussis*, an exclusively human pathogen. This disease, though preventable by vaccination, remains one of the top 10 causes of death worldwide in childhood. Moreover, in countries with highly vaccinated populations, a pertussis resurgence has been reported (2, 13, 14, 19, 36, 40, 43, 50). Several explanations have been suggested for this resurgence in such populations: low efficacy of the vaccine used, waning immunity in the absence of both natural and vaccinal boosters after 18 months of age, and adaptation of the circulating *B. pertussis* population (24, 27, 31, 39, 41, 46, 51). One explanation is based on studies of *B. pertussis* strains, first from The Netherlands and then from other countries, that focused on

genomic analysis by pulsed-field gel electrophoresis (PFGE) and on sequences of the genes that code for two immunogens, pertactin (*prn*) and the S1 subunit of pertussis toxin (*ptxS1*) (8, 19, 21, 26, 37, 40, 41). Data from these studies have been interpreted to suggest that vaccination selects for strains that have different genotypes from those used to make the vaccine, allowing the spread of escape mutants (15, 34, 40). This vaccine-driven antigenic evasion seems not to be exclusive to pertussis, since a growing body of evidence indicates it exists for at least three other diseases: hepatitis B virus, *Streptococcus pneumoniae*, and *Haemophilus influenzae* infections (5, 7, 10, 11, 44, 47, 48).

In Argentina during previous years, an increase in the pertussis incidence was registered by the Public Health Ministry, even before improvements in diagnostic methodology were introduced. In that country, whole-cell pertussis vaccine was introduced in the 1960s, and it is still the only kind of pertussis vaccine being used. Since 1985, the vaccination schedule has been 2, 4, 6, and 18 months of age, with a school entry booster at 5 to 6 years of age. Molecular characterization of *B. pertussis*

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isolates ( $n = 28$ ) obtained in Argentina from 1997 to 2003 was done (19). The isolates appeared to be genotypically different from the vaccine strain in use. The isolates were comprised of *ptxS1A* (100%) and *prn2* (93%) or *prn1* or *prn7* (7%) variants. These allelic variants are different from those present in the vaccine strain (*ptxS1B* or *ptxS1D* and *prn1*) but identical to those found in European and North American countries.

Though more epidemiological and molecular data should be collected in order to describe more precisely the pertussis disease scenario, it is clear that the control of pertussis requires improvement. Currently used pertussis vaccines in many countries are based on strains, or proteins obtained from strains, isolated before 1957, which are different from the currently circulating bacteria.

Thus, novel proteins from contemporary isolates should be considered in the design of new acellular vaccines. Surface proteins play a fundamental role in the interaction between the bacterial cell and its environment; hence, these bacterial constituents may become components of such vaccines. Comparison of multiple exposed proteins in *B. pertussis* strains used at present in vaccine production with contemporary circulating strains may allow the identification of new immunogens that can be incorporated in a new, broadly protective vaccine.

In this study, a procedure that allows the identification of surface-accessible proteins in *B. pertussis* is described for the first time. This methodology was applied to compare enriched membrane samples obtained from well-known laboratory-adapted *B. pertussis* strains widely used for vaccine production and a *B. pertussis* local isolate. The results obtained revealed that genomic divergence that seems to have a role in pertussis protection, as demonstrated here using the accepted animal model, could be extended to the bacterial surface proteome. Thus, this new insight into proteins located on the surfaces of *B. pertussis* strains may assist in the identification of potential vaccine candidates. Overall, this report presents an attractive approach for comparison of the surfaces of *Bordetella* strains and provides a framework for future proteomic analyses.

#### MATERIALS AND METHODS

**Clinical isolates.** In this study, we characterized 57 *B. pertussis* isolates, mostly collected from patients residing in Buenos Aires (Argentina) from 1997 to 2006. Isolates from 1969, 1971, 1982, and 1989 were also included. Nasopharyngeal aspirate and Dacron swab samples were obtained from patients with a clinical diagnosis of pertussis, and Bordet-Gengou agar (BGA) supplemented with defibrinated sheep blood was used for primary culture. Inoculated plates incubated at 36°C were monitored daily for 10 days. Suspected colonies were Gram stained and tested by agglutination test with antiserum for *B. pertussis* (Murex Diagnostic, Dartford, England) and specific *B. pertussis* PCR (22, 32). Biochemical typing using the API 20 NE system (bioMérieux, Marcy l'Etoile, France) was also performed on the clinical isolates. The ages of patients ranged from 1 month to 5 years, with the median age being 4 months. Thirty-three patients from whom bacteria were isolated were less than 18 months old, and only one was 5 years old. For the other positive culture patients, no data on age were reported.

**PCR, sequencing, and PFGE.** PCR, sequencing, and PFGE were performed according to standardized recommendations for *B. pertussis* typing (38). Bacteria were cultivated on BGA containing defibrinated sheep blood at 36°C for 72 h. For PFGE, *B. pertussis* strains 134 (United States), 509 (The Netherlands and Mexico), 10536, Tohama (Japan), and 18323 (United States) were used as reference strains.

Briefly, DNA sequencing of relevant regions of the *prn* and *ptxS1* genes was performed on PCR fragments as described by Mooi et al. (38). For sequencing of region 1, the primer pair AF/AR (5'-CAATGTCACGGTCCAA-3'/5'-GCAAGGTGATCGACAGGG-3') was used, and for sequencing of region 2, the primer pair BF/BR (5'-AGCTGGGCGGTCAAGGT-3'/5'-CCGATTCCAGG

CGCAACTC-3') was used. For pertussis toxin, primers S1-F2 (5'-CCCCTGCCATGGTGTGATC-3') and S1-R2 (5'-AGAGCGTCTTGGCGTCGATC-3') were used to amplify a 930-bp product that contained the complete *ptxS1* gene. S1-F2/S1-R2 were also used for sequencing (18). Mutations were confirmed by sequencing using two additional primers, S1-MF/S1-MR (5'-ACAATGCCGGCGTATCCTC-3'/5'-TTCGAAGTACGAGCTGGCGG-3').

All clinical isolates and reference strains were genotyped by PFGE using the restriction enzyme XbaI (Promega) with a CHEF-DR III apparatus (Bio-Rad Laboratories) at 6 V/cm with a 120° angle and pulses of 5 to 6 s for 19 h at 14°C.

Gels were stained for 40 min in 500 ml deionized water containing 50  $\mu$ l of a 10-mg/ml solution of ethidium bromide. Unbound ethidium bromide was removed by washing the gels in deionized water for 60 min. DNA fragments were visualized on a UV transilluminator, and the gels were photographed with a Gel Doc 2000 system.

The Tohama strain was used in each gel as an external quality control to assess whether the reproducibility of its banding pattern was consistent with those previously published and between runs.

BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) version 3.5 software was used to normalize the DNA fragment migration distances relative to those of *Salmonella enterica* serotype Braenderup strain H9812. DNA fragments were analyzed, with at least one band discrepancy as the basis for discriminating among profiles.

The unweighted pair group method using arithmetic averages was used as the clustering method, with a 1% band tolerance and 1% optimization settings with the Dice coefficient. All profiles were verified by visual comparison.

**Murine respiratory-infection model.** Female BALB/c mice at 3 to 4 weeks of age from Biol SAIC, Argentina, were used as a model of in vivo respiratory infection by *B. pertussis*. Bacteria grown on BGA were resuspended and adjusted to approximately  $10^8$  CFU ml<sup>-1</sup> in phosphate-buffered saline (PBS). Fifty microliters of a bacterial suspension of each strain studied was delivered intranasally to each mouse via an air displacement pipette. At different times postinoculation, three mice from each group were sacrificed, and the lungs were removed aseptically. The lungs were homogenized in PBS, and appropriate dilutions were plated onto BGA to determine the number of viable bacteria present in the lungs. All the experiments were performed two times, and they gave consistent results.

**Active immunization and intranasal challenge.** Female BALB/c mice were obtained at 4 weeks of age from Biol SAIC, Argentina. The animal protocol was performed according to the study by Guiso et al. (23). Groups of BALB/c mice were immunized intraperitoneally with 1/10 of the human dose of diphtheria-tetanus-whole-cell pertussis vaccine (*ptxS1B prn1*), following a two-dose schedule over a period of 2 weeks. The mice were challenged 2 weeks after the second immunization by nasal challenge with a *B. pertussis* vaccine strain (Tohama, harboring *ptxS1B* and *prn1*) or the clinical isolate *B. pertussis* 106 (*ptxS1A prn2*) delivered from a culture containing  $10^8$  bacteria per ml. We also evaluated the potential for bacterial clearance of a vaccine prepared by us containing a *B. pertussis* 106 clinical isolate (wP<sub>Bp106</sub>).

The lungs of infected animals were aseptically removed, homogenized in sterile PBS, serially diluted, and then plated on BGA plates to determine bacterial recovery at different time points during the course of infection. The outcome of the challenge was followed by performing CFU counts on lung homogenates from individual mice.

All the experiments were performed three times, and they gave consistent results.

**Statistical analysis.** Means and standard deviations were calculated from log<sub>10</sub>-transformed CFU numbers. Differences among means were assessed by two-tailed Student's *t* tests, with significance accepted as a *P* value of <0.05.

**Membrane protein enrichment.** The membrane fraction of *B. pertussis* was prepared as described previously (6) with minor modifications. *B. pertussis* cells were harvested by centrifugation (10,000  $\times$  g; 30 min; 4°C) and washed twice with low-salt washing buffer containing 3 mM KCl, 68 mM NaCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, and 9 mM NaH<sub>2</sub>PO<sub>4</sub>. The cells were suspended in 10 mM Tris-HCl (pH 8.5) supplemented with phenylmethylsulfonyl fluoride and protease inhibitor cocktail tablets (Roche Applied Science) and then disrupted with an ultrasonicator (Sonic & Materials, Inc., Danbury, CT). DNase and RNase (20  $\mu$ g/ml each) were added to the cell suspension, and the mixture was incubated at 37°C for 1 h. The unbroken cells were removed by centrifugation (12,000  $\times$  g; 30 min; 4°C), and the supernatant was retained. Total membrane proteins were then collected by centrifugation (30,000  $\times$  g; 1 h; 4°C) and resuspended in 7 M urea, 2 M thiourea, 10% isopropanol, and 2% Triton X-100. Membrane proteins were divided into aliquots and stored at -20°C until they were used.

For each strain, sample preparation, two-dimensional electrophoresis (2-DE),

and protein identification were repeated at least four times to establish reproducibility.

**Protein quantification.** Protein concentrations were determined by the Bradford method (4), with bovine serum albumin as a standard.

**2-DE.** Isoelectric focusing (IEF) was performed by using immobilized pH gradient strips (Immobiline DryStrip; Amersham Biosciences). Portions (200  $\mu$ g) of the membrane proteins were applied to 7-cm strips in a pH range from 4.0 to 7.0. The immobilized pH gradient strips were rehydrated overnight at room temperature with 125  $\mu$ l of rehydration buffer (7 M urea, 2 M thiourea, 10% isopropanol, and 2% Triton X-100) plus 1.25  $\mu$ l 28% dithiothreitol (DTT), 0.62  $\mu$ l 0.5% ampholyte (pH 4.0 to 7.0 [Amersham]), and 0.01% bromophenol blue containing 200  $\mu$ g of proteins. Three preset programs were executed with slight modifications so that the focusing conditions consisted of the conditioning step, voltage ramping, and final focusing.

The purpose of the conditioning step (500 V for 30 min) was to remove salt ions and charged contaminants, and this was followed by linear voltage ramping for 30 min at 1,000 V. In the final focusing step, the maximum voltage of the ramp step was maintained (up to 5,000 V  $\cdot$  h) and the current did not exceed 50  $\mu$ A/strip. After IEF, the strips were equilibrated in 50 mM Tris buffer (pH 8.8) containing 6 M urea, 2% sodium dodecyl sulfate, 30% glycerol, and 1% DTT, followed by another 1-h equilibration step with the same buffer supplemented with 4.5% iodoacetamide. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the Laemmli method (35) with a 12.5% resolving polyacrylamide gel without a stacking gel. Separation in the second dimension was carried out at 40 V at 4°C until the running dye reached the bottom.

**Coomassie staining and gel drying.** Proteins resolved on gels were visualized by using a colloidal Coomassie staining method (<http://prospector.ucsf.edu>) with slight modifications. Briefly, the gel was fixed in a solution containing 30% ethanol-2% phosphoric acid with double-distilled water (ddH<sub>2</sub>O) three times for 30 min each time. All incubations were performed with gentle shaking. After a fixing step, the gel was washed with a solution of 2% phosphoric acid in ddH<sub>2</sub>O three times for 20 min each time. The wash solution was discharged, and the gels were fixed again with a solution containing 2% phosphoric acid (vol/vol), 18% ethanol (vol/vol), and 15% ammonium sulfate (wt/vol) in ddH<sub>2</sub>O for 30 min. To this solution, a 1.5% volume of a suspension of 2% colloidal Coomassie (G-250) in ddH<sub>2</sub>O was added. The duration of this step was 24 to 72 h.

**Image analysis.** A gel image was obtained by scanning Coomassie-stained gels with the Fluor-S MultiImager (Bio-Rad). The image was documented through the PDQUEST 2-D gel analysis software (version 6; Bio-Rad).

**In-gel tryptic digestion of proteins.** Coomassie-stained spots were excised from 2-DE gels and transferred into microcentrifuge tubes. Plugs were washed with 100  $\mu$ l 25 mM ammonium bicarbonate for 10 min and incubated for 30 min at 60°C in 20  $\mu$ l of 5 mM DTT in 25 mM ammonium bicarbonate solution. This step was followed by another incubation for 15 min at room temperature in darkness with 20  $\mu$ l 55 mM iodoacetamide in 25 mM ammonium bicarbonate. The solution was discharged, and a new wash with 25 mM ammonium bicarbonate was performed, followed by another wash with 100  $\mu$ l acetonitrile. Then, the spots were subjected to a dehydration step three times with 25  $\mu$ l acetonitrile for 10 min at room temperature. When the gel spots became completely white, the acetonitrile was eliminated by evaporation. Spot rehydration was performed with small volumes (up to 20  $\mu$ l) of 50 mM ammonium bicarbonate containing trypsin (20  $\mu$ g/ml) and incubation for 45 min at 4°C. The tryptic digestion was carried out overnight at 37°C. After digestion, the spots were washed twice with 20  $\mu$ l acetonitrile-0.1% trifluoroacetic acid (33:66) for 10 min, and the supernatants were collected. Sample concentration was performed in a Speedvac.

**Peptide mass fingerprinting.** A matrix solution composed of  $\alpha$ -cyano-4-hydroxy cinnamic acid (0.2 g/liter) in 50% acetonitrile and 0.25% trifluoroacetic acid was prepared for peptide mass fingerprinting. A 0.4- $\mu$ l mixture of matrix and sample solutions was applied to the target well. The solution mixture was dried for 10 min at room temperature and subjected to a matrix-assisted laser desorption/ionization-time of flight mass spectrometry operation by using an Ultraflex (Bruker) with the following parameters: 20-kV accelerating voltage, 75% grid voltage, 0.02% guide wire voltage, 70-ns delay, and a mass gate of 800 to 3,000.

**Identification of proteins.** Searches for and identification of peptides were performed with a licensed version of MASCOT software (Matrix Science) in a database containing the 3,436 accession number entries derived from the complete *B. pertussis* genome sequence (downloaded from <http://www.ncbi.nlm.nih.gov/>). The MASCOT search parameters were as follows: (i) species, bacteria (eubacteria); (ii) allowed number of missed cleavages (only for trypsin digestion), one; (iii) variable posttranslational modification, methionine oxidation; (iv) fixed modification, carbamidomethylation; (v) peptide tolerance,  $\pm$ 50 ppm; (vi) peptide charge, positive; and (vii) monoisotopic peptide masses were used to search

the database, allowing a molecular mass range for 2-DE analyses of  $\pm$ 15%. Only significant hits as defined by MASCOT probability analysis were considered.

Prediction of protein localization was carried out using a PSORT algorithm available at <http://psort.nibb.ac.jp>.

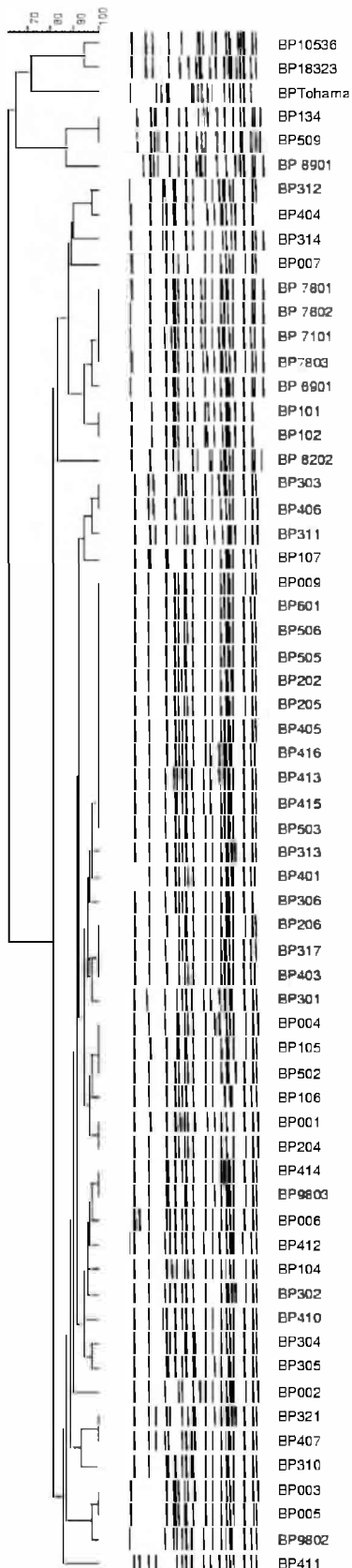
## RESULTS AND DISCUSSION

**Genotypic analysis.** Polymorphism and variation in virulence factors and strains have been observed in different countries that use whole-cell pertussis vaccines where nonvaccine strains are circulating (9, 19, 21, 25, 30, 36, 49, 50). In this study, we used PFGE and *prn* and *ptxS1* typing to add new insight into the characterization of *B. pertussis* isolates obtained in Argentina in two periods: 1969 to 1989 and 1997 to 2006.

Since it was previously reported that PFGE analysis with XbaI gave optimal discrimination (1, 28), we used this enzyme to classify the vaccine strains and local isolates into PFGE profile groups. Figure 1 shows the dendrogram of DNA profile relatedness calculated by the unweighted pair group method using arithmetic averages clustering of Dice coefficients. A total of 39 distinct PFGE profiles, named V1 to V3 for vaccine strains, R1 for the reference strain, and A1 to A35 for clinical isolates, were observed. These profiles were classified into six major groups (I to VI) based on a criterion of similarity higher than 0.70. The three groups corresponding to the vaccine strains *B. pertussis* 10536 (group I, which also includes the WHO reference strain 18323), Tohama (group II), and *B. pertussis* 134 and *B. pertussis* 509 (group III, which also includes the clinical isolate *B. pertussis* 8901) had low similarity with all Argentinean clinical isolates tested, with relatedness of 67%. Most Argentinean isolates fell in a small number of PFGE groups. Eleven isolates were segregated into PFGE group IV, one into PFGE group V, and 44 into PFGE group VI. PFGE group IV was the only one detected among the strains from earlier than 1982, while only a single 1982 isolate corresponded to PFGE group V. While 6 contemporary *B. pertussis* isolates (12%) were clustered in PFGE group IV, 44 (88%) were grouped in PFGE group VI. PFGE group IV seems to have been dominant till at least 1982, and group VI has been dominant from 1998/2000 to 2006 (Fig. 1).

This lower-than-expected level of *B. pertussis* population diversity may be a consequence of sequential bottlenecks or fitness differences between the strains that favored only those variants able to evade the immunity induced by vaccination or with increased virulence. That is, circulating bacteria could have overcome the strong bottleneck of the immunity induced by the vaccine strain by accumulating mutations that allowed them to evade host responses.

Differences between circulating bacteria and vaccine strains were also observed when the sequences of *ptxS1* and *prn* alleles (38) were analyzed. Studies of *ptxS1* subunit polymorphism showed that 53 *B. pertussis* isolates contained the *ptxS1A* allele and only 4 had non-*A* *ptxS1*. By contrast, the vaccine strains used in Argentina contain *ptxS1B* or *ptxS1D* alleles. For pertactin, a significant correlation was observed between the PFGE groups and *prn* alleles. All 11 of the isolates from group IV contained *prn1*, and all isolates from group VI had the *prn2* allele. It seems that *prn2* has replaced the *prn1* present in old isolates.



PFGE groups or subgroups	Profile	Year of collection	<i>prn</i>	<i>ptxS1</i>
I	V1		1	no A
I	R1	1947	6	E
II	V2	1954	1	B
III	V3	1950	1	B
III	V3		7	D
III	A1	1989	1	no A
IV	A2	2003	1	A
IV	A3	2004	1	A
IV	A4	2003	1	A
IV	A5	2000	1	A
IV	A6	1978	1	A
IV	A6	1978	1	A
IV	A6	1971	1	A
IV	A6	1978	1	A
IV	A7	1969	1	A
IV	A8	2001	1	A
IV	A8	2001	1	A
V	A9	1982	1	no A
VI	A10	2003	2	A
VI	A10	2004	2	A
VI	A11	2003	2	A
VI	A12	2001	2	A
VI	A13	2000	2	A
VI	A13	2006	2	A
VI	A13	2005	2	A
VI	A13	2005	2	A
VI	A13	2002	2	A
VI	A13	2002	2	A
VI	A13	2004	2	A
VI	A13	2004	2	A
VI	A13	2004	2	A
VI	A13	2005	2	A
VI	A14	2003	2	A
VI	A15	2004	2	A
VI	A16	2003	2	A
VI	A17	2002	2	A
VI	A17	2003	2	A
VI	A17	2004	2	A
VI	A18	2003	2	A
VI	A19	2000	2	A
VI	A19	2001	2	A
VI	A19	2005	2	A
VI	A20	2001	2	A
VI	A21	2000	2	A
VI	A21	2002	2	A
VI	A22	2004	2	A
VI	A22	1998	2	A
VI	A23	2000	2	A
VI	A24	2004	2	A
VI	A25	2001	2	A
VI	A26	2003	2	A
VI	A27	2004	2	A
VI	A28	2003	2	A
VI	A29	2003	2	A
VI	A30	2000	2	no A
VI	A31	2004	2	no A
VI	A31	2004	2	A
VI	A32	2003	2	A
VI	A33	2000	2	A
VI	A33	2000	2	A
VI	A34	1998	2	A
VI	A35	2004	2	A

FIG. 1. Genomic analysis of Argentinean *B. pertussis* isolates. On the left are shown chromosomal DNA profiles obtained after digestion with XbaI.

In the United Kingdom and Finland, a change from *prn1* to *prn2* was observed in isolates from as early as 1982, in The Netherlands from 1990, and in Taiwan from 1997 (16, 21, 36, 41). These same variations seem to support a possible explanation that the impact of the vaccination-induced immune responses to these epitopes that have been recorded were strong enough to structure the population into discrete combinations of epitopes or antigenic types. By contrast, under weak immune selection, a greater antigenic and genetic variability of the bacterial population is expected, as occurs, for example, with the poorly immunogenic meningococcus serogroup B capsular polysaccharide, which shows high genetic variability (3).

Though the genotypic divergences between the vaccine strains and the circulating bacterial population are clear, important questions remain. Among them is whether the *B. pertussis* population genotypic variation has affected vaccine efficacy and contributed to resurgence. Though it seems to be too early to predict this, since the shift in virulent *B. pertussis* alleles has continued over the years, it is important to begin to analyze the consequence of the structural genomic variation in pertussis protection using the accepted animal model.

**Animal studies.** To analyze the implications of divergence between vaccine strains and clinical isolates for protection, we used an intranasal mouse challenge model. Briefly, this model consists of two immunizations of mice (3- to 4-week-old BALB/c mice) with either current pertussis whole-cell vaccine or a vaccine prepared by us containing the representative *B. pertussis* 106 clinical isolate. This isolate was selected to represent the circulating bacterial population because it comes from the most differentiated and major PFGE group (PFGE group VI). The immunized mice were then intranasally challenged with sublethal doses of either a *B. pertussis* vaccine strain or the representative clinical isolate, *B. pertussis* 106. Control animals were vaccinated with PBS. Two hours later and on days 5 and 8 after the challenge, the lungs were collected for bacterial counts. As expected, significant differences between immunized animals and the control group were observed ( $P < 0.001$ ) (Table 1). Adequate elimination rates were observed in mice immunized and challenged with the same kind of strain (immunization and challenge with a vaccine strain). However, a lower potential of protective immunity induced by whole-cell vaccination was observed in elimination rates of the clinical *B. pertussis* isolate *B. pertussis* 106 (*prn2 ptxS1A*) from the lungs than for the vaccine strain Tohama (*prn1 ptxS1B*) ( $P < 0.001$ ) (Table 1). Furthermore, when mice were immunized with a vaccine containing a clinical isolate and challenged with a vaccine strain or antigenically divergent clinical isolates, lower clearance efficiency for the vaccine strain was induced ( $P < 0.002$ ) as early as day 5 after challenge (Table 1).

These results could not be attributed to differences in colonization abilities between the vaccine strain and the clinical isolate, since the number of colonies recovered at each time in infection experiments were the same for both strains (not shown). Overall, these results clearly demonstrate that the efficacies of whole-cell vaccines depend on the relatedness between the vaccine strains and the circulating strains. In addition, the present observations support the hypothesis of the role of divergence of circulating strains in pertussis resurgence.

TABLE 1. Lung clearance efficiency of diphtheria–tetanus–whole-cell pertussis vaccine and wP<sub>Bp106</sub> in intranasal-challenge model experiments

Immunization	<i>B. pertussis</i> challenge strain/isolate	Bacteria recovered after challenge (log <sub>10</sub> CFU/lung ± SD) on day <sup>a</sup> :		
		0	5	8
PBS	Tohama	6.70 ± 0.13	6.45 ± 0.30	6.05 ± 0.28
DTwP	Tohama	6.45 ± 0.73	5.20 ± 0.04	— <sup>b</sup>
wP <sub>Bp106</sub>	Tohama	6.90 ± 0.8	3.20 ± 0.19	ND
PBS	106	6.79 ± 0.13	6.51 ± 0.30	5.86 ± 0.28
DTwP	106	6.45 ± 0.02	5.41 ± 0.26	5.34 ± 0.05
wP <sub>Bp106</sub>	106	6.80 ± 0.04	1.99 ± 0.74	ND

<sup>a</sup> Values shown are means of three or four animals sacrificed at each time point. ND, not determined.

<sup>b</sup> CFU were not detected in the lung samples.

**Proteomic analysis.** For further characterization of bacterial divergence, we compared the bacterial surfaceomes of three known *B. pertussis* strains that are widely used for vaccine production and that clustered in different PFGE groups: *B. pertussis* 10536 (PFGE group I), Tohama I (PFGE group II), and 509 (PFGE group III), and extended this analysis to the representative local isolate, *B. pertussis* 106 (PFGE group VI). Several protocols have been developed for the preparation and analysis of membrane proteins, but none of them have yet been used for *Bordetella*. The procedure employed here allowed us to obtain an enriched fraction of surface proteins as established by PSORT analysis. Of the 54 proteins successfully identified, 9 were predicted to be associated with the external membrane, 6 had periplasmic localization, 6 had cytoplasmic/membrane localization (5 harbored signal peptide), 16 had an unknown origin (3 had signal peptide), and 17 had a cytoplasmic localization (Table 2). In the last group, some of the proteins, such as EF-Tu, ET TS, enolase, 60-kDa chaperonin, and some dehydrogenases, may be associated with membranes, as was observed in *Neisseria meningitidis* (17).

Some of the proteins separated by 2-DE were present as multiple spots exhibiting variability in pI values (horizontal spot patterns) (Fig. 2). Charge variants included EF-Tu, 60-kDa chaperonin, outer membrane porin protein precursor, serum resistance protein, and serine protease. These may represent natural isoforms or an artifact caused by sample preparation or 2-DE. Similarly, serum resistance protein resolved in multiple spots of differing masses and pIs, suggesting possible protein processing, degradation, and/or modifications (Fig. 2).

Few proteins could be detected as differential among the three analyzed vaccine strains. Leu-Ile-Val protein precursor (spot 17; BP1285), DNA direct RNA  $\alpha$  subunit polymerase (spot 18; BP3642), and a two-component sensor protein (spot 31; BP2483) could be detected in Tohama and *B. pertussis* 509; however, they were not detected in strain 10536. Putative penicillin binding protein precursor (spot 50; BP0102) and hypothetical protein BP3128 (spot 39; BP3128) were detected in *B. pertussis* 509 and 10536 but were not evident in Tohama, and putative binding protein-dependent transport protein (spot 10; BP3322) was not observed in *B. pertussis* 509, whereas putative molybdopterin oxidoreductase (spot 22; BB0468) was clearly detected in *B. pertussis* 10536. Furthermore, putative ABC transport ATP binding protein (spot 8; BP3757) and putative

TABLE 2. Surface proteome of *B. pertussis* vaccine strains Tohama, 10536, and 509 and Argentinean clinical isolate 106

Order	Protein name/function	Gene locus <sup>a</sup>	Mass (kDa)	pI	Protein localization <sup>b</sup>	Presence of gene in strain <sup>c</sup> :			
						Tohama	509	10536	106
1	Competence lipoprotein precursor	BP1146	29.8	5.0	Unknown	Yes	Yes	Yes	Yes
2	Conserved hypothetical protein	BP3441	19.8	5.1	Cytoplasmic/membrane <sup>d</sup>	Yes	Yes	Yes	Yes
3	Adhesin	BP2667	263.6	9.7	Outer membrane	Yes	Yes	Yes	Yes
4	Putative lipoprotein	BP1296	30.6	7.4	Unknown	Yes	Yes	Yes	Yes
5	Succinate dehydrogenase catalytic subunit	BP2360	27.2	6.2	Cytoplasmic	Yes	Yes	Yes	Yes
6	Outer membrane protein OMPQ	BP3405	39.1	5.7	Outer membrane <sup>e</sup>	Yes	Yes	Yes	Yes
7	Outer membrane porin protein precursor	BP0840	41	5.4	Outer membrane	Yes	Yes	Yes	Yes
8	Putative ABC transport ATP binding protein	BP3757	29.6	5.1	Unknown	Yes <sup>e</sup>	Yes	Yes	Yes
9	Putative periplasmic solute binding protein	BP1487	40	7.8	Unknown <sup>c</sup>	Yes	Yes	Yes	Yes
10	Putative binding protein-dependent transport protein	BP3322	40.9	6.94	Periplasmic	Yes	No	Yes	Yes
11	Putative membrane protein	BP1440	33.4	5.3	Unknown <sup>e</sup>	Yes <sup>e</sup>	Yes	Yes	Yes <sup>e</sup>
12	Antioxidant protein	BP0965	23.7	5.7	Cytoplasmic	Yes	Yes	Yes	Yes
13	Serin protease	BP2434	52.1	8.8	Periplasmic	Yes	Yes	Yes	Yes
14	Chaperonin, 60 kDa	BP3495	57.4	4.9	Cytoplasmic <sup>d</sup>	Yes	Yes	Yes	Yes
15	Serum resistance protein	BP3494	103.3	7.1	Outer membrane	Yes	Yes	Yes	Yes
16	EF Tu	BP0007	42.9	5.1	Cytoplasmic <sup>d</sup>	Yes	Yes	Yes	Yes
17	Leu-Ile-Val protein precursor	BP1285	39.6	6.8	Periplasmic	Yes	Yes	No	No
18	DNA direct RNA $\alpha$ subunit polymerase	BP3642	36.1	5.7	Cytoplasmic	Yes	Yes	No	Yes
19	Pertactin	BP1054	93.4	10	Outer membrane	Yes	Yes	Yes	Yes
20	Putative quino protein	BP2196	40	8.7	Unknown	Yes	Yes <sup>e</sup>	Yes	Yes
21	Putative extracellular solute binding protein	BP3862	57.3	9.7	Cytoplasmic/membrane <sup>e</sup>	Yes	Yes	Yes	No
22	Putative molibdopterin oxidoreductase	BB0468	121.6	7.3	Periplasmic	No	No	Yes	No
23	Outer membrane protein A precursor	BP0943	20.9	9.2	Outer membrane <sup>e</sup>	Yes	Yes	Yes	Yes
24	Superoxide dismutase	BP2761	21.2	6.5	Unknown	Yes	Yes	Yes	Yes
25	Putative exported protein	BP2513	34.9	10.2	Outer membrane	Yes	Yes	Yes	Yes
26	Ubiquinol cytochrome <i>c</i> reductase iron sulfur subunit	BP0277	22.8	5.2	Cytoplasmic/membrane <sup>e</sup>	Yes	Yes	Yes	Yes
27	Putative exported protein	BP2755	189	6.2	Cytoplasmic/membrane <sup>e</sup>	Yes	Yes	Yes	No
28	3-Oxoacyl-(acyl carrier protein) synthase	BP2439	43.6	5.7	Cytoplasmic	No	No	No	Yes
29	Putative bacterial secretion system protein	BP3794	29.4	6.8	Unknown <sup>c</sup>	Yes	Yes	Yes	Yes
30	Enolase	BP2386	45.9	4.5	Cytoplasmic <sup>d</sup>	Yes	Yes	Yes	Yes
31	Two-component sensor protein	BP2483	97.4	8.7	Cytoplasmic/membrane	Yes	Yes	No	No
32	Serum resistance protein	BP3494	103.3	7.1	Outer membrane	Yes	Yes	Yes	Yes
33	Succinate dehydrogenase flavo subunit	BP2361	64.8	6.5	Cytoplasmic	Yes	Yes	Yes	Yes
34	Putative ABC transport solute binding protein	BP2747	40.6	6.5	Cytoplasmic/membrane <sup>e</sup>	Yes	Yes	Yes	Yes
35	ATP synthase subunit B	BP3288	50.5	4.7	Cytoplasmic	Yes	Yes	Yes	Yes
36	EF Ts	BP1420	30.9	5.1	Cytoplasmic <sup>d</sup>	Yes	Yes	Yes	Yes
37	Ribose phosphate pyrophosphokinase	BP3125	34.1	5.1	Unknown	No	No	No	Yes
38	Dihydroliipoamide dehydrogenase	BP0995	62.3	5.8	Cytoplasmic	No	No	No	Yes
39	Hypothetical protein	BP3128	68.5	6.1	Unknown	No	Yes	Yes	Yes
40	Hypothetical protein	BP3515	35.9	6.6	Unknown	Yes	Yes	Yes	Yes
41	Polysaccharide biosynthesis protein	BP3150	46.7	5.6	Cytoplasmic	No	No	No	Yes
42	Capsular polysaccharide biosynthesis protein	BP1630	37.3	5.7	Unknown	No	No	No	Yes
43	Putative L-lactate dehydrogenase	BP0379	37.2	5.8	Unknown	No	No	No	Yes
44	Hypothetical protein	BP2964	48.5	6.2	Cytoplasmic	Yes	Yes	No	Yes <sup>e</sup>
45	Hypothetical protein	BP1203	42.7	6	Unknown	Yes	Yes	Yes	Yes
46	Putative sigma factor regulatory protein	BP2435	39.2	9.6	Periplasmic	No	No	No	Yes
47	Enoyl-acyl carrier protein	BP3215	27.6	5.8	Unknown	Yes	Yes	Yes	Yes
48	Lipoprotein	BP2750	23.1	7.7	Unknown	Yes	Yes	Yes	Yes
49	Alkyl hydroperoxide reductase	BP3552	20.1	4.9	Cytoplasmic	Yes	Yes	Yes	Yes
50	Putative penicillin binding protein precursor	BP0102	44.8	7.9	Periplasmic	No	Yes	Yes	Yes
51	Dihydroliipoamide dehydrogenase	BP1126	50.1	6.7	Cytoplasmic	No	No	No	Yes
52	Trigger factor	BP1774	47.5	4.9	Cytoplasmic	Yes	Yes	Yes	Yes
53	Putative type III secretion protein	BP2235	63.3	5.9	Outer membrane	No	No	No	Yes
54	Hypothetical protein	BP3559	37.9	4.7	Cytoplasmic	Yes	Yes	Yes	Yes

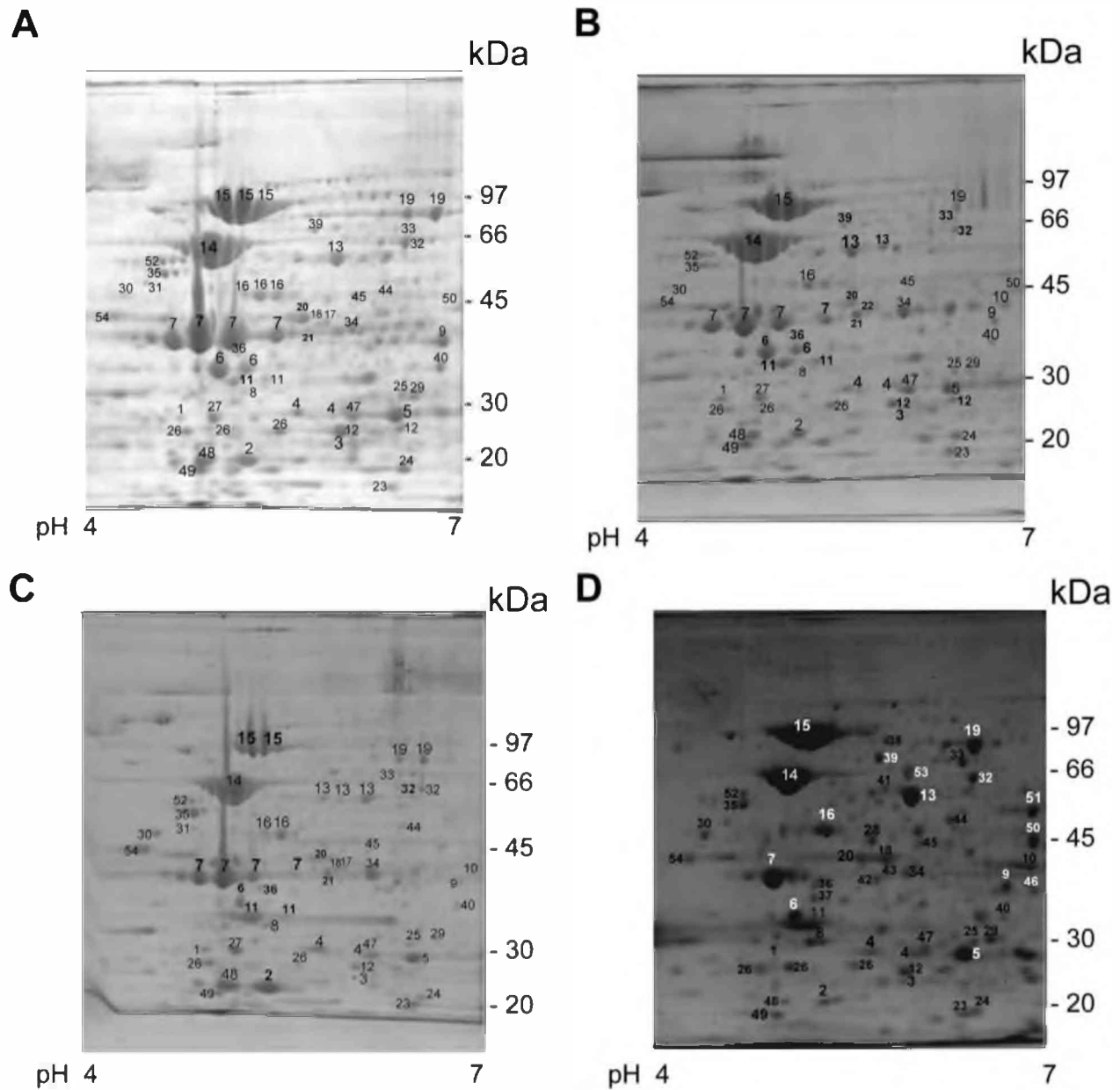
<sup>a</sup> Gene loci are named according to NCBI (<http://www.ncbi.nlm.nih.gov/>).

<sup>b</sup> Protein localization is as predicted by PSORT (<http://psort.nibb.ac.jp/>).

<sup>c</sup> Signal peptide present.

<sup>d</sup> Membrane associated in other bacteria.

<sup>e</sup> Greater quantity.



- |  |  |   |
|--|--|---|
| 1- competence lipoprotein precursor <i>bp1146</i>                      | 18- DNA direct RNA $\alpha$ subunit polymerase <i>bp3642</i>           | 34- putative ABC transport solute binding protein <i>bp2747</i> |
| 2- conserved hypothetical protein <i>bp3441</i>                        | 19- pertactin <i>bp1054</i>  | 35- ATP synthase subunit B <i>bp3288</i>                        |
| 3- adhesin <i>bp2667</i>   | 20- putative quinone protein <i>bp2196</i>                             | 36- elongation factor TS <i>bp1420</i>                          |
| 4- putative lipoprotein <i>bp1296</i>                                  | 21- putative extracellular solute binding protein <i>bp3862</i>        | 37- ribose phosphate pyrophosphokinase <i>bp3125</i>            |
| 5- succinate dehydrogenase catalytic subunit <i>bp2360</i>             | 22- putative molybdopterine oxidoreductase <i>bb0468</i>               | 38- dihydrolipoamide dehydrogenase <i>bp0995</i>                |
| 6- outer membrane protein CMPQ <i>bp3405</i>                           | 23- outer membrane protein A precursor <i>bp0943</i>                   | 39- Hypothetical protein <i>bp3126</i>                          |
| 7- outer membrane porin protein precursor <i>bp0840</i>                | 24- superoxide dismutase <i>bp2761</i>                                 | 40- hypothetical protein <i>bp3515</i>                          |
| 8- putative ABC transporter, ATP binding protein <i>bp3757</i>         | 25- putative exported protein <i>bp2513</i>                            | 41- polysaccharide biosynthesis protein <i>bp3150</i>           |
| 9- putative periplasmic solute binding protein <i>bp1487</i>           | 26- ubiquinol cytochrome C reductase iron sulfur subunit <i>bp0277</i> | 42- capsular polysaccharide biosynthesis protein <i>bp1630</i>  |
| 10- putative binding protein dependent transport protein <i>bp3322</i> | 27- putative exported protein <i>bp2755</i>                            | 43- putative L lactate dehydrogenase <i>bp0379</i>              |
| 11- hypothetical protein <i>bp1440</i>                                 | 28- 3-oxoacyl-(acyl carrier protein) synthase <i>bp2439</i>            | 44- hypothetical protein <i>bp2964</i>                          |
| 12- antioxidant protein <i>bp0965</i>                                  | 29- putative bacterial secretion system protein <i>bp3794</i>          | 45- hypothetical protein <i>bp1203</i>                          |
| 13- serin protease <i>bp2434</i>                                       | 30- endase <i>bp2386</i>   | 46- putative sigma factor regulatory protein <i>bp2435</i>      |
| 14- chaperonin 60 kDa <i>bp3495</i>                                    | 31- Two component sensor protein <i>bp2483</i>                         | 47- enoyl acyl carrier protein <i>bp3215</i>                    |
| 15- serum resistance protein <i>bp3494</i>                             | 32- serum resistance protein <i>bp3494</i>                             | 48- lipoprotein <i>bp2750</i>                                   |
| 16- elongation factor Tu <i>bp0007</i>                                 | 33- succinate dehydrogenase flavo subunit <i>bp2361</i>                | 49- alkyl hydroperoxide reductase <i>bp3552</i>                 |
| 17- leu-Ile-Val protein precursor <i>bp1285</i>                        |  | 50- putative penicillin binding protein precursor <i>bp0102</i> |
|  |  | 51- dihydrolipoamide dehydrogenase <i>bp1126</i>                |
|  |  | 52- trigger factor <i>bp1774</i>                                |
|  |  | 53- putative type III secretion protein <i>bp2235</i>           |
|  |  | 54- hypothetical protein <i>bp3559</i>                          |

FIG. 2. Separation and identification of membrane-related proteins in *B. pertussis* enriched membrane samples using two dimensional polyacrylamide gel electrophoresis, followed by matrix-assisted laser desorption ionization-time of flight mass spectrometry. (A) *B. pertussis* 509. (B) *B. pertussis* 10536. (C) *B. pertussis* Tohama. (D) *B. pertussis* 106. All samples were resolved by IEF (pH 4 to 7). Protein spots were visualized by colloidal Coomassie staining.

membrane protein (spot 11; BP1440) were detected in strain Tohama in smaller quantities than in strains 509 and 10536, and putative quinoprotein (spot 20; BP2196) was detected at a high level in *B. pertussis* 509 but in small quantities in *B. pertussis* 10536 and Tohama.

While a number of protein spots were common among the vaccine strains tested and the clinical isolate *B. pertussis* 106, there were multiple spots unique to *B. pertussis* 106, i.e., ribose phosphate pyrophosphokinase (spot 37; BP3125), dihydrolipoamide dehydrogenase (spot 38; BP0995), polysaccharide biosynthesis protein (spot 41; BP3150), capsular polysaccharide biosynthesis protein (spot 42; BP1630), putative L-lactate dehydrogenase (spot 43; BP0379), a hypothetical protein (spot 44; BP2964, which is present in greater quantity than was observed in Tohama and 10536), putative sigma factor regulatory protein (spot 46; BP2435), dihydrolipoamide dehydrogenase (spot 51; BP1126), and putative type III secretion protein (spot 53; BP2235). The presence of the last protein is an interesting result, since it was previously reported that the type III secretion system is expressed only in *Bordetella bronchiseptica*. This result is in agreement with the work presented by K. Mills at the Eighth International Symposium: Saga of Genus *Bordetella*, 1906 to 2006, at the Institut Pasteur, Paris, France. He described an effector of the type III secretion system that was found solely in clinical isolates and not in laboratory-adapted *B. pertussis* strains.

Though most of the proteins identified in *B. pertussis* 106 are involved in carbohydrate metabolism, some of them were recently described in other gram-negative bacteria as having roles in pathogenicity (12) and immunogenicity (33).

One of these enzymes is the dihydrolipoamide dehydrogenase (Lpd), which is an essential functional subunit of the multienzyme pyruvate dehydrogenase complex, with a predicted molecular mass of 50.37 kDa. The pyruvate deshydrogenase complex is comprised of multiple copies of the ternary complex of Lpd, dihydrolipoamide transacetylase, and pyruvate dehydrogenase enzymes and is involved in the oxidation of pyruvate. The dihydrolipoamide dehydrogenase enzyme allows the continued function of the pathway by maintaining the correct redox state of the enzymes involved in the bioconversion. For *Mycobacterium*, it was reported that Lpd has an important role in host colonization and pathogenesis, and in *H. influenzae* type B, a transposon insertion in *lpdA*, a dihydrolipoamide dehydrogenase gene homolog, converts the virulent bacteria into attenuated bacteria. When the *H. influenzae* LpdA mutant was used to challenge 5-day-old infant rats intraperitoneally, it was unrecoverable compared to the wild-type recovery rate (29). These evidences point out that the Lpd identified in *B. pertussis* 106 may have a role in pathogenicity that needs to be investigated.

In *B. pertussis* 106, two other differing spots were detected: polysaccharide biosynthesis protein (spot 41; BP3150) and capsular polysaccharide biosynthesis protein (spot 42; BP1630). Since capsules are often key contributors to the abilities of pathogens to withstand host defense mechanisms (42), the expression of these proteins in *B. pertussis* 106 may be a consequence of its need to evade the host immune response induced by vaccination. A locus that is likely to encode a type II polysaccharide capsule (20) comprising three regions that are involved in export/modification, biosynthesis, and transport

was previously reported to be present in the *Bordetella* genome sequence (45). In *B. pertussis* strain Tohama, it was reported that the central part of the locus is intact but that the 3' region of one gene has undergone an inversion event and, at the 5' end of the locus, an insertion element-mediated rearrangement has deleted part of region 1 (which contains genes that are involved in export/modification) and moved two of the export genes to a different part of the chromosome. It will be interesting to analyze the structure of this locus in *B. pertussis* 106.

This study not only shows that the previously observed genomic divergences between the Argentinean circulating *B. pertussis* population and vaccine strains is now registered (by the Public Health Ministry), but more importantly, that these genomic differences have implications for pertussis protection and could also be extended to the protein expression patterns.

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