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ORIGINAL ARTICLE

Genotypic and phenotypic characterization of *Bordetella pertussis* strains used in different vaccine formulations in Latin America

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Abstract

Aim: To characterize *Bordetella pertussis* vaccine strains in comparison with current circulating bacteria.

Methods and Results: Genomic and proteomic analyses of *Bp*137 were performed in comparison with other vaccine strains used in Latin America (*Bp*509 and *Bp*10536) and with the clinical Argentinean isolate *Bp*106. Tohama I strain was used as reference strain. Pulse-field gel electrophoresis (PFGE) and pertussis toxin promoter (*ptx*P) sequence analysis revealed that *Bp*137 groups with *Bp*509 in PFGE group III and contains *ptx*P2 sequence. Tohama I (group II) and *Bp*10536 (group I) contain *ptx*P1 sequence, while *Bp*106 belongs to a different PFGE cluster and contains *ptx*P3. Surface protein profiles diverged in at least 24 peptide subunits among the studied strains. From these 24 differential proteins, *Bp*10536 shared the expression of ten proteins with Tohama I and *Bp*509, but only three with *Bp*137. In contrast, seven proteins were detected exclusively in *Bp*137 and *Bp*106.

Conclusions: *Bp*137 showed more features in common with the clinical isolate *Bp*106 than the other vaccine strains here included.

Significance and Impact of the Study: The results presented show that the old strains included in vaccines are not all equal among them. These findings together with the data of circulating bacteria should be taken into account to select the best vaccine to be included in a national immunization programme.

Introduction

Pertussis or whooping cough is an immune-preventable respiratory disease that is still endemic worldwide among infants. This age group is most at risk of morbidity, hospitalization and mortality. Estimates from WHO suggest that in 2008, about 16 million cases of pertussis occurred world-wide, 95% of which were in developing countries, and that about 195 000 children died from the disease (World Health Organization 2010). The best way to prevent this highly contagious disease is to get vaccinated. Two types of pertussis vaccines are available: whole-cell (wP) vaccines based on killed aetiological pathogen (*Bordetella pertussis*) and acellular (aP) vaccines based on highly purified, selected bacterial components. Although for paediatric population, wP or aP vaccines could be used, for adolescent and adults, only aP vaccine with lower dose of immunogens is recommended to reduce the reactogenicity associated with the other vaccine formulations (World Health Organization 2010).

The optimal pertussis immunization schedule and the appropriate time for booster dose in a country are normally assessed based on its current epidemiological situation. Because of that, epidemiological surveillance of pertussis is encouraged worldwide. Moreover, the reported shift in the antigenic characteristics of *Bord. pertussis* circulating strains (Mooi *et al.* 1998; Hozbor *et al.* 2009) makes such surveillance crucial to evaluate the

potential impact of bacterial shift on the overall immunity of a population. To control the increasing number of pertussis cases, many countries that do not produce vaccines must import the vaccine doses required to handle the demands of its population. In countries where wP vaccines are still being used, the selection of the vaccine to be imported is a challenge in itself because not all vaccines are formulated with the same strain or the same combination of strains. Latin American countries are using wP vaccines that contain among others the *Bord. pertussis* strains *Bp*10536, *Bp*509 and *Bp*137. In our previous work, we have characterized the first two vaccine strains (*Bp*10536 and *Bp*509) and have observed not only differences between them but also a representative isolate of the currently circulating bacterial population. *Bp*137 strain has been included in a Brazilian vaccine successfully used in their national vaccination programme for more than 17 years (Pereira *et al.* 2005). However, the properties of this strain are scarcely studied. In this work, we present the results obtained from proteomic and genomic studies on this strain and their comparison with those from other vaccine strains. Results from the current clinical isolate *Bp*106 were also included.

Materials and methods

Bacterial strains and growth conditions

The strains of *Bord. pertussis* used in this study were Tohama I (Kasuga *et al.* 1954a,b,c) obtained from the collection of the Pasteur Institute, France, *Bp*509 (van Hemert 1969) obtained from the Netherlands Vaccine Institute, and *Bp*10536 (Stainer and Scholte 1970) and *Bp*137 (Pereira *et al.* 2005) obtained from the National Administration of Laboratories and Institutes of Health. The last three strains are widely used in wP vaccines in Latin America (Table 1). The Argentinean clinical isolate, *Bp*106, which was collected in 2001 from an infant patient residing in Buenos Aires, was also included (Bottero *et al.* 2007). The strains and isolates were cultured on Bordet–Gengou agar (BGA, Difco) supplemented with 1% glycerol, Bacto-peptone (Difco) 10 g l^{-1} and 10% (v/v) defibrinated sheep blood and incubated at 36°C for 3 days. Then, the bacteria were replated in the same medium for 24 h. Bacterial suspensions prepared from these plates were used for genomic analysis [PCR, sequencing and pulse-field gel electrophoresis (PFGE)].

For proteomic experiments, subcultures were grown in Stainer–Scholte liquid medium (Stainer and Scholte 1970) for 20 h at 36°C until the optical density at 650 nm reached 1·0.

PCR, sequencing and PFGE

PCR, sequencing and PFGE were performed as previously described (Mooi *et al.* 2000, 2009; Hardwick *et al.* 2002b; van Loo *et al.* 2002; Fiett *et al.* 2003; Advani *et al.* 2004; Schouls *et al.* 2004; Borisova *et al.* 2007; Bottero *et al.* 2007). The sequences of the primers used to amplify and sequence the promoter region of pertussis toxin (*ptx*P), subunit A of pertussis toxin (*ptx*A), pertactin (*prn*), and type 2 (*fim*2) and type 3 (*fim*3) fimbriae are given in Table 2.

The obtained *Xba*I PFGE profiles were analysed using BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) software version 3.5. The unweighted pair group method with arithmetic mean (UPGMA) algorithm was used as the clustering method, with a 1% band tolerance and 1% optimization settings with the Dice's coefficient. The band pattern of each strain was verified by visual comparison. PFGE profiles were classified into groups based on a criterion of similarity higher than 82%.

Table 1 Vaccine strains used in this study

Vaccine strain	Origin of the strain	Year of isolation	wP vaccine–manufacturing countries in Latin America	
			Before 1996	At present
Tohama I	Japan	1954	Chile	None
*Bp*509	the Netherlands	1950	Cuba Mexico Venezuela	Cuba Mexico Venezuela
*Bp*10536	USA	Before 1940	Argentina Colombia	None
*Bp*137	USA	No data available	Ecuador Uruguay Brazil	Brazil Ecuador

Table 2 Primers used in this study

Gene	Primer sequence	References
*ptx*P	F: 5′-AATCGTCCTGCTCAACCGCC-3′ R: 5′-GGTATACGGTGGCGGGAGGA-3′	Schouls *et al.* (2004), Mooi *et al.* (2009)
*ptx*A	F: 5′-CCCCTGCCATGGTGTGATC-3′ R: 5′-TCAATTACCGGAGTTGGGCG-3′	Fiett *et al.* (2003)
prn	F: 5′-CAATGTCACGGTCCAA-3′ R: 5′-GCAAGGTGATCGACAGGG-3′	Mooi *et al.* (2000)
fim2	F: 5′-GCGCCGGGCCCTGCATGCAC-3′ R: 5′-GGGGGGTTGGCGATTTCCAGTTCTC-3′	Van Loo and Mooi (2002), Borisova *et al.* (2007)
fim3	F: 5′-GACCTGATATTCTGATGCCG-3′ R: 5′-AAGGCTTGCCGGTTTTTTTTGG-3′	Borisova *et al.* (2007)

Serotyping

Serotype analysis was performed using an agglutination assay with monoclonal antibodies against type 2 fimbriae (Fim2; NIBSC, 04/154) and type 3 fimbriae (Fim3, NIBSC, 04/156) according to EU pertstrain group recommendations (http://www.eupertstrain.org). Briefly, 15 μl of bacterial suspension in PBS was mixed on slide with an equal volume of 1/10 dilution of monoclonal antibodies against Fim2 and 1/100 dilution of monoclonal antibodies against Fim3. If the agglutination reaction was obtained with either Fim2, Fim3, or both antibodies, the serotype was defined as Fim2, Fim3 or Fim2,3, respectively. If no reaction was detected, the serotype was defined as untypeable. Autoagglutination was examined with phosphate-buffered saline in parallel with monoclonal antibodies.

Membrane protein enrichment for two-dimensional polyacrylamide gel electrophoresis (2-DE)

Membrane fractions were prepared as described previously (Bottero *et al.* 2007). Briefly, *Bord. pertussis* cells were harvested by centrifugation (10 000 ***g***; 30 min; 4°C) and washed twice with low-salt washing buffer containing 3 mmol l^{-1} KCl, 68 mmol l^{-1} NaCl, 1·5 mmol l^{-1} KH_2PO_4 and 9 mmol l^{-1} NaH_2PO_4. The cells were suspended in 10 mmol l^{-1} Tris–HCl (pH 8·5) supplemented with phenylmethylsulphonyl fluoride and protease inhibitor cocktail tablets (Roche Applied Science, Buenos Aires, Argentina) and then disrupted with an ultrasonicator (Sonics & Materials, Inc., Danbury, CT, USA). DNase and RNase (20 μg ml^{-1} 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 ***g***; 30 min; 4°C), and the supernatant was retained. Total membrane proteins were then collected by centrifugation (30 000 ***g***, 1 h; 4°C) and resuspended in 7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 10% isopropanol and 2% Triton X-100. Membrane proteins were divided into aliquots and stored at −20°C.

Sample preparation, 2-DE and protein identification were repeated at least four times for each strain.

Protein quantification

Protein concentrations were determined by the Bradford's method (Bradford 1976) with bovine serum albumin (Sigma) as a standard.

2-DE

The method previously described by Bottero *et al.* (2007) was followed. Seven-centimetre Immobiline DryStrip (IPG, pH 4–7; Amersham Biosciences) dissolving 200 μg of the membrane proteins in a volume of 125 μl of rehydration buffer (7 mol l^{-1} urea, 2 mol l^{-1} thiourea, 10% isopropanol and 2% Triton X-100) plus 1·25 μl 28% dithiothreitol (DTT), 0·62 μl 0·5% ampholyte (pH 4·0–7·0 [Amersham]) and 0·01% bromophenol blue was rehydrated overnight at room temperature. Three preset programmes were executed with slight modifications so that the focusing conditions consisted of the conditioning step, voltage ramping and final focusing. After IEF, the strips were equilibrated in 50 mmol l^{-1} Tris buffer (pH 8·8) containing 6 mol l^{-1} urea, 2% sodium dodecyl sulphate, 30% glycerol and 1% DTT, followed by another 1-h equilibration step with the same buffer supplemented with 4·5% iodoacetamide. SDS-PAGE was performed according to (Laemmli 1970) 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 of the gel.

Proteins were visualized using a colloidal Coomassie staining method (http://prospector.ucsf.edu) with the modifications described previously (Bottero *et al.* 2007). A gel image was captured in a UVP Bioimaging system Epi Chemi3 Darkroom with a Hamamatsu Photonic systems camera, model 1394 C8484-51-03G, controlled by Labworks image acquisition and analysis software version 4.6.00.0. The 8-bit grey-scale tif files obtained were later processed with the Image Master 2D Platinum software ver. 6.0 (GE Heathcare Argentina S.A., CABA, Argentina).

MALDI-TOF-MS analysis and database search

Coomassie-stained spots were excised from 2-DE gels for tryptic in-gel digestion and MALDI-TOF-MS with an Ultraflex (Bruker) (Bottero *et al.* 2007). Peptide mass fingerprint (PMF) data were searched against the NCBI database in MASCOT server (http://www.matrixscience.com) for sequence match. The MASCOT search parameters were as follows: (i) species, bacteria (eubacteria); (ii) allowed number of missed cleavages (only for trypsin digestion), 1; (iii) variable post-translational modification, methionine oxidation; (iv) fixed modification, carbamidomethylation; (v) peptide tolerance, ±50 ppm; (vi) peptide charge, +; and (vii) monoisotopic peptide masses that were used to search the database, allowing a molecular mass range for 2-DE analyses of ±15%. Only significant hits as defined by MASCOT probability analysis were considered. Prediction of protein localization was carried out using a PSORTb.2, PSORTb.3 algorithm available at http://psort.nibb.ac.jp.and Proteome Analyst (PA) (Lu *et al.* 2004).

Results

Genotypic analysis

Chromosomal DNA samples from *Bp*137 and two other vaccine strains (*Bp*10536 and *Bp*509) used in some Latin America countries were digested with *Xba*I and examined by PFGE. The profiles obtained were compared with that from the reference strain Tohama I (Fig. 1a). The profiles were distributed in three groups classified according to a criterion of similarity higher than 80%. The vaccine strain *Bp*137 grouped with *Bp*509 in PFGE group III. The similarity between these strains was 83%. Group I included *Bp*10536, and group II was composed of the Japanese vaccine strain Tohama I.

The representative isolate *Bp*106 collected after the introduction of a massive vaccination programme in Argentina is clearly separated from vaccine strains as we previously reported (Bottero *et al.* 2007).

Regarding the genotypification of well-known polymorphic sequences described for virulence factors of *Bord. pertussis*, vaccine strains *Bp*137 and *Bp*509 present pertussis toxin promoter *ptx*P2 and the allele *fim*2-2. These genotypes are different from those of the other vaccine strains (Fig. 1b).

In contrast, the representative clinical isolate *Bp*106 contained *ptx*P3, *ptx*A1, *prn*2, *fim2*-1 and *fim3*-B alleles. In fact, we observed this genotype in the majority of the current members of our collection of circulating clinical isolates (data not shown). Regarding the *fim*2 and *fim3* alleles, 97% of the collection, including the *Bp*106 representative strain, is *fim*2-1 and 76% has the variant B for the *fim*3 allele. In relation to the *fim*3 allele, the vaccine strains included in our study have the variant A.

The Fim serotypes are Fim2 for Tohama I strain and Fim2,3 for *Bp*10536, *Bp*137 and *Bp*509. In our study, the Fim serotype for *Bp*106 and for 97% of clinical isolates of our collection was Fim3.

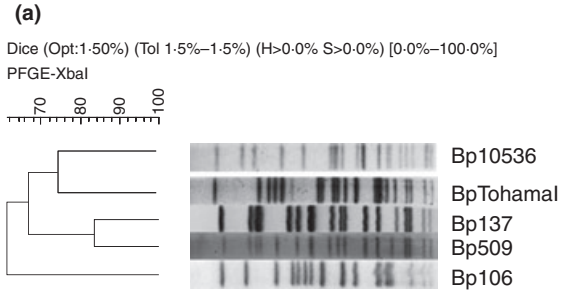
Proteomic analysis

We characterized *Bp*137 strain by proteomic analysis and compared its surface proteome with the proteomes of the other strains previously reported but repeated here (Bottero *et al.* 2007; Supporting Information, Fig. S1). In the analysis, we also included the data of human and murine immunoproteomes already performed (Altindis *et al.* 2009; Zhu *et al.* 2010; Tefon *et al.* 2011).

The 2-DE profile of *Bp*137 revealed more than 90 protein spots from which 50 proteins were successfully identified (Fig. 2). For this work, we have repeated the 2-DE of surface proteins of the other four strains (*Bp*10536, *Bp*509, Tohama I and *Bp*106). In all instances, we have confirmed previously published data, but in addition, we have identified more spots (64 spots in total). Of the total identified peptide subunits, 12 were predicted to be associated with the external membrane/extracellular localization, ten had periplasmic localization, nine had cytoplasmic membrane localization, eight had an unknown or undefined origin and 25 had a cytoplasmic localization (Table 3). As observed for the other vaccine strains, 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 the following proteins: EF-Tu, 60-kDa chaperonin, outer membrane porin protein precursor, serum resistance protein and serine protease. These may represent natural isoforms or an artefact caused by sample preparation or 2-DE.

From the proteins identified by MALDI–TOF-MS, 14 are involved in small-molecule metabolism (BP2360, BP0277, BP2439, BP2386, BP3288, BP3125, BP0995, BP0379, BP3215, BP1126, BP0844, BP1499, BP0843 and BP0047), seven are associated with macromolecule biosynthesis and degradation (BP2434, BP0007, BP3642, BP2361, BP1420, BP1455 and BP2470), 15 are classified in the category cell structure (BP1146, BP1296, BP3405,

(a)



(b)

Strain/Isolate	Allelic variant of					Fimbrial Serotype
	ptxP	*ptxA*	*prn*	*fim2*	*fim3*	
TohamaI	1	2	1	1	A	2
*Bp*509	2	4	7	2	A	2, 3
*Bp*10536	1	2	1	1	A	2, 3
*Bp*137	2	4	1	2	A	2, 3
*Bp*106	3	1	2	1	B	3

Figure 1 (a) Genomic analysis of *Bordetella pertussis* strains used for vaccine production. The chromosomal DNA profiles obtained after digestion with *XbaI* are shown on the left side and the identifier of strains on the right side. (b) Characteristics of vaccine strains used in this work.

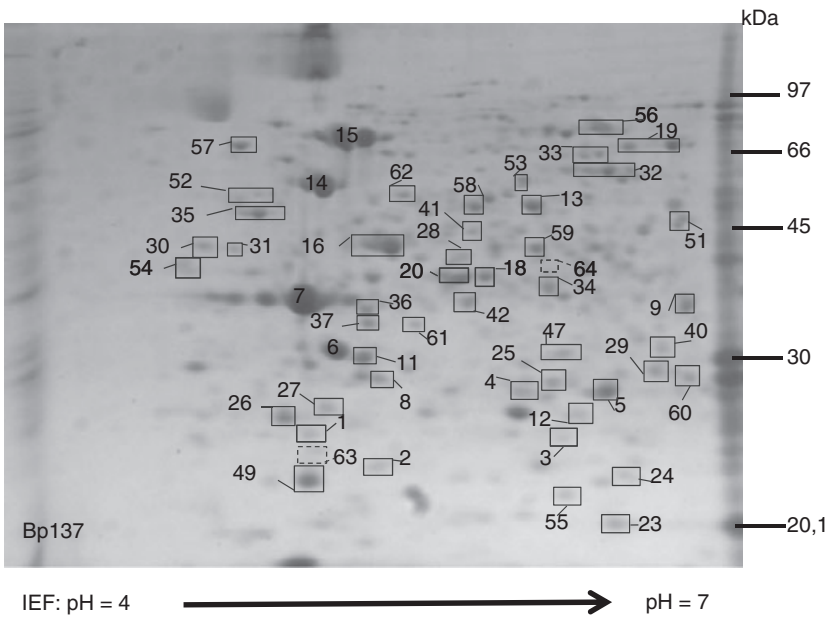


Figure 2 2-D proteome of *Bordetella pertussis* vaccine strain Bp137. Preparations of membrane-enriched protein samples were separated by IEF at pH 4–7 in the first dimension and then by 12·5% SDS-PAGE in the second dimension. Protein spots were visualized by colloidal Coomassie staining. The spot numbers refer to the identified peptide subunits by MALDI-TOF.

BP0840, BP1440, BP3862, BP0943, BP2513, BP2755, BP3150, BP1630, BP2750, BP3559, BP3077 and BP1485), 14 are associated with cellular processes (BP3757, BP1487, BP3322, BP0965, BP3495, BP1285, BP2761, BP3794, BP2747, BP3552, BP1774, BP2235, BP2499 and BP2744), two have general regulatory roles (BP2483 and BP2435), three are associated with phages, transposons and pathogenicity islands (BP2667, BP3494 and BP1054) and, finally, six have unknown function (BP3441, BP2196, BP3128, BP3515, BP2964 and BP1203) according to Riley categories (Riley 1993).

Twenty of the 64 identified proteins were not detected in at least one of the strains studied, and four proteins were detected only in the local isolate *Bp*106 (Table 3). Tohama I and *Bp*509 have very similar protein profiles with only one differential subunit peptide (spot 10). However, these two strains share the expression of only three of the 24 differential proteins with *Bp*137. Interestingly, we note that seven of 24 peptide subunits were expressed exclusively by the vaccine strain *Bp*137 and the clinical isolate *Bp*106. Peptide subunit Bp2235 (spot53), a potential protein of type III secretion system (TTSS), belongs to this group of seven subunits. Two other proteins identified only in *Bp*106 and *Bp*137, but not detected in the rest of the vaccine strains, are BP3150 and BP1630, assigned to polysaccharide biosynthesis and capsule biosynthesis (spot 41 and spot 42, respectively, in *Bp*137).

Human immunoproteomic data recently published (Zhu *et al.* 2010) include 16 of the 64 polypeptides here identified, indicating that they are immunogenic (Table 3). Other ten were detected to be reactive against murine immune serum. Five of them were reactive against both sera. Three of the five are present in all the strains here included and correspond to well-known antigens of *Bord. pertussis*: 60-kDa chaperonin (spot 14), pertactin (spot 19) and serum resistance protein (spot 32) (Altindis *et al.* 2009; Zhu *et al.* 2010; Tefon *et al.* 2011) (Table 3). Other proteins such as BP1285, BP3642 and BP0844 are among the differential proteins here detected.

Discussion

Here, we showed that the PFGE of the *Bord. pertussis* strain *Bp*137 and two other strains included in wP vaccines in Latin America were distributed in three groups classified according to a criterion of similarity higher than 80%. Although this observation of the vaccine strain PFGE profiles is similar to that previously reported in other countries (Caro *et al.* 2005), it is still important for our region. The current PFGE classifies strains that were not studied before and that are currently included in the national immunization schedules of Latin American countries (e.g. the Brazilian vaccine strain *Bp*137 and strain *Bp*10536, which is included in vaccines used in Argentina). The representative isolate *Bp*106, collected after the introduction of generalized vaccination in Argentina, is clearly separated from vaccine strains as we previously reported (Bottero *et al.* 2007).

Table 3 Surface proteome of *Bordetella pertussis* vaccine strains and an Argentinean clinical isolate Bp106. Numbers in parentheses indicate corresponding spot number of Fig. 2

GI:	Gene locus†	Localization‡	Protein name/function	MW (kDa)	pI	Spot detection in strain (spot number in Bottero *et al.* 2007 or this work)				Murine serum reactive	Human serum reactive	Spot detection in strain (spot number in Bottero *et al.* 2007)*
						Bp137	Bp509	Bp10536	Tohama I			Bp106
33592278	*bp1146*	Outer membrane	Competence lipoprotein precursor	29·8	5·0	Yes(1)	Yes	Yes	Yes			Yes
33594323	*bp3441*	Cytoplasmic membrane	Conserved hypothetical protein	19·8	5·1	Yes(2)	Yes	Yes	Yes			Yes
33593636	*bp2667*	Outer membrane	Adhesin	263·6	9·7	Yes(3)	Yes	Yes	Yes			Yes
33592419	*bp1296*	Unknown	Putative lipoprotein	30·6	7·4	Yes(4)	Yes	Yes	Yes			Yes
33593352	*bp2360*	Not defined	Succinate dehydrogenase catalytic subunit	27·2	6·2	Yes(5)	Yes	Yes	Yes			Yes
33594289	*bp3405*	Outer membrane	Outer membrane protein OMPQ	39·1	5·7	Yes(6)	Yes	Yes	Yes		Yes	Yes
33592006	*bp0840*	Outer membrane	Outer membrane porin protein precursor	41·0	5·4	Yes(7)	Yes	Yes	Yes		Yes	Yes
33594616	*bp3757*	Cytoplasmic membrane	Putative ABC transport ATP binding protein	29·6	5·1	Yes(8)	Yes	Yes	Yes			Yes
33592580	*bp1487*	Periplasmic	Putative periplasmic solute binding protein	40·0	7·8	Yes(9)	Yes	Yes	Yes		Yes	Yes
33594215	*bp3322*	Periplasmic	Putative binding protein-dependent transport protein	40·9	6·9	No	No	Yes(10)	Yes(10)			Yes(10)
33592538	*bp1440*	Cytoplasmic membrane	Putative membrane protein	33·4	5·3	Yes(11)	Yes	Yes	Yes			Yes
33592121	*bp0965*	Cytoplasmic	Antioxidant protein	23·7	5·7	Yes(12)	Yes	Yes	Yes			Yes
33593418	*bp2434*	Periplasmic	Serine protease	52·1	8·8	Yes(13)	Yes	Yes	Yes	Yes		Yes
33594370	*bp3495*	Cytoplasmic	Chaperonin 60 kDa	57·4	4·9	Yes(14)	Yes	Yes	Yes	Yes	Yes	Yes
33594369	*bp3494*	Outer membrane	Serum resistance protein	103·3	7·1	Yes(15)	Yes	Yes	Yes	Yes	Yes	Yes
33591281	*bp0007*	Cytoplasmic	Elongation factor Tu	42·9	5·1	Yes(16)	Yes	Yes	Yes		Yes	Yes
33592409	*bp1285*	Periplasmic	Leu/Ile/Val protein precursor	39·6	6·8	No	Yes(17)	No	Yes(17)	Yes	Yes	No
33594507	*bp3642*	Cytoplasmic	DNA direct RNA α subunit polymerase	36·1	5·7	Yes(18)	Yes	No	Yes	Yes		Yes
33592195	*bp1054*	Outer membrane	Pertactin	93·4	10·0	Yes(19)	Yes	Yes	Yes	Yes	Yes	Yes
3593200	*bp2196*	Outer membrane	Putative quino protein	40·0	8·7	Yes(20)	Yes	Yes	Yes			Yes
33594713	*bp3862*	Cytoplasmic membrane	Putative extracellular solute binding protein	57·3	9·7	No	Yes(21)	Yes(21)	Yes(21)			No
33599458	*bb0468*	Periplasmic	Putative molybdopterin oxidoreductase	121·6	7·3	No	No	Yes(22)	No			No

Table 3 (*Continued*)

GI:	Gene locus†	Localization‡	Protein name/function	MW (kDa)	pI	Spot detection in strain (spot number in Bottero *et al.* 2007 or this work)				Murine serum reactive	Human serum reactive	Spot detection in strain (spot number in Bottero *et al.* 2007)*
						Bp137	Bp509	Bp10536	Tohama I			Bp106
33592100	*bp0943*	Outer membrane	Outer membrane protein A precursor	20·9	9·2	Yes(23)	Yes	Yes	Yes			Yes
33593721	*bp2761*	Periplasmic	Superoxide dismutase	21·2	6·5	Yes(24)	Yes	Yes	Yes	Yes		Yes
33593496	*bp2513*	Periplasmic	Putative exported protein	34·9	10·2	Yes(25)	Yes	Yes	Yes			Yes
33591513	*bp0277*	Cytoplasmic membrane	Ubiquinol cytochromo C reductase iron sulfur subunit	22·8	5·2	Yes(26)	Yes	Yes	Yes			Yes
33593715	*bp2755*	Cytoplasmatic/ membrane	Putative exported protein	189·0	6·2	Yes(27)	Yes	Yes	Yes			No
33593423	*bp2439*	Cytoplasmatic	3-oxoacyl-(acyl carrier protein) synthase	43·6	5·7	Yes(28)	No	No	No			Yes
33594649	*bp3794*	Unknown/multiple localization	Putative bacterial secretion system protein	29·4	6·8	Yes(29)	Yes	Yes	Yes		Yes	Yes
33593375	*bp2386*	Cytoplasmic	Enolase	45·9	4·5	Yes(30)	Yes	Yes	Yes			Yes
33593466	*bp2483*	Cytoplasmic membrane	Two-component sensor protein	97·4	8·7	Yes(31)	Yes	No	Yes			No
33594369	*bp3494*	Outer membrane	Serum resistance protein	103·3	7·1	Yes(32)	Yes	Yes	Yes	Yes	Yes	Yes
33593353	*bp2361*	Cytoplasmic membrane	Succinate dehydrogenase flavo subunit	64·8	6·5	Yes(33)	Yes	Yes	Yes		Yes	Yes
33563780	*bp2747*	Periplasmic	Putative ABC transport solute binding protein	40·6	6·5	Yes(34)	Yes	Yes	Yes		Yes	Yes
33594186	*bp3288*	Cytoplasmic	ATP synthase subunit B	50·5	4·7	Yes(35)	Yes	Yes	Yes	Yes		Yes
33592518	*bp1420*	Cytoplasmic	Elongation factor Ts	30·9	5·1	Yes(36)	Yes	Yes	Yes		Yes	Yes
33594046	*bp3125*	Cytoplasmic	Ribose phosphate pyrophosphokinase	34·1	5·1	Yes(37)	No	No	No			Yes
33592145	*bp0995*	Cytoplasmic	Dihydrolipoamide dehydrogenase	62·3	5·8	No	No	No	No			Yes(38)
33594049	*bp3128*	Unknown/multiple localization	Hypothetical protein	68·5	6·1	No	Yes(39)	Yes(39)	Yes(39)			Yes(39)
33594387	*bp3515*	Cytoplasmic	Hypothetical protein	35·9	6·6	Yes(40)	Yes	Yes	Yes			Yes
33594071	*bp3150*	Cytoplasmic	Polysaccharide biosynthesis protein	46·7	5·6	Yes(41)	No	No	No			Yes
33592714	*bp1630*	Cytoplasmic	Capsular polysaccharide biosynthesis protein	37·3	5·5	Yes(42)	No	No	No			Yes
33594122	*bp0379*	Cytoplasmic	Putative L lactacto dehydrogenase	37·2	5·8	No	No	No	No		Yes	Yes(43)

Table 3 (*Continued*)

GI:	Gene locus†	Localization‡	Protein name/function	MW (kDa)	pI	Spot detection in strain (spot number in Bottero *et al.* 2007 or this work)				Murine serum reactive	Human serum reactive	Spot detection in strain (spot number in Bottero *et al.* 2007)*
						Bp137	Bp509	Bp10536	Tohama I			Bp106
33593899	*bp2964*	Cytoplasmic	Hypothetical protein	48·5	6·2	No	Yes(44)	No	Yes(44)			Yes(44)
33592332	*bp1203*	Unknown	Hypothetical protein	42·7	6·0	No	Yes(45)	Yes(45)	Yes(45)			Yes(45)
33593419	*bp2435*	Periplasmic	Putative sigma factor regulatory protein	39·2	9·6	No	No	No	No			Yes(46)
33594122	*bp3215*	Cytoplasmic membrane	Enoyl-acyl carrier protein	27·6	5·8	Yes(47)	Yes	Yes	Yes			Yes
33593710	*bp2750*	Unknown	Lipoprotein	23·1	7·7	No	Yes(48)	Yes(48)	Yes(48)			Yes(48)
33594422	*bp3552*	Cytoplasmic	Alkyl hydroperoxide reductase	20·1	4·9	Yes(49)	Yes	Yes	Yes			Yes
33591361	*bp0102*	Periplasmic	Putative penicillin binding protein precursor	44·9	7·8	No	No	No	No		Yes	Yes(50)
33571906	*bp1126*	Cytoplasmic	2-oxoglutarate dehydrogenase complex. E3 component	50·3	6·3	Yes(51)	No	No	No			Yes
33592841	*bp1774*	Cytoplasmic	Trigger factor	47·5	4·9	Yes(52)	Yes	Yes	Yes			Yes
3593235	*bp2235*	Outer membrane	Putative type III secretion system	63·3	5·9	Yes(53)	No	No	No			Yes
33564503	*bp3559*	Not defined	Hypothetical protein	37·9	4·7	Yes(54)	Yes	Yes	Yes			Yes
33592552	*bp1455*	Cytoplasmic	Probable phosphoglycerate mutase 2	23·8	5·9	Yes(55)	Yes	Yes	Yes			Yes
33594004	*bp3077*	Outer membrane	Putative outer membrane protein	77·7	6·1	Yes(56)	Yes	Yes	Yes			Yes
39931027	*bp2499*	Cytoplasmic	Molecular chaperone DnaK	69·7	4·9	Yes(57)	Yes	Yes	Yes	Yes		Yes
33592578	*bp1485*	Extracellular	Putative membrane protein	51·6	6·8	Yes(58)	Yes	Yes	Yes			Yes
33592010	*bp0844*	Cytoplasmic	NADH dehydrogenase delta subunit	47·7	5·8	Yes(59)	Yes	No	Yes		Yes	Yes
33593704	*bp2744*	Not defined	Putative ABC transport protein. ATP binding component	29·1	6·3	Yes(60)	Yes	Yes	Yes			Yes
33592591	*bp1499*	Cytoplasmic	Glutathione synthetase	34·7	5·4	Yes(61)	No	No	No			Yes
33593453	*bp2470*	Cytoplasmic	Seryl-tRNA synthetase	50·0	5·4	Yes(62)	Yes	Yes	Yes			Yes
33592009	*bp0843*	Cytoplasmic	NADH dehydrogenase subunit C	24·1	5·1	No	Yes(63)	Yes(63)	Yes(63)			Yes(63)
33591314	*bp0047*	Cytoplasmic	Homoserine *O*-acetyltransferase	44·9	5·7	No	Yes(64)	No	Yes(64)			Yes(64)

*Numbers in parentheses correspond to spot number in this work or from Bottero *et al.* (2007).

†Gene loci are named according to NCBI (http://www.ncbi.nlm.nih.gov/).

‡Protein localization is as predicted by PSORT (http://psort.nibb.ac.jp).

As expected, the above-mentioned *Bord. pertussis* wP vaccine strains contain the characteristic *ptx*A, *prn* and *fim*3 gene alleles of the old *Bord. pertussis* strains (Fig. 1b) (Cassiday *et al.* 2000; Gzyl *et al.* 2001; Hardwick *et al.* 2002a; Fiett *et al.* 2003). The vaccine strains *Bp*137 and *Bp*509, however, present different characteristics from those of the other vaccine strains: pertussis toxin promoter *ptx*P2 and the allele *fim*2-2 instead *ptx*P1 and *fim*2-1. The *ptx*P2 allele was found in the Netherlands at a frequency of 43% and in the United States at 29% during the prevaccination period. In the Netherlands, this allele was also detected during the 1999–2000 period, but at a very low frequency (0·003%). Bart *et al.* (2010) showed that strains that harbour this *ptx*P2 allele represented a distinct lineage that diverged from other strains relatively early in the evolutive history of *Bord. pertussis*. The *ptx*P2 and also *ptx*P1 strains are nearly completely replaced in the late 1990s by the *ptx*P3 strains. In the Netherlands, the increase in the frequency of *ptx*P3 strains was associated with the resurgence of pertussis. The *ptx*P3 strains produced more Ptx than the *ptx*P1 strain, and epidemiological data suggest that *ptx*P3 strains are more virulent. The *ptx*P3 strains have spread worldwide, being the predominant allele in our country (Mooi *et al.* 2009; Bart *et al.* 2010).

Regarding circulating bacteria, we observed that *Bp*106, as well as the majority of the current members of our collection, contains *ptx*P3, *ptx*A1, *prn*2, *fim2*-1 *and fim3*-B alleles (data not shown). The replacement of *ptx*P1, *ptx*A2 or *ptx*A4, *prn*1 or *prn*7 strains by *ptx*P3, *ptx*A1 and *prn*2 strains in recent times is a global phenomenon that has been observed in other countries (van Gent *et al.* 2009; Kallonen and He 2009; Mooi 2010; Advani *et al.* 2011).

Regarding the *fim*2 allele, 97% of the collection, including the *Bp*106 representative strain, is *fim*2-1. This finding agrees with observations made in the UK, where *fim*2-1 has been the prevalent allele since 1920, and in the Netherlands, where it has been the prevalent allele since 1965 (Van Loo and Mooi 2002; Packard *et al.* 2004). In relation to the *fim*3 allele, vaccine strains included in our study have the variant A, which was found in 24% of the isolates of our collection. The representative local strain, *Bp*106, has the allele B, similar to 76% of the circulating bacteria. This finding agrees with results from Finland prior to 1999, Canada prior to 1990 and Russia prior to 1969, as all isolates in those countries at those times contained the variant A. Isolates obtained from those countries after those years contained the predominant allele B (Tsang *et al.* 2004; Kallonen and He 2009).

The Fim serotypes are Fim2 for Tohama I strain and Fim2,3 for *Bp*10536, *Bp*137 and *Bp*509. In our study, the Fim serotype for *Bp*106 and for 97% of clinical isolates was Fim3. The serotype for these circulating bacteria correlated with observations in other populations where Fim3 is the most frequent (Tsang *et al.* 2004; Heikkinen *et al.* 2008; Kallonen and He 2009; Kurova *et al.* 2010; Zhang *et al.* 2010; Advani *et al.* 2011).

Regarding the proteomic analysis here performed, the 2-DE profile of *Bp*137 revealed more than 90 protein spots from which 50 proteins were successfully identified (Fig. 2). Sixteen polypeptides from the total identified seem to be immunogenic in humans as it was recently published (Zhu *et al.* 2010).

Comparative analysis of the proteomes showed that *Bp*137 and *Bp*106 present seven proteins that are not detected in the other strains. One of these seven proteins is BP2235, which is a potential protein of TTSS (spot 52). This result is striking because previously we found that the TTSS is expressed in bacteria that have recently been in contact with the host, whereas in laboratory-adapted vaccine strains, this expression would not occur (Gaillard *et al.* 2011). In contrast to those findings, here we observed the expression of TTSS components in the vaccine strain *Bp*137, even when this strain is adapted to growth in laboratory conditions. This result suggests that the expression of TTSS in this strain is governed by a different regulatory mechanism than in other vaccine strains. Whatever the molecular mechanism, whose identification is not within the scope of this work, the expression of TTSS components in *Bp*137 is a desirable feature in a vaccine strain, not only because the TTSS is immunogenic but also because it shares a property with circulating clinical isolates (Fennelly *et al.* 2008; Medhekar *et al.* 2008; Zongfu Wu *et al.* 2008).

Two other proteins identified only in *Bp*106 and *Bp*137 but not detected in the rest of the vaccine strains are BP3150, which is assigned to polysaccharide biosynthesis, and BP1630, which is assigned to capsule biosynthesis (spot 41 and spot 42, respectively, in *Bp*137). Bacterial capsules allow pathogens to evade host defences. The expression of capsule proteins in these strains, therefore, could indicate the need to overcome the host immune response induced by vaccination.

Our results show that, among the vaccine strains studied here, the strain *Bp*137 is the one that shares the highest number of proteins detected in the surface proteome with the representative circulating bacteria *Bp*106. Interestingly, some of these common proteins have immunogenic properties. Based on these results and taking into account the previous reports showing that phenotypic and genotypic divergence between strains could have an impact in protection (King *et al.* 2001; Bottero *et al.* 2007), we suggest that vaccines containing *Bp*137 could be appropriate to improve the control of pertussis in our region.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Proteome reference map of *Bordetella pertussis* vaccine strains *B*p509, Tohama I, *Bp*10536 and clinical isolate *Bp*106.

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