genomics profiling revealed that the genome of *B. pertussis* Tohama differs from clinical isolates in four regions (RD11 to RD14) [25]. In contrast, progressive gene loss mediated by homologous recombination between IS*481* insertion sequence elements has been observed among recently circulating strains of *B. pertussis* isolates [26,27]. IS*481* is present in multiple copies on the *B. pertussis* chromosome, and it plays a critical role in *B. pertussis* evolution through genomic rearrangement.

Proteomic analysis has been widely applied to comparisons of protein expression among different strains, and information accumulated from genomic studies of *Bordetella* spp. facilitates comparative proteomic approaches to the investigation of *B. pertussis* clinical strains [6,28]. In the present study, a proteomic approach was employed to identify the protein(s) involved in the genetic shift from vaccine-type to nonvaccine-type in *B. pertussis* strains. The protein profile analyses identified one differentially expressed protein, the T3SS effector BteA (alias BopC) [29,30], between the strain types. BteA is a 68 kDa cytotoxic effector that has been identified in *B. bronchiseptica* but not in the *B. pertussis* human pathogen. Here we studied the differential expression of BteA protein in *B. pertussis* clinical strains and identified a specific IS*481* insertion in the 5′ untranslated region (5′-UTR) of *bteA* in vaccine-type strains.

Results

Identification of BteA in *B. pertussis* nonvaccine-type strain

A comparative proteomic analysis of two clinical strains was performed to investigate the shift of *B. pertussis* strains from vaccine-type to nonvaccine-type. Figure 1 shows 2-dimensional electrophoretic (2-DE) maps of total protein expressed in the nonvaccine-type clinical strain BP235 and the vaccine-type BP233. Among >600 protein spots detected on the 2-DE gel, one was notably absent in the 2-DE map of BP233. The protein spot was observed in other nonvaccine-type strains (BP157, BP159, BP162 and BP228), but not in other vaccine-type strains (BP155, BP156, BP232 and BP243). The protein represented by the spot was identified by LC-MS/MS analysis using tryptic digests. The MS/MS of the protein digests provided four peptide sequences (RPDEFAAR, FDALR, ITALNLR and TQTQLLALQR) that matched the *B. pertussis* hypothetical protein BP0500 (NCBI accession: NP_879352). Hypothetical protein BP0500 was identified as the T3SS effector BteA, since the sequence is highly conserved with 98% amino acid identity to the BteA (BopC) of *B. bronchiseptica* [29,30].

High expression of BteA protein in nonvaccine-type strains

Immunoblots of *B. pertussis* clinical strains using anti-BteA antiserum detected high levels of a protein of ~68 kDa in all nonvaccine-type clinical strains (BP157, BP159, BP162, BP228 and BP235), whereas BteA expression was greatly reduced in the vaccine-type clinical strains (BP155, BP156, BP232, BP233 and BP243). Additional products of >200 kDa were also detected in the nonvaccine-type clinical strains. These high molecular mass signals appear to be the protein bands that have been reported as a multimeric complex of BteA in *B. bronchiseptica* [29,30] (see Figure S1). T3SS function in the nonvaccine-type strains was confirmed

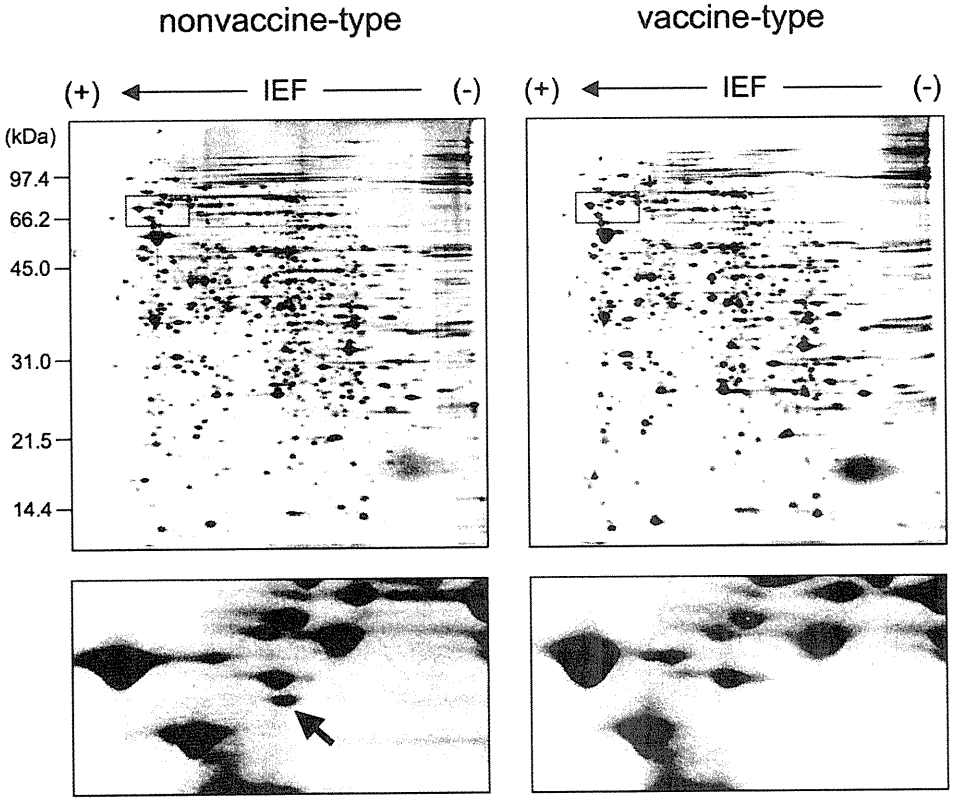

Figure 1. Comparative proteomic analysis of *B. pertussis* nonvaccine-type and vaccine-type strains. Total protein (10 μg) from the nonvaccine-type and vaccine-type clinical strains was separated by 2-D gel electrophoresis and silver stained. The left upper panel shows the protein profile of the nonvaccine-type BP235. The right upper panel shows the protein profile of the vaccine-type BP233. The red-boxed areas are enlarged (lower panels). The arrow in left lower panel indicates the spot that was identified as type III effector BteA by LC-MS/MS analysis.
doi:10.1371/journal.pone.0017797.g001

by using whole cell protein extracts for immunoblots of BtcA (the BtcA chaperone) [29,31] and BopD (the T3SS translocon) [32]. BtcA and BopD polypeptides were detected in both strain types, but the BtcA signals produced by the nonvaccine-type strains were apparently lower than those of the vaccine-type strains (Figure 2). The reason for the different expression is not clear. In contrast, adenylate cyclase toxin (ACT), another *Bordetella* spp. virulence factor, was detected at similar levels in both strain types.

In order to confirm BteA secretion by *B. pertussis* strains, BteA polypeptide in the culture supernatants (CS) was subjected to immunoblot analysis. BteA was detected in secreted proteins from the nonvaccine-type clinical strain BP159 at 12, 24 and 48 h, whereas the signal was very low in the vaccine-type clinical strain BP155 over the 48-h time period (Figure 3). Conversely, signals corresponding to PT-S1 subunit and FHA polypeptides were detected in the supernatants of both cultures throughout the sampling period, although silver staining revealed small differences in their protein profiles after 24 h in culture.

Transcription of *bteA*

bteA gene expression in *B. pertussis* strains was investigated with conventional RT-PCR and quantitative RT-PCR. *bteA* was transcribed in both the nonvaccine-type (BP157, BP159, BP162, BP228 and BP235) and vaccine-type (BP155, BP156, BP232, BP233 and BP243) clinical strains (Figure 4A). Similarly, *btcA* transcripts were detected in both strain groups. RT-PCR experiments lacking reverse transcriptase showed no specific product for *bteA* amplification, confirming negligible genomic DNA contamination in the RNA preparations. Quantitative RT-PCR (qRT-PCR) showed an average *bteA* transcript level of 0.146 (±1SD range, 0.107 to 0.184) in nonvaccine-type strains and 0.095 (±1SD range, 0.076 to 0.113) in vaccine-type clinical strains, a difference that was not statistically significant ($P = 0.11$) (Figure 4B).

IS*481* insertion in the *bteA* 5′-UTR in vaccine-type strains

Sequencing of the *bteA* 5′-UTR of the five vaccine-type strains (BP155, BP156, BP232, BP233 and BP243), revealed a 1,043-bp insertion sequence (IS*481*) −136 bp upstream of the *bteA* start codon (Figure 5A). IS*481a*, which is newly identified in *B. pertussis*, showed 99% nucleotide sequence identity with IS*481* of *B. pertussis* Tohama. The CCTAAC sequence in the *bteA* 5′-UTR is an insertion site of IS*481a* and is duplicated by the insertion, although the 6-bp consensus recognition sequence of IS*481* has been reported as NCTAGN [33]. IS*481* insertions were not found in the nonvaccine-type clinical strains, which had nucleotide sequences that were 99% identical to that of *B. pertussis* Tohama. In the *bteA* 5′-UTR of the nonvaccine-type strains (BP157, BP159, BP162 BP228 and BP235), one single nucleotide polymorphism (A→G) was observed at 207 bp upstream of the *bteA* translation start site (Figure 5B).

The *bteA* 5′-UTR was PCR-amplified from chromosomal DNA of other *B. pertussis* strains to confirm insertion of IS*481*. Among 61 vaccine-type clinical strains, 60 (98%) produced amplicons of ~3.1 kb, a size indicative of an IS*481* insertion in the *bteA* 5′-UTR. One strain (BP121) had a product of ~2.1 kb, corresponding to the predicted size of the native 5′-UTR (data not shown). Of the 23 nonvaccine-type strains examined, all generated ~2.1 kb amplicons, confirming the absence of the IS*481* insertion.

Determination of the *bteA* transcription start site

5′-RACE mapping was used to identify the *bteA* transcription start site in vaccine-type strain BP155. Nucleotide sequences of the 5′-RACE PCR products revealed two transcription start sites, P1 and P2, located −68 and −147 bp from the *bteA* translation start codon (Figure 5A). The P1 start site (+1) was located within the *bteA* 5′-UTR, whereas the P2 start site (−79) was located within IS*481a*. Only the P1 start site was also found in the nonvaccine-type strain BP159 (Figure 5B). IS*481* contains an outward-facing

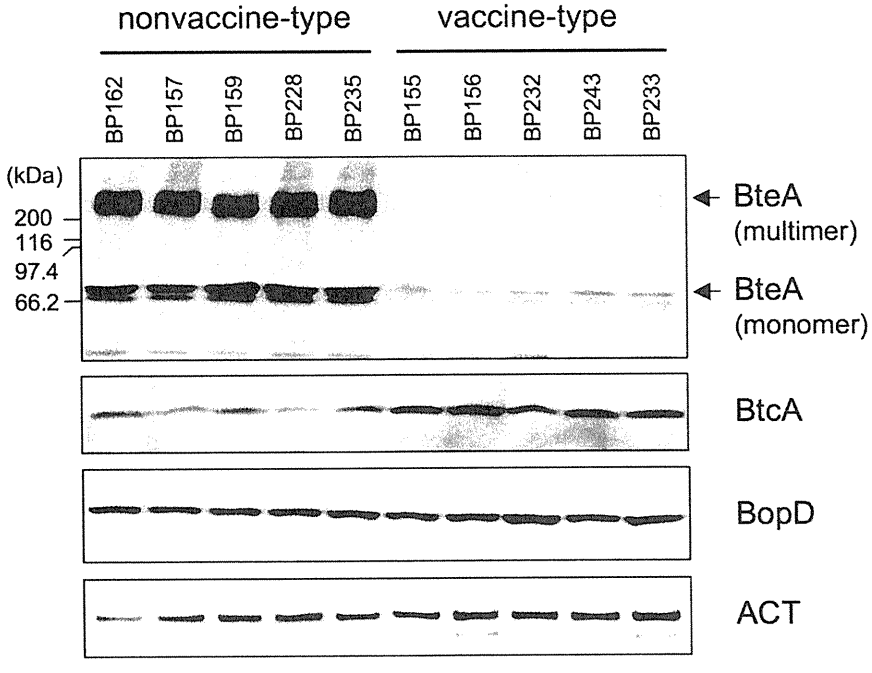

Figure 2. Expression of BteA, BtcA, BopD and ACT proteins in *B. pertussis* nonvaccine-type and vaccine-type strains. The nonvaccine-type clinical strains (BP157, BP159, BP162, BP228 and BP235) and vaccine-type clinical strains (BP155, BP156, BP232, BP233 and BP243) were cultured in modified SS medium for 18 h. Total protein extracted from bacterial cells was subjected to immunoblot analysis with anti-BteA, anti-BtcA, anti-BopD or anti-ACT antiserum. For BteA detection, 10 μg of total protein was loaded in each lane.
doi:10.1371/journal.pone.0017797.g002

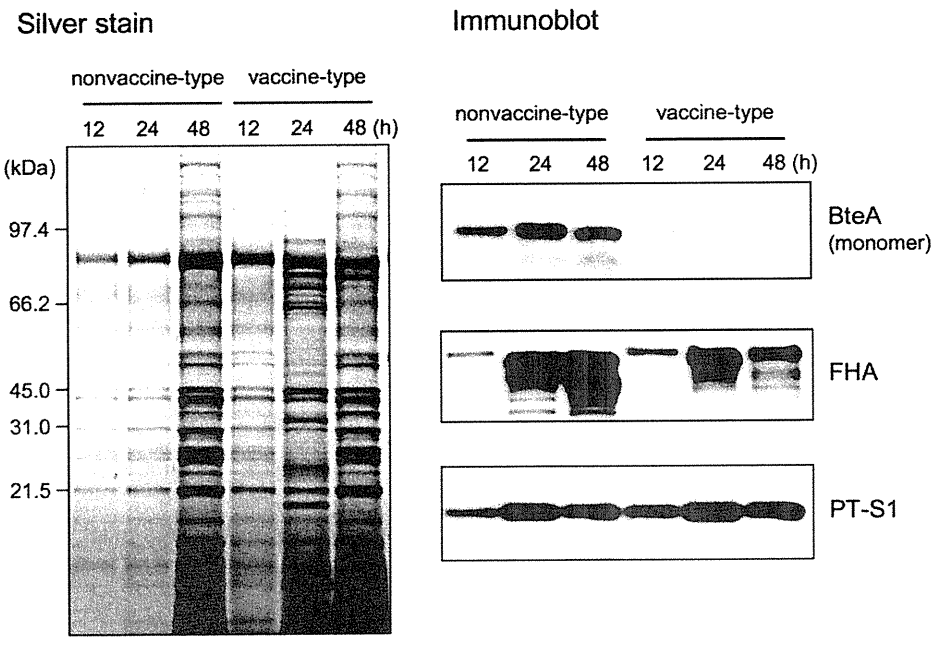

Figure 3. BteA secretion from *B. pertussis* nonvaccine-type and vaccine type strains. Strains BP235 (nonvaccine-type) and BP233 (vaccine-type) were cultured in modified SS medium, and the culture supernatants (CS) were collected at 12, 24 and 48 h. Protein samples prepared by precipitation with 10% trichloroacetic acid were separated by 12.5% SDS-PAGE followed by silver staining (left panel). BteA, FHA and PT secretions were analyzed by immunoblots using anti-BteA, anti-FHA or anti-PT antiserum (right panels). For BteA detection, the equivalent of 200 µl of CS was loaded in each lane.
doi:10.1371/journal.pone.0017797.g003

promoter at one end that is responsible for transcription of the flanking catalase gene (*katA*) in *B. pertussis* [34]. However, the P2 start site is different from the *katA* transcription start site. The transcription start site of *btcA*, also determined by 5′-RACE, was mapped to a T residue 31 bp upstream of the *btcA* translation start codon in both the vaccine-type and nonvaccine-type strains (Figure 5B).

Primer extension analysis was also performed in an attempt to resolve the *bteA* transcription start sites. However, the start sites could not be ascertained, probably due to low amounts of *bteA* transcript in *B. pertussis*.

IS*481a*-promoter transcript is the major *bteA* transcript in the vaccine-type strain

Expression of the IS*481a*-promoter transcript (P2 transcript) in *B. pertussis* vaccine-type strain BP155 was analyzed by qRT-PCR with TaqMan probes (Figure 5A). The P2 transcript and total *bteA* (P1 + P2) transcripts were determined individually and the ratio of P2 transcript to total *bteA* transcript was calculated. Based on four independent experiments, the ratio (P2 transcript/P1 + P2 transcripts) was estimated to be 0.88 (±1SD range, 0.70 to 1.09), indicating that the P2 transcript is the major *bteA* transcript in the vaccine-type strain (data not shown).

BteA expression in *B. pertussis* BteA mutants

To clarify the effect of the IS*481* insertion on BteA expression, four BteA mutants (Δ*bteA*-BP155, ΔIS*481*-BP155, Δ*bteA*-BP157 and +IS*481*-BP157) were constructed from *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type) by homologous recombination (Figure 6A). The Δ*bteA*-BP155 and Δ*bteA*-BP157 mutants had a 178-bp deletion in the 5′ region of *bteA*. In the ΔIS*481*-BP155 mutant, a 2.2-kb insertion containing an intact *bteA* 5′-UTR (derived from *B. pertussis* Tohama) replaced the native *bteA* 5′-UTR + IS*481a* gene. In contrast, +IS*481*-BP157 mutant had a 3.2-kb insertion containing a *bteA* 5′-UTR + IS*481a* (derived from *B. pertussis* BP155) instead of its own *bteA* 5′-UTR. Consequently, ΔIS*481*-BP155 had an IS*481a* deletion from the *bteA* 5′-UTR, whereas the +IS*481*-BP157 mutant had an IS*481a* insertion in the *bteA* 5′-UTR. The *btcA*–*bteA* region of the mutants was verified by DNA sequence analysis.

BteA expression in the bacterial cells and CS after 24 h in culture was analyzed by immunoblot with anti-BteA antiserum (Figure 6B). In ΔIS*481*-BP155 bacterial cells and CS, BteA polypeptide(s) corresponding to ~68 kDa and >200 kDa were detected at the same level as was observed in the BP157 wild-type strain. In contrast, the signals of BteA polypeptide(s) from +IS*481*-BP157 mutant were very low in both bacterial cells and CS. Similarly, BteA polypeptide(s) were not detected in either Δ*bteA*-BP155 or Δ*bteA*-BP157. These results clearly showed that BteA protein expression is down-regulated by the IS*481* insertion in *B. pertussis*, and that the anti-BteA antiserum is highly specific to BteA.

Discussion

The BteA effector (alias BopC) is required for the induction of necrotic cell death during *B. bronchiseptica* infections, and is thought to play a pivotal role in T3SS-mediated cell death [29,30,35]. BteA is also involved in dephosphorylation of tyrosine-phosphorylated proteins (PY) of host cells [30], and its 130-amino acid N-terminal sequence is associated with target lipid rafts [31]. BteA is the only cytotoxic effector that has been identified in *Bordetella* spp. In *B. pertussis*, low-passage clinical strains have an ability to express a functionally active T3SS; however, BteA protein had not been detected in the clinical and common laboratory-adapted strains by MALDI-TOF mass spectrometry [22]. Here we demonstrate that BteA protein is highly expressed in *B. pertussis* nonvaccine-type

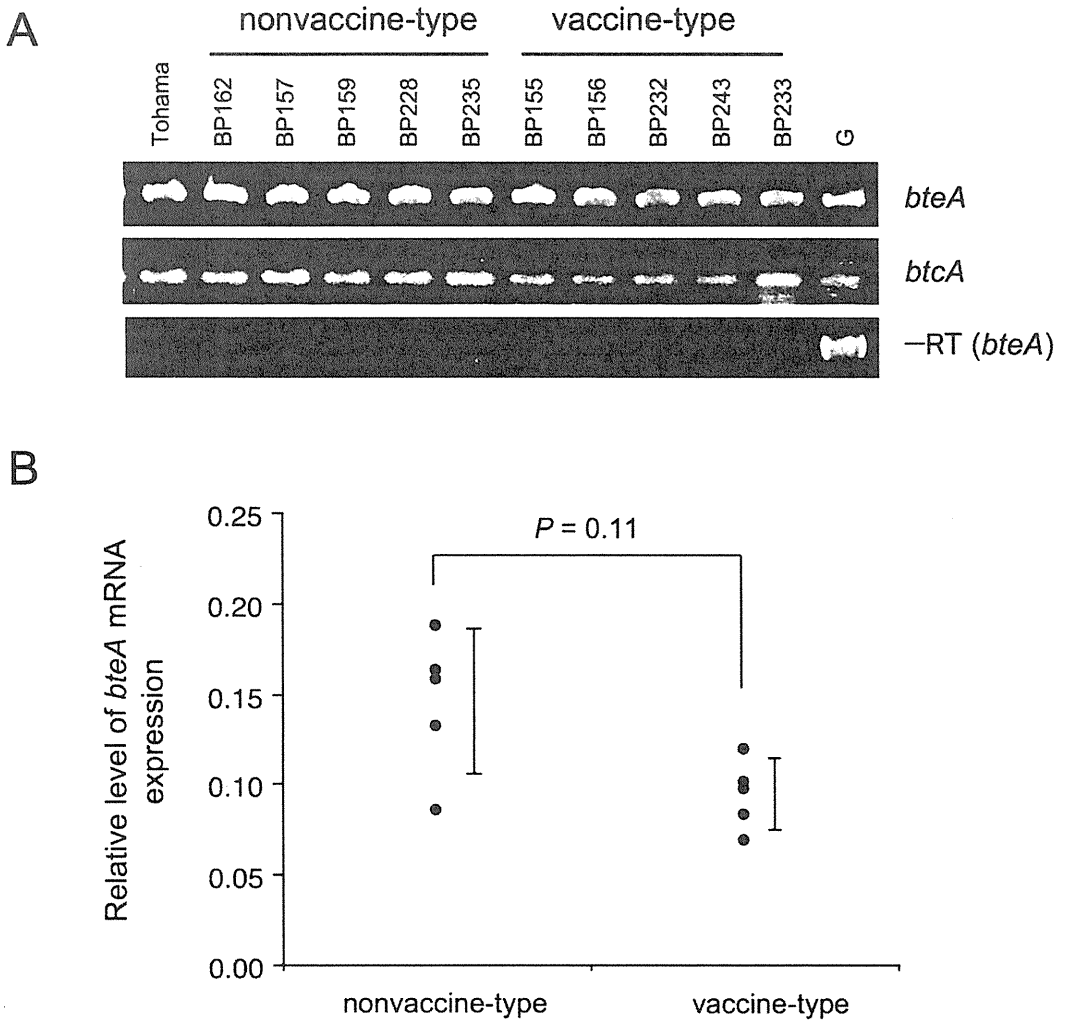

Figure 4. RT-PCR analysis of *bteA* transcript in *B. pertussis* nonvaccine-type and vaccine-type strains. (A) RT-PCR with primers specific for *bteA* and *btcA*. cDNA made from total RNA of nonvaccine-type (BP157, BP159, BP162, BP228 and BP235) and vaccine-type (BP155, BP156, BP232, BP233 and BP243) clinical strains was used as templates for PCR. Genomic DNA (G) from *B. pertussis* strain Tohama was used as a positive control. A mock reaction for *bteA* (−RT) consisted of reactions lacking reverse transcriptase. (B) Quantitative RT-PCR analysis of *bteA* transcript levels in the nonvaccine-type and vaccine-type clinical strains listed in (A). The *recA* transcript was used as a reference. Each point represents one strain and vertical bars indicate standard deviations.
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strains but not in the vaccine-type strains, and that BteA protein expression is down-regulated by IS*481a* insertion in the vaccine-type strains. We provide the first evidence that BteA protein expression is type-dependent due to the IS*481a* insertion in *B. pertussis* clinical strains.

In Japan, *B. pertussis* circulating strains began to change from vaccine-type to nonvaccine-type in the mid-1990s [8], and the reported incidence of adult cases of pertussis has dramatically increased since 2002 [36]. The genetic divergence in *B. pertussis* circulating strains has also been observed in many other countries. A possible explanation for the genetic divergence is that the type shift is a result of vaccine-driven evolution [12–15]. More recently, Mooi et al. [37] reported that expansion of *B. pertussis* strains with increased PT production has contributed to the resurgence of pertussis in the Netherlands. Here we showed prominent expression of the T3SS effector protein BteA in the nonvaccine-type strains, and that PT and ACT (important virulence factors of *B. pertussis*) are expressed at the same level in both the nonvaccine and vaccine-type strains. Besides vaccine-driven evolution, our findings could provide another possible explanation for the type shift from vaccine-type to nonvaccine-type, i.e., the augmented expression of BteA protein in *B. pertussis* nonvaccine-type strains may be involved in the type shift.

B. bronchiseptica BteA has *in vitro* cytotoxic activity against cultured mammalian cells [18,22,29,30]. In this study, we determined the cytotoxicity of *B. pertussis* BteA mutants by measuring the release of lactate dehydrogenase (LDH) from L2 rat lung epithelial cells, J774 mouse macrophage-like cells, or HeLa cells. However, even BteA-expressing strains (Δ IS*481*-BP155 and wild-type BP157) showed low cytotoxicity (<10%), and consequently no statistically significant differences in cytotoxicity were observed among the wild-type and mutant strains. *B. pertussis* is known to have a lower *in vitro* cytotoxicity than *B. bronchiseptica* [18,22], which is consistent with the extremely low secretion of BteA in *B. pertussis* as compared to *B. bronchiseptica* (Figure S1). Therefore, a more sensitive and quantitative assay is required to determine the BteA-dependent cytotoxicity of *B. pertussis*.

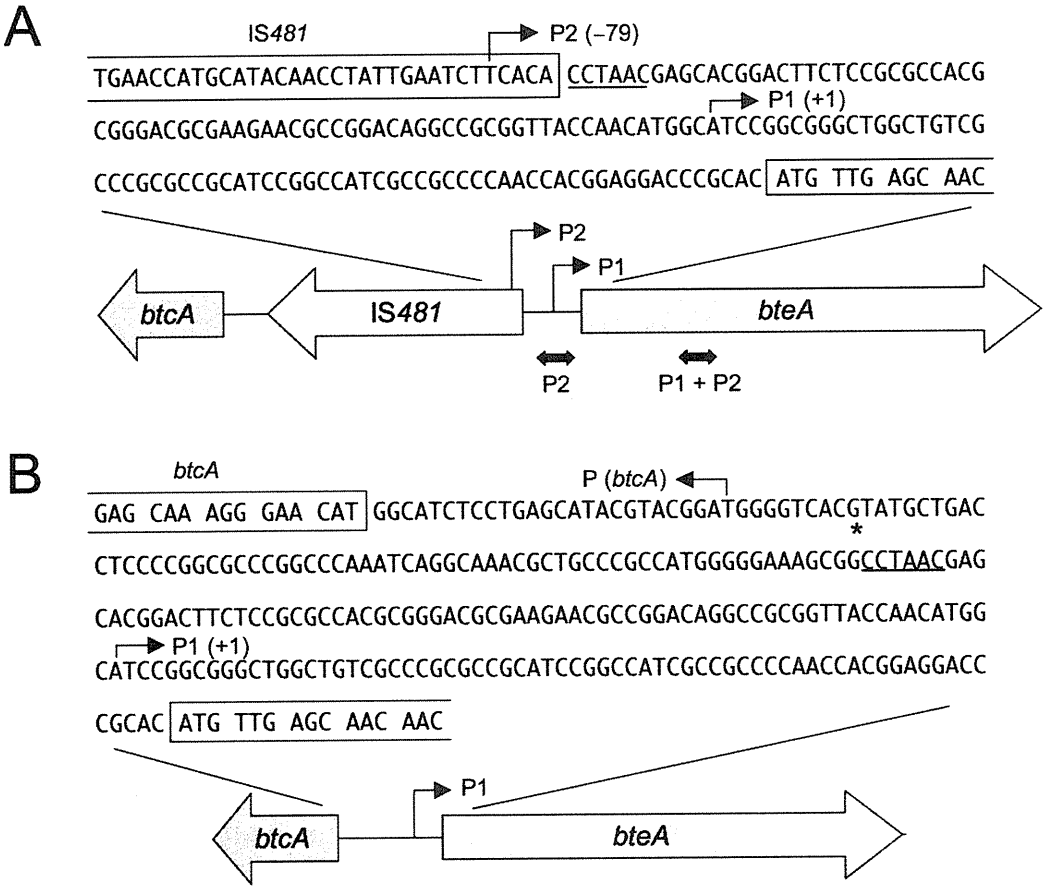

Figure 5. Physical maps of the *btcA*–*bteA* region of *B. pertussis* vaccine-type and nonvaccine-type strains. (A) The vaccine-type clinical strain BP155. The location of IS*481a* is represented by a gray arrow on the physical map. The recognition sequence of IS*481a* is underlined. The two mapped transcriptional start sites (P1 and P2) of *bteA* are shown by arrows. Region amplified by qRT-PCR to determine the IS*481a*-promoter (P2) and total (P1 + P2) transcripts are shown by two-headed arrows below the physical map. (B) The nonvaccine-type clinical strain BP159. The mapped transcriptional start sites of *bteA* (P1) and *btcA* [P (*btcA*)] are shown by arrows. The single nucleotide polymorphism (A→G) at −207 bp from the *bteA* translation start codon is indicated by an asterisk.
doi:10.1371/journal.pone.0017797.g005

IS*481* belongs to the recently defined IS*481* family [38], and 238 copies of IS*481* are present in the *B. pertussis* Tohama genome [39]. In *B. pertussis* clinical strains, IS*481* is also present in multiple copies on the chromosome and it plays a critical role in *B. pertussis* evolution [26,40]. Many IS elements have been shown to activate the expression of neighboring genes. IS*481* contains an outward-facing promoter that is located in close proximity to the left terminal inverted repeat, and this promoter is responsible for the transcription of *katA* in certain *B. pertussis* strains [34]. Here we identified an IS*481a* insertion in the *bteA* 5′-UTR in *B. pertussis* vaccine-type clinical strains and detected a high level of *bteA* transcripts from the IS*481a* promoter (P2) compared with its own promoter (P1). However, the vaccine-type strains showed a low level of BteA protein expression, suggesting that insertion of IS*481a* represses P1 promoter activity, and that P2 transcript has a low translational efficiency from the additional nucleotide sequence (79 nucleotides) at its 5′ end. Use of a cell-free coupled transcription-translation system revealed that the additional nucleotide sequence is involved in down-regulation of transcription and/or translation (Figure S2). The 5′-UTR of bacterial mRNAs can bear regulatory elements that are involved in down- or up-regulation of translation [41]. The regulatory mechanisms in this region are controlled by RNA-binding proteins, small noncoding RNAs and structural rearrangements with the 5′-UTR. In addition, a 5′ stem-loop structure that sequesters the ribosomal binding site has been shown to be involved in translational regulation. Bioinformatic analysis uncovered a predicted stem-loop structure in the *bteA* 5′-UTR of P2 transcript (Figure S2).

In this study, the 5′-UTRs of five *B. pertussis* vaccine-type clinical strains were sequenced; all had an insertion of an IS*481a* in the *bteA* 5′-UTR, both transcribed in the same direction. In one of the vaccine-type strains, BP155, the major *bteA* mRNA was transcribed from P2 in the IS*481a*-promoter. These observations raise the possibilities that (i) the P2 transcript is translated into BteA under certain environmental conditions, and (ii) the P2 transcript is translated into another novel protein by translational frame-shifting. BteA is known to be regulated by the BvgAS system and an extracytoplasmic function (ECF) sigma factor BtrS in *B. bronchiseptica* [18,29]. In *B. pertussis*, it has been suggested that expression of the T3SS translocon Bsp22 is blocked by post-transcriptional regulation [18]. However, the molecular details of the regulatory mechanism are still unclear. Further studies are needed to determine the down-regulation of BteA protein in *B. pertussis* vaccine-type clinical strains.

In conclusion, *B. pertussis* vaccine-type strains have been replaced with the nonvaccine-type strains in many countries, and the resurgence of pertussis has been observed in several

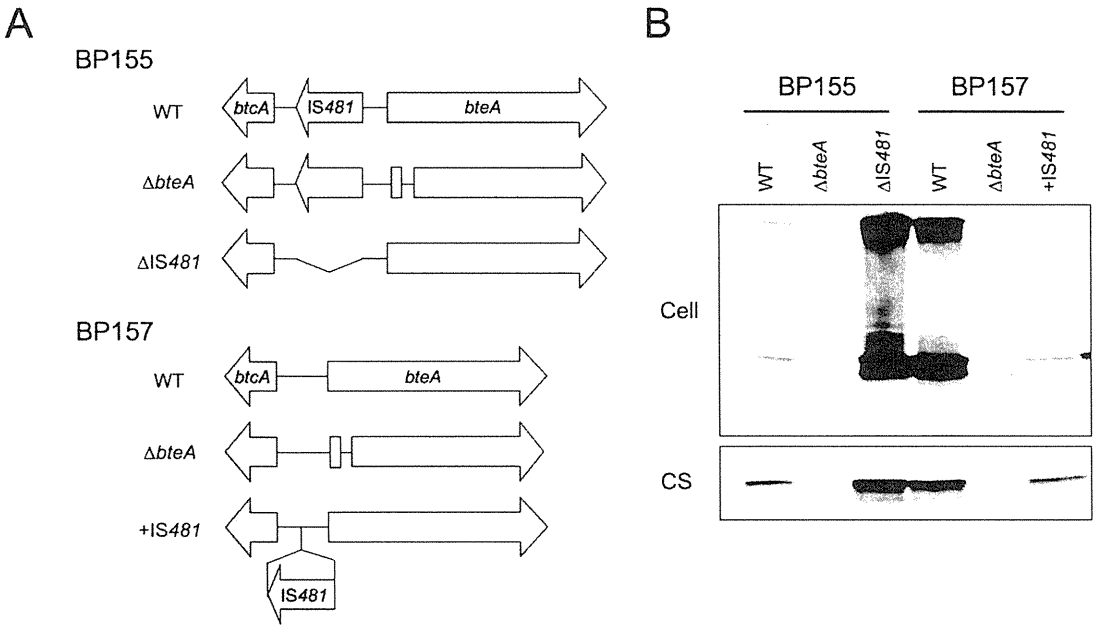

Figure 6. Construction and characterization of *B. pertussis* BteA mutants. (A) Physical map of the *btcA*−*bteA* region of BteA mutants derived from *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type). WT, wild-type; Δ*bteA*, a 178-bp deletion around the 5′ region of *bteA*; Δ*IS481*, *IS481a* deletion from the *bteA* 5′-UTR; +*IS481*, *IS481a* insertion in the *bteA* 5′-UTR. (B) Expression of BteA protein in the BteA mutants. The mutants were cultured in modified SS medium for 24 h. Total protein from the bacterial cells (Cell) and culture supernatants (CS) was analyzed with immunoblot using anti-BteA antiserum.
doi:10.1371/journal.pone.0017797.g006

nations. In Japanese *B. pertussis* clinical strains, the T3SS effector BteA is highly expressed in nonvaccine-type strains as compared with the vaccine-type strains. Our findings indicate that augmented expression of BteA protein in *B. pertussis* circulating strains could play a key role in the type shift. However, it is unclear whether BteA protein is implicated in the resurgence of pertussis. Further studies are needed to determine the expression of BteA protein in *B. pertussis* circulating strains on a global scale.

Materials and Methods

Bacterial strains and growth conditions

B. pertussis clinical strains were selected from the laboratory collection of the National Institute of Infectious Diseases, Tokyo, Japan. The selection criteria included the time and geographic location of isolation, and their *ptxA* and *prn* alleles. A total of 10 clinical strains from 2002 to 2004 in Japan were included. Of the 10 clinical strains, 5 harbored *ptxA1* and *prn2* alleles (BP157, BP159, BP162, BP228 and BP235; nonvaccine-type strains), while the others carried *ptxA2* and *prn1* (BP155, BP156, BP232, BP233 and BP243; vaccine-type strains). All strains were cultured on Bordet-Gengou agar (BG agar, Difco) supplemented with 1% glycerol and 15% defibrinated horse blood or in modified Stainer-Scholte (SS) medium [42].

Two-dimensional gel electrophoresis (2D-PAGE)

2D-PAGE was performed based on the O'Farrell method [43] with minor modifications. *B. pertussis* clinical strains grown on BG agar plates were resuspended in casamino acid solution (1% casamino acid, 0.6% NaCl, pH 7.1). Bacterial cells were precipitated by centrifugation (12,000 × *g*, 10 min) and resuspended in SDS-lysis buffer (62.5 mM Tris-HCl, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8) by sonication. Total protein was extracted by boiling for 3 min, followed by centrifugation. A portion (10 μg, approximately 2 μl) of the protein solution was mixed with 20 μl of sample buffer [8.5 M urea, 2% Nonidet P-40, 2% Ampholine (pH 3.5 to 10)], and applied to an isoelectric focusing tube gel (2.0 mm inside diameter by 12.0 cm) containing 4% polyacrylamide, 8.5 M urea, 2% Nonidet P-40, and 2% Ampholine (pH 5 to 7 and pH 3.5 to 10 in a ratio of 1:4). Proteins were focused at 10°C for 17 h (1 h at 200 V, 2 h at 400 V, and 14 h at 800 V) with 10 mM H_3PO_4 (anolyte) and 20 mM NaOH (catholyte). In the second dimension, the electrofocused tube gel was electrophoresed in 12% SDS-PAGE. The separated polypeptides were visualized by silver staining and analyzed with the PDQuest 2-D Analysis Software (Bio-Rad, Hercules, CA). The Lowry assay was used to measure protein concentrations in a trichloroacetic acid (TCA) pellet (resuspended in 1 N NaOH) using bovine serum albumin as a standard.

Protein identification

2D-PAGE gels were stained with silver nitrate without glutaraldehyde fixation [44], and protein spots of interest were excised. Proteins were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, and digested with sequencing grade-modified trypsin (Promega, Madison, WI). Digested peptides were separated on a C18 capillary column (0.2 by 50 mm, Michrom Bioresources, CA) equipped with a Chorus 220 solvent delivery system and an HTC PAL auto-sampler system (CTC Analytics AG, Zwingen, Switzerland). Separated peptides were analyzed by the Finnigan LCQ^{TM} Deca XP ion trap mass spectrometer (Thermo Fisher Scientific Inc., MA) with electrospray ionization (ESI) interface using the Nanosprayer FS (GL Sciences Inc., Japan). To identify peptides, data files were generated from the MS/MS scans by Bioworks 3.0 using the SEQUEST algorithm (threshold, 10^5; minimum group scan 2, Xc >1.0, Thermo Fisher Scientific) and searched against the complete amino acid database derived from the *B. pertussis* Tohama genome database.

Antibody production against recombinant BteA, BtcA and ACT

The BteA gene (NCBI accession: NP_879352) was amplified by PCR from *B. pertussis* Tohama DNA using BteA-F and BteA-R primers, and cloned into the XmnI/HindIII sites of pMal-c2X (New England Biolabs, Beverly, MA) to generate a maltose binding protein (MBP) fusion with BteA. Production of this fusion protein was induced in *E. coli* BL21 with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and subsequently purified using amylose resin (New England Biolabs) and Resource Q (Amersham Pharmacia Biotech, Uppsala, Sweden) columns. A two-step PCR was carried out to amplify recombinant BtcA (NCBI accession: NP_879351). The first PCR was performed using the BtcA-BteA-F3 and BtcA-BteA-R3 primers (Table S1), which amplified the region between positions 165122 and 167190 of the *B. pertussis* Tohama genome (GenBank accession: BX640412). In the second PCR, *btcA* was amplified from the first PCR product with the 5-BtcA and 3-BtcA primers (Table S1) and cloned into the NdeI/HindIII sites of pCold II DNA (TAKARA Bio Inc.). His-tagged BtcA was induced in *E. coli* BL21 with 0.5 mM IPTG at 15°C and purified using the HisTrap FF Crude Kit (GE Healthcare UK Ltd.). A recombinant catalytic domain of *B. pertussis* adenylate cyclase toxin (ACT) was a gift from Mineo Watanabe.

Antibodies against MBP-BteA, BtcA and ACT were generated in mice at Nippon Biotest Laboratories, Inc. (Tokyo, Japan). The MBP-BteA antiserum was pre-absorbed with MBP2 protein (New England BioLabs) and the resulting antiserum was used.

Immunoblot analysis

B. pertussis clinical strains were inoculated in modified SS medium with a starting optical density of 0.2 at 600 nm, and further cultured with shaking at 36°C. Total protein was extracted with SDS-lysis buffer, and culture supernatant (CS) proteins were precipitated with 10% TCA. Protein samples were subjected to SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad) and incubated with anti-BteA, anti-BtcA, anti-BopD [32], anti-ACT, anti-FHA, or anti-PT antiserum. Antigen-antibody complexes were visualized using horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad, Hercules, CA) and ECL Western Blotting Detection Reagents (GE Healthcare).

DNA sequencing

The region between the *btcA* and *bteA* gene corresponding to positions 165122 to 168021 of *B. pertussis* Tohama (GenBank accession: BX640412) was amplified in vaccine-type and non-vaccine-type clinical strains with the appropriate primers and sequenced. Sequencing reactions were carried out with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA), and the products were sequenced on an ABI PRISM 3130*xl* Genetic Analyzer (Applied Biosystems).

Transcriptional analyses

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) and treated with RNase-free DNase (Promega) to degrade contaminating DNA. Reverse transcriptase-PCR (RT-PCR) was performed with btcA RT-R and btcA RT-R primers (Table S1) using the TAKARA One Step RNA PCR Kit (AMV, TAKARA Bio Inc.). PCR was performed with the following conditions: one cycle of 50°C for 30 min, 95°C for 2 min; 25 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 1 min; and a final incubation at 72°C for 10 min. Primer sets, btcA RT-F/btcA RT-R and btcA RT-F/btcA RT-R, were used for *bteA* and *btcA* amplification, respectively (Table S1). Products were analyzed by electrophoresis on a 1.5% agarose gel. Reverse transcriptase was omitted from the negative control reaction mixtures.

For quantitative RT-PCR (qRT-PCR), 5 μg of RNA was reverse transcribed into cDNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) with random hexamer primers. Relative levels of total *bteA* and *recA* transcripts were determined using TaqMan probes (btcA- and recA-probes, Table S1) and *Premix Ex Taq*™ (Perfect Real Time, TAKARA Bio Inc.) with the ABI PRISM 7500 Sequence Detection System (Applied Biosystems). The qRT-PCR conditions were 30 s at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression of *recA* was used as an internal control [45]. All samples were run in triplicate and *bteA* transcript (P1 + P2 transcripts) was normalized to the *recA* transcript for each sample. The *bteA* IS*481a*-promoter transcript (P2 transcript) was determined using a TaqMan probe (IS481-btcA probe). The qRT-PCR conditions were 30 s at 95°C, followed by 40 cycles of 95°C for 15 s and 55°C for 1 min. The ratio of P2 transcript to total *bteA* transcript (P2 transcript/P1 + P2 transcripts) was estimated from four independent experiments. The regions amplified by qRT-PCR are shown in Figure 6A.

Mapping transcriptional start sites

5′ rapid amplification of cDNA ends (5′-RACE) was performed using 5′-Full RACE Core Set (TAKARA Bio Inc.) according to the manufacturer's instructions. Reverse transcription was executed at 55°C using a 5′ phosphorylated RT primer (btcA-RT, Table S1). The first PCR used primers bteA-S1 (S1) and bteA-A1 (A1) primers, and bteA-S2 (S2) and bteA-A2 (A2) for the second (Table S1). PCR products were cloned into the pT7Blue T-vector (Novagen, Madison, Wis.) and transformed into *E. coli* XL1-Blue, which were plated on LB agar plates. Several clones were sequenced. The transcription start site of *btcA* was located using 5′-RACE with five primers, btcA-RT (5′ phosphorylated primer), btcA-S1 (S1), btcA-A1 (A1), btcA-S2 (S2) and btcA-A2 (A2) (Table S1).

Generation of BteA mutants

Four BteA mutants, Δ*bteA*-BP155, Δ*bteA*-BP157, ΔIS*481*-BP155 and +IS*1481*-BP157, were constructed by homologous recombination as described previously with minor modifications [30] (Figure 6A).

BteA-deficient mutants: A 2.2-kbp DNA fragment containing a 5′ portion of the *bteA* gene was amplified by PCR with the B1-bteA and B2-bteA primers (Table S1) using the *B. pertussis* Tohama genomic DNA as the template. The PCR product was cloned into the pDONR221 vector (Invitrogen) to obtain pDONR-*bteA* by means of adaptor PCR and site-specific recombination techniques with the Gateway Cloning System (Invitrogen). Inverse PCR was then carried out with R1-bteA and R2-bteA primers (Table S1) using circular pDONR-*bteA* as the template. The R1-btcA and R2-bteA primers contained a BamHI site. The resulting PCR product was digested with BamHI and self-ligated to obtain pDONR-Δ*bteA*, which contained a 178-bp deletion around the 5′ region of *bteA*. pDONR-Δ*bteA* was mixed with pABB-CRS2 [46] to obtain pABB-Δ*bteA* using the Gateway Cloning System. pABB-Δ*bteA* was then introduced into *E. coli* SM10λ*pir* and transconjugated into streptomycin (SM)-resistant *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type) clinical strains. The resultant mutant strains were designated Δ*bteA*-BP155 and Δ*bteA*-BP157.

IS481-deletion mutant: pABB-*bteA* was constructed from pDONR-*bteA*. pABB-*bteA* was introduced into *E. coli* SM10λ*pir* and transconjugated into SM-resistant *B. pertussis* vaccine-type BP155. The resultant mutant strain was designated ΔIS*481*-BP155.

*IS*481-*insertion mutant*: a 3.2-kbp DNA fragment (*bteA*+IS*481*) containing the *bteA* 5′-UTR and IS*481a* was amplified with the B1-bteA and B2-bteA primers (Table S1) using *B. pertussis* BP155 genomic DNA as the template. pABB-*bteA*+IS*481* was constructed from pDONR-*bteA*+IS*481* and transconjugated into SM-resistant *B. pertussis* nonvaccine-type BP157 via *E. coli* SM10λ*pir*. The resultant mutant strain was designated +IS*481*-BP157.

Statistical analysis

The Student's *t*-test was employed. A value of $P<0.05$ was considered statistically significant.

Nucleotide sequence accession number

The IS*481a* sequence was deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession number AB473880.

Supporting Information

Figure S1 High secretion of BteA protein in *Bordetella bronchiseptica*. *B. bronchiseptica* (BB R05), *B. pertussis* BP155 (vaccine-type) and BP157 (nonvaccine-type) were cultured in modified SS medium for 24 h. Total protein extracted from the bacterial cells (Cell) and culture supernatants (CS) was separated by SDS-PAGE followed by silver staining (left panel). Immunoblots were incubated with anti-BteA, anti-BteA or anti-BopD antiserum (right panel). For BteA detection, 0.5 μg of total protein (for Cell) and 5 μl of CS were loaded in the indicated lanes. The amount of total protein loaded was one-twentieth of that in Figure 2, and the loaded CS volume was one-fortieth of that in Figure 3.
(TIF)

Figure S2 *In vitro* transcription-translation analysis of a *bteA* 5′-UTR deletion series. (A) *bteA* 5′-UTR deletion genes were PCR-amplified using *B. pertussis* BP155 (vaccine-type) as the template. Proteins were synthesized using the WakoPURE System (Wako Pure Chemical Industries, Ltd.). The 5′-UTR deletion genes harbored the T7 promoter at their 5′ end. (B) Expression of BteA protein in an *in vitro* transcription-translation system (WakoPURE System). The synthesized product was analyzed with immunoblots using anti-BteA antiserum. NC, negative control. (C) A predicted stem-loop structure in the 5′-UTR of *bteA* mRNA (P2 transcript). The RNA secondary structure was analyzed by CentroidFold (http://www.ncrna.org/centroidfold). The schematic shows a simplified map. TIR, translation initiation region.
(TIF)

Table S1 Primers and probes in this study.
(XLS)

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Author Contributions

Conceived and designed the experiments: H-JH KK. Performed the experiments: H-JH KK. Analyzed the data: H-JH KK. Contributed reagents/materials/analysis tools: AK AA YA. Wrote the paper: KK.

References

1. Mattoo S, Cherry JD (2005) Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin Microbiol Rev 18: 326 382.
2. Andrews R, Herceg A, Roberts C (1997) Pertussis notifications in Australia, 1991 to 1997. Commun Dis Intell 21: 145 148.
3. de Melker HE, Schellekens JF, Neppelenbroek SE, Mooi FR, Rümke HC, et al. (2000) Reemergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. Emerg Infect Dis 6: 348 357.
4. Hellenbrand W, Beier D, Jensen E, Littmann M, Meyer C, et al. (2009) The epidemiology of pertussis in Germany: past and present. BMC Infect Dis 9: 22.
5. Tanaka M, Vitek CR, Pascual FB, Bisgard KM, Tate JE, et al. (2003) Trends in pertussis among infants in the United States, 1980-1999. JAMA 290: 2968 2975.
6. Bottero D, Gaillard ME, Fingermann M, Weltman G, Fernández J, et al. (2007) Pulsed-field gene electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical *Bordetella pertussis* strains. Clin Vaccine Immunol 14: 1490 1498.
7. Fry NK, Neal S, Harrison TG, Miller E, Matthews R, et al. (2001) Genotypic variation in the *Bordetella pertussis* virulence factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. Infect Immun 69: 5520 5528.
8. Kodama A, Kamachi K, Horiuchi Y, Konda T, Arakawa Y (2004) Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates in Japan. J Clin Microbiol 42: 5453 5457.
9. Litt DJ, Neal SE, Fry NK (2009) Changes in genetic diversity of the *Bordetella pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination coverage and emergence of a single dominant clonal type. J Clin Microbiol 47: 680 688.
10. Tsang RSW, Lau AKH, Sill ML, Halperin SA, Van Caeseele P, et al. (2004) Polymorphisms of the fimbria *fim3* gene of *Bordetella pertussis* strains isolated in Canada. J Clin Microbiol 42: 5364 5367.
11. van Amersfoorth SCM, Schouls LM, van der Heide HGJ, Advani A, Hallander HO, et al. (2005) Analysis of *Bordetella pertussis* populations in European countries with different vaccination policies. J Clin Microbiol 43: 2834 2843.
12. Borisova O, Kombarova SY, Zakharova NS, van Gent M, Aleshkin VA, et al. (2007) Antigenic divergence between *Bordetella pertussis* clinical isolates from the Moscow, Russia, and vaccine strains. Clin Vaccine Immunol 14: 234 238.
13. Cassiday P, Sanden G, Heuvelman K, Mooi F, Bisgard KM, et al. (2000) Polymorphism in *Bordetella pertussis* pertactin and pertussis toxin virulence factors in the United States, 1935-1999. J Infect Dis 182: 1402 1408.
14. He Q, Mäkinen J, Berbers G, Mooi FR, Viljanen MK, et al. (2003) *Bordetella pertussis* protein pertactin induces type-specific antibodies: one possible explanation for the emergence of antigenic variants? J Infect Dis 187: 1200 1205.
15. Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, et al. (1998) Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun 66: 670 675.
16. Cummings CA, Bootsma HJ, Relman DA, Miller JF (2006) Species- and strain-specific control of a complex, flexible regulon by *Bordetella* BvgAS. J Bacteriol 188: 1775 1785.
17. Shrivastava R, Miller JF (2009) Virulence factor secretion and translocation by *Bordetella* species. Curr Opin Microbiol 12: 88 93.
18. Mattoo S, Yuk MH, Huang LL, Miller JF (2004) Regulation of type III secretion in *Bordetella*. Mol Microbiol 52: 1201 1214.
19. Yuk MH, Harvill ET, Miller JF (1998) The BvgAS virulence control system regulates type III secretion in *Bordetella bronchiseptica*. Mol Microbiol 28: 945 959.
20. Coburn B, Sekirov I, Finlay BB (2007) Type III secretion system and disease. Clin Microbiol Rev 20: 535 549.
21. Ghosh P (2004) Process of protein transport by the type III secretion system. Microbiol Mol Biol Rev 68: 771 795.
22. Fennelly NK, Sisti F, Higgins SC, Ross PJ, van der Heide H, 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.
23. Nagamatsu K, Kuwae A, Konaka T, Nagai S, Yoshida S, et al. (2009) *Bordetella* evades the host immune system by inducing IL-10 through a type III effector, BopN. J Exp Med 206: 3073 3088.
24. Medhekar B, Shrivastava R, Mattoo S, Gingery M, Miller JF (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.
25. Caro V, Bouchez V, Guiso N (2008) Is the sequenced *Bordetella pertussis* strain Tohama I representative of the species? J Clin Microbiol 46: 2125 2128.
26. Heikkinen E, Kallonen T, Saarinen L, Sara R, King AJ, et al. (2007) Comparative genomics of *Bordetella pertussis* reveals progressive gene loss in Finnish strains. PLoS ONE 2: e904.
27. King AJ, van Gorkom T, Pennings JL, van der Heide HG, He Q, et al. (2008) Comparative genomic profiling of Dutch clinical *Bordetella pertussis* isolates using

DNA microarrays: identification of genes absent from epidemic strains. BMC Genomics 9: 311.
28. Vidakovics MLP, Paba J, Lamberti Y, Ricart CA, de Sousa MV, et al. (2007) Profiling the *Bordetella pertussis* proteome during iron starvation. J Proteome Res 6: 2518 2528.
29. Panina EM, Mattoo S, Griffith N, Kozak NA, Yuk MH, et al. (2005) A genome-wide screen identifies a *Bordetella* type III secretion effector and candidate effectors in other species. Mol Microbiol 58: 267 279.
30. Kuwae A, Matsuzawa T, Ishikawa N, Abe H, Nonaka T, et al. (2006) BopC is a novel type III effector secreted by *Bordetella bronchiseptica* and has a critical role in the type III-dependent necrotic cell death. J Biol Chem 281: 6589 6600.
31. French CT, Panina EM, Yea SH, Griffith N, Arambula DG, et al. (2009) The *Bordetella* type III secretion system effector BteA contains a conserved N-terminal motif that guides bacterial virulence factors to lipid rafts. Cell Microbiol 11: 1735 1749.
32. Nogawa H, Kuwae A, Matsuzawa T, Abe A (2004) The type III secreted protein BopD in *Bordetella bronchiseptica* is complexed with BopB for formation on the host plasma membrane. J Bacteriol 186: 3806 3813.
33. Stibitz S (1998) IS*481* and IS*1002* of *Bordetella pertussis* create a 6-base-pair duplication upon insertion at a consensus target site. J Bacteriol 180: 4963 4966.
34. DeShazer D, Wood GE, Friedman RL (1994) Molecular characterization of catalase from *Bordetella pertussis*: identification of the *katA* promoter in an upstream insertion sequence. Mol Microbiol 14: 123 130.
35. Kozak NA, Panina EM, Miller JF (2007) Type III secretion in *Bordetella* subspecies. In: Locht C, ed. *Bordetella*: molecular microbiology. Norfolk, United Kingdom: Horizon Scientific Press. pp 119 140.
36. Han H-J, Kamachi K, Okada K, Toyoizumi-Ajisaka H, Sasaki Y, et al. (2008) Antigenic variation in *Bordetella pertussis* isolates recovered from adults and children in Japan. Vaccine 26: 1530 1534.
37. Mooi FR, van Loo IHM, van Gent M, He Q, Bart MJ, et al. (2009) *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 15: 1206 1213.
38. Craig NL, Craigie R, Gellert M, Lambowitz AM (2002) Mobile DNA II. Washington DC: ASM Press. 1204 p.
39. Parkhill J, Sebaihia M, Preston A, Murphy LD, Thomson N, et al. (2003) Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Nat Genet 35: 32 40.
40. Caro V, Hot D, Guigon G, Hubans C, Arrivé M, et al. (2006) Temporal analysis of French *Bordetella pertussis* isolates by comparative whole-genome hybridization. Microbes Infect 8: 2228 2235.
41. Kaberdin VR, Bläsi U (2006) Translation initiation and the fate of bacterial mRNAs. FEMS Microbiol Rev 30: 967 979.
42. Pradel E, Guiso N, Menozzi FD, Locht C (2000) *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. Infect Immun 68: 1919 1927.
43. O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007 4021.
44. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68: 850 858.
45. Stefanelli P, Sanguinetti M, Fazio C, Posteraro B, Fadda G, 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.
46. Sekiya K, Ohishi M, Ogino T, Tamano K, Sasakawa C, et al. (2001) Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. Proc Natl Acad Sci U S A 25: 11638 11643.

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NOTE

Bactericidal activity of topical antiseptics and their gargles against *Bordetella pertussis*

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Abstract *Bordetella pertussis* is the etiological agent of whooping cough, a common cause of respiratory illness in both children and adults. In the present study, we investigated the bactericidal activity of four antiseptics—povidone–iodine (PVP-I), benzethonium chloride (BEC), chlorhexidine gluconate (CHG) and benzalkonium chloride (BAC)—against *B. pertussis* ATCC9797 and clinical isolates. Among the topical antiseptics, PVP-I, BEC, and BAC, PVP-I and BAC in particular, showed high bactericidal activity, whereas CHG had low activity. PVP-I gargle also showed high bactericidal activity, similar to topical PVP-I. However, BEC gargle had low bactericidal activity. Our results indicate that topical PVP-I and BAC, and PVP-I gargle would be useful as effective antiseptics against *B. pertussis*.

Keywords *Bordetella pertussis* · Antiseptics · Bactericidal activity · Povidone–iodine · Benzalkonium chloride

Pertussis is an acute respiratory infection caused by the gram-negative coccobacillus *Bordetella pertussis* [1]. This disease is highly communicable, with a second attack rate of up to 90% among unvaccinated household contacts. *B. pertussis* is transmitted from an infected person to susceptible persons, primarily through aerosol droplets of respiratory secretions and secondarily through direct contact with the respiratory secretions. In Japan, the incidence of pertussis has been successfully decreased through the introduction of pertussis vaccines; however, there has been an increase in adult patients with pertussis since 2002 [2]. To prevent healthcare-associated bacterial pneumonia including pertussis, hand hygiene and disinfection of medical apparatus are strongly recommended for prevention of transmission of microorganisms [3]. In addition, oropharyngeal cleaning and decontamination with antiseptics are effective ways to prevent nosocomial respiratory infection [3, 4]. Although hand hygiene and oral rinse are recommended, no reports on the bactericidal activity of antiseptics against *B. pertussis* have been published. In the present study, therefore, the bactericidal activity of commercial topical antiseptics and their gargles against *B. pertussis* ATCC and clinical strains was determined.

Ten *B. pertussis* clinical isolates, collected from 2004 to 2008 in Japan, were investigated. The isolates were selected from the National Institute of Infectious Diseases (NIID) strain collections, according to their genotype (multilocus sequence type, MLST): five isolates, MLST-1; three isolates, MLST-2; one isolate, MLST-3; one isolate, MLST-4. The MLST-1 and MLST-2 strains were commonly isolated during the past two decades in Japan, but MLST-3 and -4 strains were not [2]. *B. pertussis* ATCC9797 was used as a laboratory strain. The *B. pertussis* clinical and ATCC9797 strains were cultured on Bordet–Gengou agar containing 15% defibrinated sheep blood for 48 h at 35°C, followed by subculture for 48 h. The following topical antiseptics and gargles were tested in

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this study. The topical antiseptics were povidone–iodine (PVP-I; Isodine solution 10%; Meiji Seika Kaisha), benzethonium chloride (BEC; Hyamine solution 10%; Daiichi-Sankyo), chlorhexidine gluconate (CHG; Hibitane 20%; Dainippon Sumitomo Pharma), and benzalkonium chloride (BAC; Osvan S; Takeda Pharmaceutical). The gargle antiseptics were PVP-I (Isodine gargle solution 7%; Meiji Seika Kaisha) and BEC (Neostelin green 0.2% mouthwash solution; Nippon Shika Yakuhin).

Topical PVP-I, BEC, CHG, and BAC were diluted with sterile water at two or three concentrations according to the package insert instructions: PVP-I, 0.05–0.5%; BEC, 0.005–0.2%; CHG, 0.05–0.5%; BAC, 0.05% and 0.2%. The bacterial inoculum suspension and each antiseptic solution were mixed at 1:25 and incubated. After 0.25, 0.5, 1 and 3 min, 0.1 ml of the mixture was inoculated into 0.9 ml of neutralizer containing Tween 80, soybean lecithin, and sodium thiosulfate. Tenfold serial dilutions of each mixture were prepared, and 0.1 ml dilute solution was plated on Bordet–Gengou agar and incubated for 72 h at 35°C. The number of colonies was counted, and the number of colony-forming units (CFUs) in the mixture was calculated from the dilution rate [5].

Table 1 shows the bactericidal activity of the topical antiseptics against *B. pertussis* ATCC9797. A 0.25-min treatment with 0.05% PVP-I was found to successfully reduce the viable cells by more than 1×10^5 CFU/ml. A 0.25-min treatment with 0.05% BEC reduced the viable cells by more than 1×10^5 CFU/ml, whereas with 0.005% BEC, a 3-min treatment was required to achieve the same effect. A 0.25-min treatment with 0.05% BAC reduced the viable cells by more than 1×10^5 CFU/ml. With 0.05% or 0.2% CHG, even a 3-min treatment could not reduce the viable cells enough. To reduce the viable cells by more than 1×10^5 CFU/ml, treatment with 0.5% CHG for 3 min was required. This finding indicates that topical CHG has a lower bactericidal activity than topical PVP-I, BEC, and BAC against *B. pertussis* ATCC9797.

The bactericidal activity of topical PVP-I, BEC, and CHG against *B. pertussis* isolates was also investigated. The level of bactericidal activity is shown in Fig. 1. All isolates that received a 0.25-min treatment with 0.2% or 0.05% PVP-I had viable cells reduced by more than 1×10^5 CFU/ml. In contrast, with 0.005% BEC, a 3-min treatment was required to achieve the same effect. On the other hand, a sufficient decrease could not be achieved even when a 3-min treatment with 0.2% CHG was performed. With all the antiseptics, no marked differences were seen in terms of MLST and bactericidal effect. Table 2 shows the bactericidal activity of PVP-I and BEC gargles against *B. pertussis* ATCC9797. A 0.25-min treatment with 0.05% PVP-I gargle was found to reduce the viable cells by more than 1×10^5 CFU/ml, indicating that the PVP-I gargle had the same bactericidal activity as topical PVP-I. In contrast, with 0.2% BEC gargle, no significant decreases in viable cells were observed with 3-min treatment, although topical BEC has high bactericidal activity (Table 1).

To our knowledge, this is the first report on the bactericidal activity of antiseptics, especially PVP-I, against *B. pertussis*. Here, we show that topical PVP-I and BAC, and PVP-I gargle have high bactericidal activity compared with BEC and CHG. CHG is classified as a low-level antiseptic according to Spaulding's classification and shows variable bactericidal activity depending on the bacterial species. Furthermore, it was reported that a

Table 1 Bactericidal activity of topical povidone–iodine (PVP-I), benzethonium chloride (BEC), chlorhexidine gluconate (CHG), and benzalkonium chloride (BAC) against *Bordetella pertussis* ATCC9797

Antiseptic	Concentration (%)	Viable cells (CFU/ml)			
		0.25 min	0.5 min	1 min	3 min
PVP-I	0.5	–	–	–	–
	0.2	–	–	–	–
	0.05	–	–	–	–
BEC	0.2	–	–	–	–
	0.05	–	–	–	–
	0.005	8.5×10^6	1.3×10^6	3.0×10^3	–
CHG	0.5	1.8×10^7	1.1×10^7	1.4×10^6	–
	0.2	1.8×10^7	1.8×10^7	8.1×10^6	6.0×10^4
	0.05	3.0×10^7	2.5×10^7	1.9×10^7	1.9×10^6
BAC	0.2	–	–	–	–
	0.05	–	–	–	–

–, not detected ($<1 \times 10^2$ CFU/ml)

Initial cell concentration was $2.9–5.5 \times 10^7$ CFU/ml

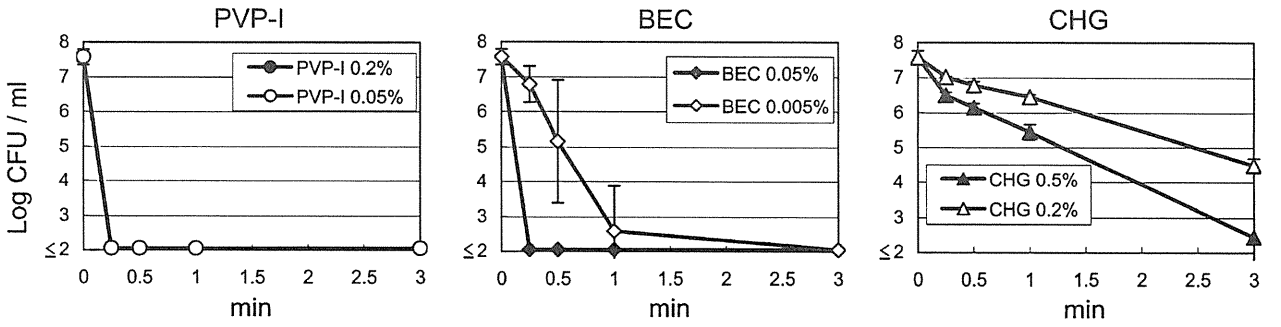

Fig. 1 Bactericidal activity of topical povidone–iodine (PVP-I), benzethonium chloride (BEC), and chlorhexidine gluconate (CHG) against *Bordetella pertussis* clinical isolates. Ten isolates [$\sim 5 \times 10^7$ colony-forming units (CFU)] were individually mixed with the topical antiseptic solution at different concentrations: PVP-I, 0.05% and 0.2%; BEC, 0.005% and 0.05%; CHG, 0.2% and 0.5%. After 0.25, 0.5, 1, and 3 min, the mixture was inoculated into a neutralizer. The number of viable cells was determined using plate count methods, and the number of CFUs in the mixture was calculated from the dilution rate. Detection limit was 1×10^2 CFU/ml

Table 2 Bactericidal activity of PVP-I and BEC gargles against *Bordetella pertussis* ATCC9797

Antiseptic	Concentration (%)	Viable cells (CFU/ml)			
		0.25 min	0.5 min	1 min	3 min
PVP-I	0.5	–	–	–	–
	0.2	–	–	–	–
	0.05	–	–	–	–
BEC	0.2	6.8×10^7	6.2×10^7	8.2×10^7	6.6×10^7
	0.05	8.5×10^7	8.9×10^7	7.5×10^7	4.4×10^7
	0.005	8.7×10^7	7.9×10^7	6.3×10^7	3.8×10^7

–, not detected ($<1 \times 10^2$ CFU/ml)

Initial cell concentration was $2.8–5.5 \times 10^7$ CFU/ml

relatively longer drug contact period is necessary for some bacterial species [6]. It is therefore not surprising that similar results were seen in this study with *B. pertussis*. It is not clear why the BEC gargle shows weak bactericidal activity. Similar findings were obtained in methicillin-resistant *Staphylococcus aureus* (MRSA) (data not shown); thus, the difference in bactericidal effect between topical BEC and BEC gargle is not considered to be an issue specific to *B. pertussis*. The BEC gargle contains several additive agents, e.g., polysorbate 80, mentha oil, spearmint oil, saccharin sodium, thymol, and sodium copper chlorophyllin. The additive agent(s) might be the cause of the low bactericidal activity against *B. pertussis*. Our findings indicate that equal effects of an active ingredient should not be expected when administered in different forms.

PVP-I is known to have potent broad-spectrum activity against bacteria, mycobacteria, fungi, and viruses [7], whereas BAC has no bactericidal activity against mycobacteria [8, 9]. Topical PVP-I and BAC have become widely used as antiseptic and disinfectant in hospitals, and PVP-I gargle is generally used for oral disinfection in Japan, especially at the time of year when the common cold and influenza are prevalent. *B. pertussis* frequently causes hospital and intrafamilial infections transmitted via aerosol droplets as well as the common cold and influenza. In light of this, gargling with PVP-I would be important among household members and hospital patients.

Adolescents and adults are assumed to be the primary reservoir of *B. pertussis* and play a crucial role in the transmission of the microbe to infants and unvaccinated children [10–12]. Macrolide antibiotics, such as erythromycin, are widely used for treatment of patients with pertussis and are currently recommended for prophylaxis in the United States as well. However, erythromycin resistance in *B. pertussis* has been reported in the United States, with an occurrence rate of <1% [13]. Fluoroquinolones are also widely used to treat respiratory tract infections in adults. These antibiotics have excellent in vitro activity against *B. pertussis*; however, several quinolone-resistant strains of *B. pertussis* were recently found in Japan [14]. Considering the mechanism of antiseptics, disinfection and gargling with PVP-I may be an effective way to eliminate *B. pertussis* regardless of drug resistance.

In conclusion, topical PVP-I and BAC, and PVP-I gargle, have high bactericidal activity against *B. pertussis*. To prevent the spread of pertussis infections, PVP-I and BAC would be useful as effective antiseptics against *B. pertussis*.

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References

1. Mattoo S, Cherry JD. Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Clin Microbiol Rev. 2005;18:326–82.
2. Han HJ, Kamachi K, Okada K, Toyoizumi-Ajisaka H, Sasaki Y, Arakawa Y. Antigenic variation in *Bordetella pertussis* isolates recovered from adults and children in Japan. Vaccine. 2008;26:1530–4.
3. Guidelines for preventing health-care-associated pneumonia, 2003. Recommendations of CDC and the Healthcare Infection Control Practices Advisory Committee. MMWR Morb Mortal Wkly Rep 2004;53(RR-3):1–36.
4. DeRiso AJ, Ladowski JS, Dillon TA, Justice JW, Peterson AC. Chlorhexidine gluconate 0.12% oral rinse reduces the incidence of total nosocomial respiratory infection and nonprophylactic systemic antibiotic use in patients undergoing heart surgery. Chest. 1996;109:1556–61.
5. Yoneyama A, Shimizu M, Tabata M, Yashiro J, Takata T, Hikida M. In vitro short-time killing activity of povidone–iodine (Isodine® Gargle) in the presence of oral organic matter. Dermatology. 2006;212:103–8.
6. Shimizu M, Okuzumi K, Yoneyama A, Kunisada T, Araake M, Ogawa H, et al. In vitro antiseptic susceptibility of clinical isolates from nosocomial infections. Dermatology. 2002;204:21–7.
7. Zamora JL. Chemical and microbiologic characteristics and toxicity of povidone–iodine solutions. Am J Surg. 1986;151:400–6.
8. Rikimaru T, Kondo M, Oizumi K. Efficacy of common antiseptics against mycobacteria. Int J Tuberc Lung Dis. 2000;4:570–6.
9. Widmer AF, Frei R. Decontamination, disinfection, and sterilization. In: Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken R, editors. Manual of clinical microbiology. 7th ed. Washington, DC: American Society for Microbiology; 1999. p. 138–64.
10. Birkebaek NH, Kristiansen M, Seefeldt T, Degn J, Møller A, Heron I, et al. *Bordetella pertussis* and chronic cough in adults. Clin Infect Dis. 1999;29:1239–42.
11. Hewlett EL, Edwards KM. Pertussis–not just for kids. N Engl J Med. 2005;352:1215–22.
12. von König CH, Halperin S, Riffelmann M, Guiso N. Pertussis of adults and infants. Lancet Infect Dis. 2002;2:744–50.
13. Wilson KE, Cassiday PK, Popovic T, Sanden GN. *Bordetella pertussis* isolates with a heterogeneous phenotype for erythromycin resistance. J Clin Microbiol. 2002;40:2942–4.
14. Ohtsuka M, Kikuchi K, Shimizu K, Takahashi N, Ono Y, Sasaki T, et al. Emergence of quinolone-resistant *Bordetella pertussis* in Japan. Antimicrob Agents Chemother. 2009;53:3147–9.

Prevalence and Genetic Characterization of Pertactin-Deficient *Bordetella pertussis* in Japan

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Abstract

The adhesin pertactin (Prn) is one of the major virulence factors of *Bordetella pertussis*, the etiological agent of whooping cough. However, a significant prevalence of Prn-deficient (Prn^-) *B. pertussis* was observed in Japan. The Prn^- isolate was first discovered in 1997, and 33 (27%) Prn^- isolates were identified among 121 *B. pertussis* isolates collected from 1990 to 2009. Sequence analysis revealed that all the Prn^- isolates harbor exclusively the vaccine-type *prn1* allele and that loss of Prn expression is caused by 2 different mutations: an 84-bp deletion of the *prn* signal sequence (*prn1*ΔSS, $n=24$) and an IS*481* insertion in *prn1* (*prn1*::IS*481*, $n=9$). The frequency of Prn^- isolates, notably those harboring *prn1*ΔSS, significantly increased since the early 2000s, and Prn^- isolates were subsequently found nationwide. Multilocus variable-number tandem repeat analysis (MLVA) revealed that 24 (73%) of 33 Prn^- isolates belong to MLVA-186, and 6 and 3 Prn^- isolates belong to MLVA-194 and MLVA-226, respectively. The 3 MLVA types are phylogenetically closely related, suggesting that the 2 Prn^- clinical strains (harboring *prn1*ΔSS and *prn1*::IS*481*) have clonally expanded in Japan. Growth competition assays in vitro also demonstrated that Prn^- isolates have a higher growth potential than the Prn^+ back-mutants from which they were derived. Our observations suggested that human host factors (genetic factors and immune status) that select for Prn^- strains have arisen and that Prn expression is not essential for fitness under these conditions.

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Introduction

Bordetella pertussis is the causative agent of pertussis or whooping cough, a highly contagious disease of the human upper respiratory tract. Adolescents and adults are its primary reservoir and play a crucial role in the transmission of the microbe to infants and unvaccinated children [1,2]. Immunization is the most effective method for the prevention and control of pertussis. In Japan, acellular pertussis (aP) vaccines were introduced in 1981 and pertussis has been controlled by means of a schedule of three primary doses and a single booster dose at ages 3, 4, 5, and 18–23 months. The vaccine coverage with three primary does has been ≥90%.

B. pertussis produces several virulence factors that contribute to its adherence to the respiratory ciliate epithelium. The virulence factors pertussis toxin (PT) and filamentous haemagglutinin (FHA) are critical antigens responsible for inducing immunity to *B. pertussis* and are included as major antigens in aP vaccines. Some aP vaccines include either the virulence factor pertactin (Prn) and/or fimbriae (Fim) as additional antigen(s). Among four currently used Japanese aP vaccines, two vaccines contain Prn (5–7.5 μg per 0.5 ml dose) and Fim2 (1 μg/dose), and others do not contain both Prn and Fim2 [3]. In contrast, aP vaccines widely used in Europe and the USA contain from 3 to 8 μg per dose of Prn: Infanrix, 8 μg; DAPTACEL, 3 μg. The three-component aP vaccine containing PT, FHA, and Prn is more effective than the two-component aP vaccine consisting of only PT and FHA [4,5]. In vaccine efficacy trials, the anti-Prn antibody level correlates with clinical protection, suggesting an important role for Prn in immunity [6]. In vitro studies also show that anti-Prn antibody is crucial for opsonophagocytosis [7].

Prn belongs to the type V autotransporter family whose members undergo autoproteolytic processing; mature Prn is a 69-kDa protein that is attached to the bacterial cell surface [8,9,10]. This protein contains an RGD (Arg-Gly-Asp) motif, which is implicated in ligand-receptor interactions in eukaryotes [11]. Prn is considered to function as an adhesin that can bind human epithelial cells; however, the host receptor for Prn has not been identified. Besides its potential role as an adhesin, *Bordetella bronchiseptica* Prn has been shown to function as a phage receptor [12,13]. During the last decade, Prn polymorphism has been described among *B. pertussis* strains circulating worldwide. Prn variation is mainly limited to 2 regions, designated as region 1 (R1) and region 2 (R2), which are composed of the repeat motifs (GGXXP)n and (PQP)n, respectively [14]. Most variations are found in R1, which is located adjacent to an RGD motif. Thirteen Prn variants have been identified so far [15,16]. In Japan, Prn1 and Prn2 variants currently predominate; however, the vaccine-

type Prn1 has been gradually replaced with the nonvaccine-type Prn2 since the mid-1990s [17]. A recent study shows that the ability of *B. pertussis* strains to colonize mouse lung decreases in the order Prn1>Prn2 and Prn3 [18].

B. pertussis Prn$^-$ isolates are present in Europe [19,20]. The Prn$^-$ isolates were collected in Italy ($n = 1$) and France ($n = 4$), and this phenotype is due to the deletion of *prn* or insertion of the IS*481* element. The IS*481* is present in multiple copies in the *B. pertussis* chromosome, causing frequent chromosomal rearrangements and deletions [21,22]. The emerging Prn$^-$ strains raise the possibility that the prevalence of Prn$^-$ strains reduces the efficacy of aP vaccines containing Prn. Here, we identified the significant prevalence of Prn$^-$ strains recently circulating in Japan. To obtain detailed insights into these strains with respect to their genetic, temporal, and geographical characteristics, we performed sequence analysis and multilocus variable-number tandem repeat analysis (MLVA). Using an in vitro growth competition assay, we attempted to gain insights into the biological mechanisms responsible for the prevalence of Prn$^-$ strains.

Results

Identification of Prn$^-$ isolates

B. pertussis Prn expression was analyzed by immunoblotting with anti-Prn1 antiserum. Figure 1 shows a representative blot of 6 Prn-positive and 4 negative isolates. Total 33 Prn$^-$ isolates were identified among 121 *B. pertussis* isolates collected in 1990–2009 in Japan, which we acquired from the National Institute of Infectious Diseases (NIID), Japan. Interestingly, all Prn$^-$ isolates harbor vaccine-type *prn1* and *ptxA2* alleles. The expression of other virulence factors PT, FHA, and Fim3 was detected in the recent Prn$^-$ isolates (collected in 2005–2009) by immunoblotting and serotyping. Detailed information on these 121 isolates is listed in Table S1.

Sequence analysis of Prn$^-$ isolates

To investigate the molecular basis for the loss of Prn expression in Prn$^-$ isolates, we sequenced the Prn gene of all 33 Prn$^-$ isolates. Two independent mutations were detected, which had caused the loss of Prn1: a deletion of the *prn1* signal sequence (*prn1*ΔSS) and an IS*481* insertion, *prn1*::IS*481* (Figure 2). The *prn1* signal sequence, which plays an important role in localizing Prn to the *B. pertussis* outer cell membrane, was deleted in 24 (73%) out of 33 Prn$^-$ isolates. All 24 isolates harboring *prn1*ΔSS had the same 84-bp deletion, resulting in the deletion of 28 amino acid residues (Val^{9}–Trp^{36}) (Figure 2A). Secondary structure analysis also showed that the deleted DNA sequence is predicted to form a hairpin-loop structure (Figure S1). In contrast, 9 (27%) of 33 Prn$^-$ isolates were shown to contain the IS*481* insertion in *prn1*. Eight IS*481* sequences were specifically inserted in the 5′–3′ orientation between a 6-bp direct repeat (ACTAGG, 1593–1598 bp), and 1 was oriented in the opposite direction (Figure 2B).

Temporal and geographical characterization in Prn$^-$ isolates

Figure 3 shows the temporal trend of the frequency of Prn$^-$ strains among 121 *B. pertussis* isolates according to the year of collection. The frequencies of Prn$^-$ isolate harboring *prn1*ΔSS were 0, 0, 27 and 25% in the periods 1990–1994, 1995–1999, 2000–2004 and 2005–2009, respectively. In contrast, the frequencies of Prn$^-$ isolates harboring *prn1*::IS*481* were 0, 5, 11 and 7% in 1990–1994, 1995–1999, 2000–2004 and 2005–2009, respectively. Notably, the total percentage of the Prn$^-$ isolates significantly increased from the 2000s, i.e., 0% in 1990–1994, 5% in 1995–1999, 38% in 2000–2004 and 32% in 2005–2009.

During 1990 to 2000, 5 Prn$^-$ isolates (*prn1*ΔSS, 4 isolates; *prn1*::IS*481*, 1 isolate) were collected only in the Kanto district. Thereafter, Prn$^-$ isolates were collected in several areas during

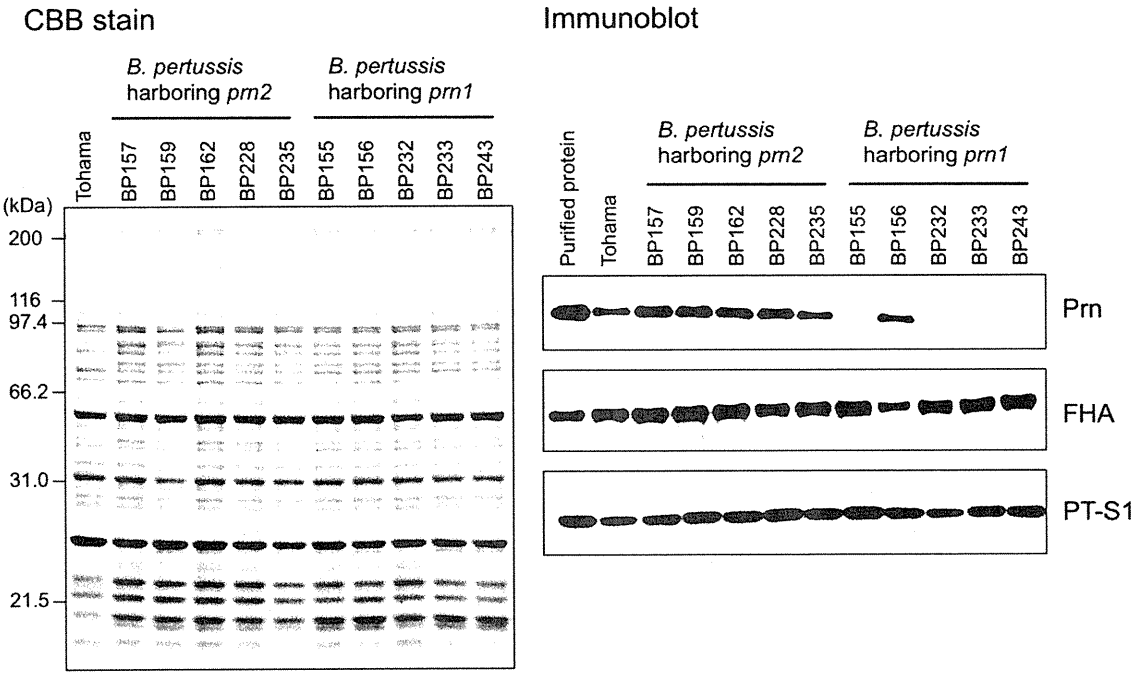

Figure 1. Prn expression in *B. pertussis* clinical isolates. The isolates harboring *prn2* allele (BP157, BP159, BP162, BP228, and BP235) and *prn1* allele (BP155, BP156, BP232, BP233, and BP243) were cultured on CSM plates. Total protein (10 μg) extracted from the bacterial cells was separated by SDS-PAGE followed by CBB R-250 staining (left panel). Immunoblots (1 μg protein/lane) were incubated with anti-Prn1, anti-PT or anti-FHA antiserum (right panel). Ten ng of purified Prn1, PT, or FHA and total protein (1 μg) from *B. pertussis* Tohama were run on the gel as positive controls.
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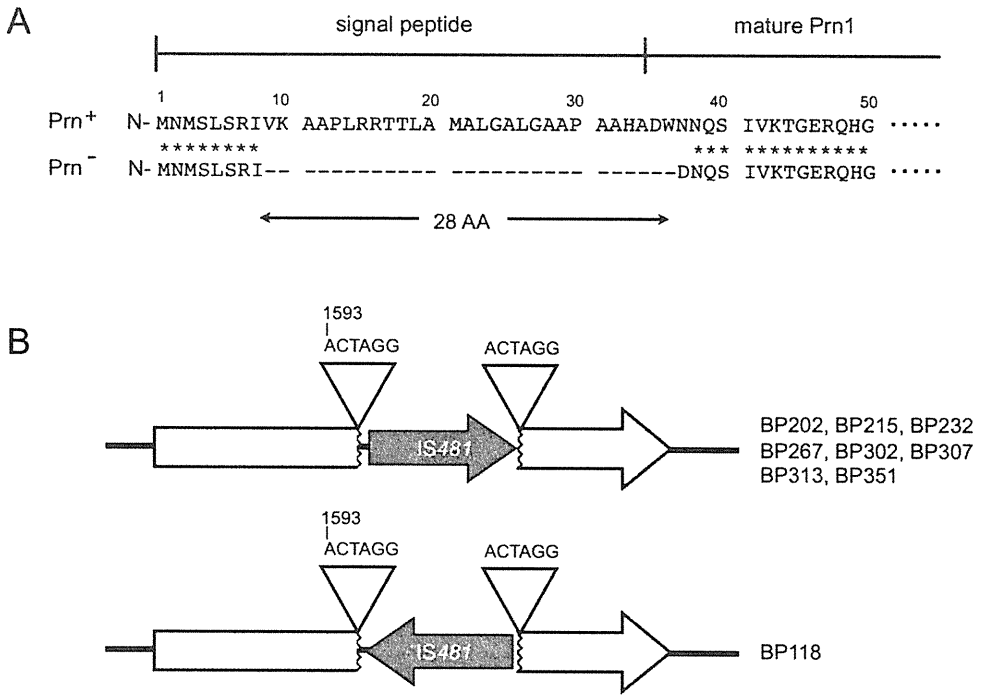

Figure 2. Molecular mechanisms of loss of Prn expression. (A) Deletion of the Prn signal sequence (*prn1*ΔSS). Prn$^-$ isolates ($n = 24$) have an 84-bp deletion, resulting in a 28-amino acid deletion (Val^9 to Trp^{36}) in the N-terminal region. (B) IS*481* insertion mutation in Prn1 gene (*prn1*::IS*481*). Eight Prn$^-$ isolates have an IS*481* insertion in the forward direction at the 6-bp direct repeats (ACTAGG, 1593–1598 bp) of *prn1*, and 1 isolate had the insertion in the reverse.
doi:10.1371/journal.pone.0031985.g002

2001 to 2009 (Figure 4). In the period from 2000 to 2009, 20 Prn$^-$ isolates harboring *prn1*ΔSS were collected from Tohoku, Kanto, Chubu, Kinki, and Kyushu districts, and 8 Prn$^-$ isolates harboring *prn1*::IS*481* were collected from Kanto, Chubu, Kinki, Shikoku, and Kyushu. These findings indicate that Prn$^-$ isolates were present nationwide since 2000.

Molecular epidemiology of Prn$^-$ isolates

Thirty-three Prn$^-$ and 88 Prn$^+$ isolates collected by the NIID between 1990 and 2009 were subjected to MLVA. Among the 121 isolates, 33 different MLVA types were identified, of which 10 were novel (MLVA-223 to -227, -229, -230, -233, -234, and -248) (Figure 5 and Table S1). Twenty-six of these MLVA types were present at low frequencies (each, ≤2% of all isolates). Thirty-three Prn$^-$ isolates belonged to only 3 MLVA types; 24 isolates (73%) were MLVA-186, 6 isolates (18%) were MLVA-194, and 3 isolates (9%) were MLVA-226. MLVA-186 was the predominant type (frequency, 35% of all isolates), whereas MLVA-194 and MLVA-226 were minor (frequency, 5% and 2%, respectively). The 3 MLVA types were closely related phylogenetically. When categorized by their mutations, 24 Prn$^-$ isolates harboring *prn*ΔSS

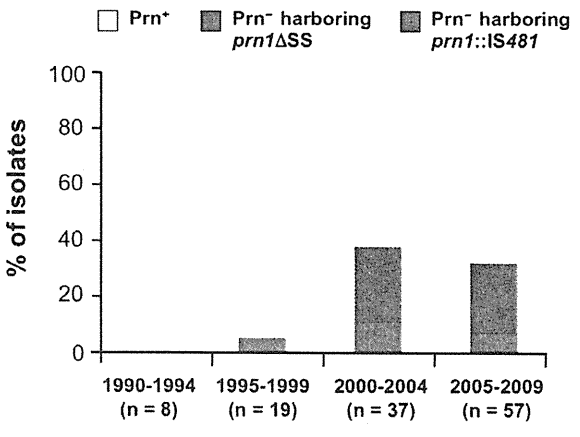

Figure 3. Temporal trend of the occurrence of Prn$^-$ isolates in Japan. The frequencies of Prn$^-$ isolates harboring *prn1*ΔSS and *prn1*::IS*481* were based on 121 *B. pertussis* isolates collected during 1990–2009. Prn$^+$ indicates Prn-expressing isolate.
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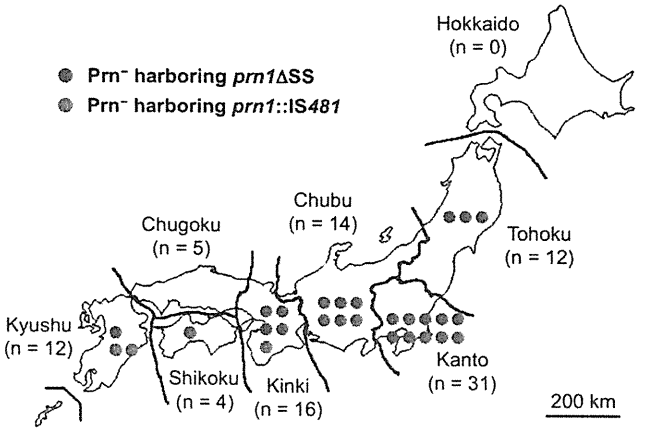

Figure 4. Geographical distribution of Prn$^-$ isolates in Japan during 2001–2009. Blue and red circles indicate Prn$^-$ isolates harboring *prn1*ΔSS and *prn1*::IS*481*, respectively. Numbers of isolates tested are indicated in parentheses.
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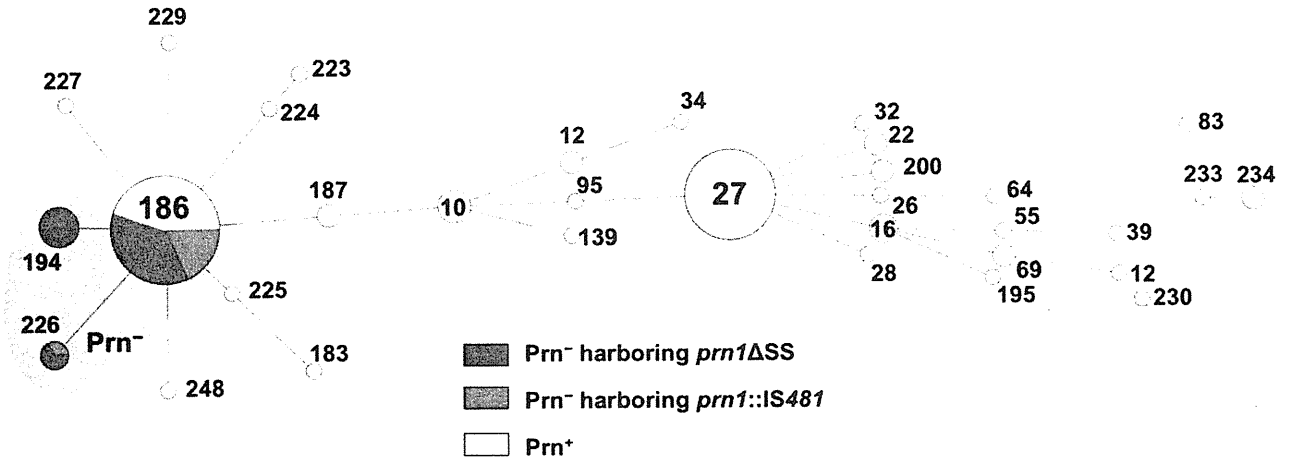

Figure 5. Minimum spanning tree of MLVA of Prn⁻ and Prn⁺ isolates. Total 121 *B. pertussis* isolates, collected during 1990–2009 in Japan, were subjected to MLVA: Prn⁻ isolate harboring *prn1*ΔSS, 24 isolates; Prn⁻ isolate harboring *prn1*::IS*481*, 9 isolates; Prn⁺ isolate, 88 isolates. Each circle in the tree represents a different MLVA type with the MLVA type number. The distance between neighboring genotypes is expressed as the similarity value. Prn⁻ isolates belong to MLVA-186, -194, and -226.
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belonged to MLVA-186 ($n=16$), MLVA-194 ($n=6$), and MLVA-226 ($n=2$); 9 Prn⁻ isolates harboring *prn1*::IS*481* belonged to MLVA-186 ($n=8$) and MLVA-226 ($n=1$). Thus, MLVA-186 and MLVA-226 were common to both the Prn⁻ isolates, whereas only the Prn⁻ isolate harboring *prn1*ΔSS was typed as MLVA-194.

Growth advantage of Prn⁻ isolates

We investigated the growth advantage of Prn⁻ isolates by an in vitro growth competition assay. For this purpose, we constructed 2 Prn⁺ back-mutants (Prn⁺-BP59Sm^r and Prn⁺-BP202Sm^r) that were derived from *B. pertussis* isolates BP59 (*prn1*ΔSS) and BP202 (*prn1*::IS*481*), which expressed Prn at a level similar to that of the *B. pertussis* vaccine strain Tohama (Figure S2). *B. pertussis* Tohama produced Prn1 at levels similar to those of other Prn⁺ isolates (Figure 1), indicating that the Prn⁺ back-mutants expressed Prn1 at the same levels as those of naturally occurring Prn⁺ isolates. The Prn⁺ back-mutants also produced PT and FHA at levels equivalent to their parental strains. Moreover, the expression of Fim2 and/or Fim3 was confirmed in the Prn⁺ back-mutants by serotyping (data not shown).

Figure 6 shows the growth characteristics of the Prn⁻ strain. When the Prn⁺-BP202Sm^r back-mutant was co-cultured with its parent, Prn⁻-BP202Sm^r, the percentage of Prn⁻-BP202Sm^r cells increased markedly with time and then reached 100% after 72 h. Similarly, the percentage of Prn⁻-BP59Sm^r increased, reaching 71% and 78% at 72 and 144 h, respectively. These results indicate that Prn⁻ strains have higher growth rates in vitro than their Prn⁺ back-mutants. Surprisingly, when the Prn⁻ and Prn⁺ strains were individually cultured in mSS broth, no significant differences were observed in their growth rates (data not shown). Furthermore, no revertant Prn⁻ strains arising from the Prn⁺ back-mutants were observed under the individual culture conditions.

Discussion

Here, we demonstrate that *B. pertussis* Prn⁻ isolates, generated by 2 different mutations, *prn*ΔSS and *prn*::IS*481*, have significantly increased in Japan since the early 2000s. The emerging Prn⁻ isolates were found nationwide in the 2000s and were found to specifically harbor the vaccine-type *prn1* allele. The rate of Prn⁻ isolation from 2005 to 2009 was 32% (18/57). We believe that this rate is accurate because we investigated all of the isolates (collected in 2005–2009) present in the NIID strain collections except for epidemiologically related cases. Recently, Prn⁻ mutants were also isolated in France at a rate of 5.6% [20], which is significantly lower than for Japan. Taken together, our findings confirm the high prevalence of Prn⁻ strains in the Japanese *B. pertussis* population and raise the question of the pathogenic role of Prn1 in *B. pertussis* infections.

MLVA analysis revealed that various Prn⁻ isolates have high genetic similarity. The Prn⁻ isolates are mainly of the MLVA type 186. The MLVA type has been found in specific countries, Japan and Hong Kong, China [23]. The data on the geographic distribution of Prn⁻ isolates lend support to our hypothesis that the Prn⁻ isolate harboring *prn1*ΔSS or *prn1*::IS*481* has clonally expanded. Interestingly, 3 of 4 French Prn⁻ isolates have an

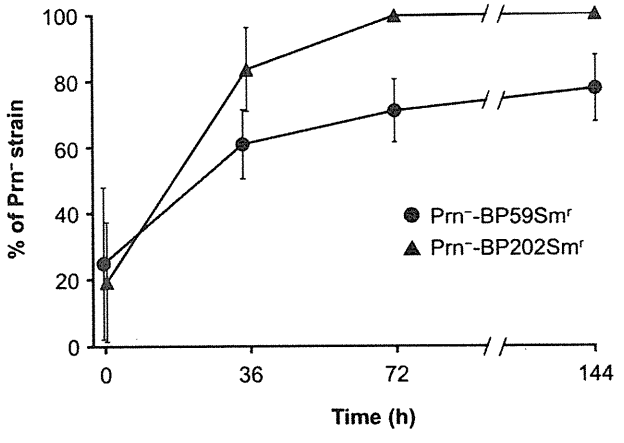

Figure 6. Population dynamics of Prn⁻ strains in in vitro growth competition assay. Prn⁺ back-mutants and parental Prn⁻ strains were mixed in the ratio 4:1 (Prn⁺-BP59Sm^r versus BP59Sm^r or Prn⁺-BP202Sm^r versus BP202Sm^r) and cocultured in mSS broth at 36°C. The bacterial cultures were collected at 0, 36, 72 and 144 h, and plated on CSM agar plates. The representation of Prn⁻ strains among 40 colonies was examined by colony-PCR. Data are means and standard deviations from 3 independent experiments.
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IS*481* insertion in *prn* at the same position as the Japanese Prn$^-$ isolates,and the other French Prn$^-$ isolate has a larger *prn* deletion (2.4-kbp) involving the 5′-upstream region and signal sequence [20]. Unlike Japanese Prn$^-$ isolates, all French Prn$^-$ isolates harbor nonvaccine-type *prn2* alleles, indicating that the Japanese Prn$^-$ isolates are genetically distinguishable from the French Prn$^-$ isolates.

Japanese Prn$^-$ isolates harboring *prn1*ΔSS had an 84-bp deletion in the *prn* signal sequence. The deleted DNA sequence is predicted to form a hairpin loop structure, suggesting that the DNA loop might be excised from the *prn1* by DNA repair enzyme(s) (Figure S1). Although the deleted sequence does not affect the translational reading frame of Prn1 gene, a truncated Prn1 precursor was not detected in the Prn$^-$ isolates (Figure 1). Interestingly, in vitro transcriptional-translation analysis revealed that the *prn1*ΔSS gene could be transcribed and translated as a truncated Prn1 precursor (data not shown). This suggests immediate degradation of truncated Prn1 in the bacterial cell. In contrast, Prn$^-$ isolates harboring *prn1*::IS*481* were disrupted by an IS*481*-insertion at a 6-bp direct repeat (ACTAGG, 1593–1598 bp) in *prn1*. The direct repeats in *prn1* conform to the recognition sequence of IS*481*, NCTAGN [24]. IS*481* is present in multiple copies in the *B. pertussis* chromosome and the number of lost genes increased with time by IS*481*-dependent rearrangement [22,25]. Taken together with information published on French Prn$^-$ isolates, 3 different mechanisms, IS*481* insertion and 2 *prn* deletions (84-bp and 2.4-kb), have contributed to the loss of Prn expression in *B. pertussis*. These gene disruptions strongly suggest that human host factors (genetic factors and immune status) that select for Prn$^-$ strains have arisen.

Prn's adhesin properties have been investigated both in vitro and in vivo [26,27,28,29]. A recent study showed that *B. pertussis* Prn$^-$ mutants colonized less well than Prn$^+$ strains in mice [18]. It is also known that Prn prevents either bacterial adherence or internalization or both, to human monocyte-derived dendritic cells [30] and that it also plays a role in resistance to neutrophil-mediated clearance [31]. Further, *B. bronchiseptica* Prn is required for optimal colonization of the swine respiratory tract [32]. Prn may therefore play a crucial role in bacterial adhesion and in survival and colonization in humans. However, here we have discovered a high prevalence of Prn$^-$ isolates in recent *B. pertussis* populations in Japan. This observation strongly suggests that loss of Prn does not significantly reduce bacterial fitness in the present environment. Prn is highly conserved among the *Bordetella* species. Surprisingly, Prn$^-$ isolates of the human pathogen *Bordetella parapertussis* have also been found recently in France [33]. This finding supports our hypothesis that the role of Prn in fitness (or transmission) has diminished in some hosts.

We demonstrate here that Prn$^-$ strains have a higher growth potential than their Prn$^+$ back-mutants in vitro (Figure 6). The increased growth advantage of Prn$^-$ strains provides knowledge about their biological properties. The most likely explanation for prevalence of Prn$^-$ strains is vaccine-driven selection. Prn is an important antigenic component of most current aP vaccines, and it plays a role in eliciting protective immunity [4,6,7,34,35], leading to the suggestion that Prn$^-$ strains have escaped the immune response to Prn. The herd immunity by aP vaccines could exert selective pressure for pathogen evolution, like the emergence of the PT promoter (*ptxP3*) lineage that produces higher levels of PT [36]. In fact, Prn1 strains might be more fit in unvaccinated than in vaccinated populations [18]. In Japan, four currently used vaccines are produced from *B. pertussis* vaccine strain Tohama; two vaccines contain Prn1 and others do not contain it [3]. The aP vaccines that can be used interchangeably for routine immunization of infants have been introduced in Japan since 1981. Subsequently, Prn1 clinical strains have been gradually replaced by Prn2 strains since the mid-1990s [17], and Prn$^-$ strains significantly increased since the early 2000s (as shown here). These observations suggest the interesting possibility that Prn$^-$ strains may have increased fitness in vaccinated populations, i.e., Prn1 strains are most affected by vaccination with aP vaccines containing Prn1, whereas Prn2 strains producing non-vaccine type Prn are not. However, in the present study, the vaccination status of the majority of patients infected with Prn$^-$ strain was unknown (Table S1). Thus, the relationship between Prn$^-$ strains and vaccine efficacy is currently unclear. Further studies now underway on patients' background are needed to verify the hypothesis.

In conclusion, Prn$^-$ strains have significantly increased in *B. pertussis* populations since the early 2000s in Japan. *B. pertussis* Prn$^-$ strains have also been found in France, as well as among isolates of the human pathogen *B. parapertussis*. These observations suggest that Prn expression may be not essential for fitness of *Bordetella* species in the recent host environment and that Prn$^-$ strains may be fit in humans immunized with aP vaccines. Further analyses and global surveillance are required to elucidate the emergence of Prn$^-$ strains.

Materials and Methods

Bacterial strains

We studied 121 *B. pertussis* clinical isolates collected from 1990 to 2009 in Japan (Table S1). The isolates were selected from the National Institute of Infectious Diseases (NIID) strain collections to reflect the same temporal distribution of the *prn* allele [17,37]. Seventy-nine isolates harbor the vaccine-type *prn1* allele, and 41 and 1 isolates harbor nonvaccine-type *prn2* and *prn9* allele, respectively. All the isolates were epidemiologically unrelated cases of pertussis. The isolates were cultured on Bordet-Gengou agar (Difco) supplemented with 1% glycerol and 15% defibrinated horse blood and incubated at 36°C for 2–3 days.

Immunoblotting and serotyping

B. pertussis isolates were subcultured on cyclodextrin solid medium (CSM) [38]. Total protein was extracted from bacterial cells with SDS-lysis buffer (62.5 mM Tris-HCl, 1% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8). Protein samples (1 μg protein) were subjected to 10% SDS-PAGE, transferred to nitrocellulose membranes (Bio-Rad), and incubated with anti-Prn1, anti-FHA, or anti-PT antiserum. Antigen-antibody complexes were visualized using horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) and ECL Western Blotting Detection Reagents (GE Healthcare) and the blots imaged using a LAS-3000 (Fujifilm, Tokyo, Japan). The anti-Prn1 antiserum was generated in mice with purified Prn1 derived from *B. pertussis* strain Tohama.

Serotyping of *B. pertussis* isolates was performed in a microplate agglutination assay using ati-Fim2 and anti-Fim3 monoclonal antibodies [39]. The anti-Fim2 (NIBSC 04/154) and anti- Fim3 (NIBSC 04/156) antibodies were obtained from the National Institute for Biological Standard and Control. *B. pertussis* strain 18323 expressing both Fim2 and Fim3 was used as a positive control [40].

DNA sequencing

DNA sequencing of PCR fragments representing relevant regions of *prn* was performed as described [14,41]. Sequence reactions were carried out with a BigDye® Terminator v3.1 Cycle

Sequencing Kit (Applied Biosystems), and the products were sequenced using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The complete open reading frames of all Prn^- isolates ($n = 33$) were determined.

MLVA

MLVA typing was performed as described previously [23,42]. Six variable-number tandem-repeat loci (VNTR1, 3a, 3b, 4, 5, and 6) were amplified by PCR, and the fragments were separated using an ABI PRISM 3130xl Genetic Analyzer with GeneScan™-600LIZ® (Applied Biosystems) as an internal lane size standard. For each VNTR locus, the size of the PCR product was converted to a number of repeat units as alleles using GeneMapper software ver.4.0 (Applied Biosystems). Each MLVA type was assigned as described earlier [23,43], and novel MLVA type numbers were assigned by Dr. F. Mooi, Netherlands Centre for Infectious Diseases Control, National Institute for Public Health and the Environment, The Netherlands.

Minimum spanning trees were generated from the 6 MLVA loci using the FPQuest Software (Bio-Rad). Links were generated between MLVA types with a categorical comparison algorithm, with the following rules in priority order: (1) Link types must have the maximum number of single-locus variants (SLVs), (2) types must have the maximum number of SLVs and double-locus variants, and (3) types must have the maximum number of entries.

Generation of Prn^+ back-mutants

Two Prn^+ back-mutants (Prn^+-BP59Sm^r and Prn^+-BP202Sm^r) were constructed from Prn^- isolates BP59 (*prn1*ΔSS) and BP202 (*prn1*::IS*481*) by double cross-over homologous recombination, respectively [44]. To construct the Prn^+-BP59Sm^r back-mutant, a 2.4-kbp DNA fragment (prnA) encoding the intact *prn* signal sequence was amplified by PCR with attB1-sigF and attB2-sigR primers (Table S2) using *B. pertussis* Tohama genomic DNA as the template. The resulting PCR product was cloned into pDONR221 to obtain pDONR-prnA using the adaptor PCR method in the Gateway cloning system (Invitrogen). The pDONR-prnA and pABB-CRS2 [45] were combined to obtain pABB-prnA using the Gateway cloning system. pABB-prnA was introduced into *E. coli* SM10λ*pir* and transconjugated into strain BP59Sm^r (streptomycin-resistant, Sm^r). The resulting mutant was designated Prn^+-BP59Sm^r.

To construct the Prn^+-BP202Sm^r back-mutant, a 2.3-kbp DNA fragment (prnB) encoding intact *prn* gene was PCR-amplified using attB1-ISF and attB2-ISR primers (Table S2). Plasmid pABB-prnB was constructed from pDONR-prnB and then transconjugated into strain BP202Sm^r via *E. coli* SM10λ*pir*. The resulting mutant was designated Prn^+-BP202Sm^r.

To confirm site-specific recombination, the *prn* of Prn^+ back-mutants was sequenced, confirming that the pABB vector sequence was entirely removed from *prn* of both Prn^+ back-mutants.

In vitro growth competition assay

Prn^+ back-mutants (Prn^+-BP59Sm^r and Prn^+-BP202Sm^r) and their parental Prn^- strains (BP59Sm^r and BP202Sm^r) were inoculated into modified Stainer-Scholte (mSS) broth [46], and cultured with shaking at 36°C. After 24 h, the culture solutions were diluted to an optical density (650 nm) of 0.2 with mSS broth. The Prn^+ back-mutant (2.4 ml) and its parental Prn^- strain (0.6 ml) were mixed at the ratio 4:1 and co-cultured with shaking at 36°C. The bacterial cultures (30 µl) were subcultured once in fresh mSS broth (3 ml) for 72 h.

The bacterial cultures were collected at 0, 36, 72, and 144 h, diluted with 1% casamino acid solution containing 0.6% NaCl, pH 7.1, and plated on CSM agar plates. After incubation for 3–4 days, 40 colonies were checked for *prn* size by colony-PCR performed as follows: 94°C for 2 min; 30 cycles of 98°C for 10 s, 55°C for 30 s, and 68°C for 3 min; and final incubation at 72°C for 5 min. Primer sets, PrnF and 1053R, and PrnF and PrnR, were used for strains BP59Sm^r (*prn1*ΔSS) and BP202Sm^r (*prn1*::IS*481*), respectively (Table S2).

Nucleotide sequence accession number

The nucleotide sequence data reported in this study have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under accession numbers AB670735 to AB670737.

Supporting Information

Figure S1 A hairpin loop structure in the signal sequence (SS) of Prn gene. Twenty-four Prn^- isolates harboring *prn1*ΔSS have an 84-bp deletion at position 26–109 bp, corresponding to the hairpin loop. The schematic shows a simplified map.
(TIF)

Figure S2 Expression of Prn, PT, and FHA in Prn^+ back-mutants derived from Prn^- isolates. Prn^+ back-mutants (Prn^+-BP59Sm^r and Prn^+-BP202Sm^r) were constructed from streptomycin-resistant Prn^- isolates, BP59Sm^r (*prn1*ΔSS), and BP202Sm^r (*prn1*::IS*481*), respectively. Total protein (1 µg) extracted from the bacterial cells was subjected to SDS-PAGE and analyzed by immunoblotting with anti-Prn1, anti-PT or anti-FHA antiserum. Total protein (1 µg) from *B. pertussis* Tohama was run on the gel as a positive control. PT-S1 indicates the S1 subunit of PT.
(TIF)

Table S1 Characteristics of *B. pertussis* isolates.
(XLSX)

Table S2 PCR primers in this study.
(XLSX)

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Author Contributions

Conceived and designed the experiments: NO KK. Performed the experiments: NO H-JH HT-A YN KK. Analyzed the data: NO KK. Contributed reagents/materials/analysis tools: YA KS KK. Wrote the paper: NO KK.

References

1. von König CH, Halperin S, Riffelmann M, Guiso N (2002) Pertussis of adults and infants. Lancet Infect Dis 2: 744–750.
2. Hewlett EL, Edwards KM (2005) Clinical practice. Pertussis–not just for kids. N Engl J Med 352: 1215–1222.
3. Okada K, Komiya T, Yamamoto A, Takahashi M, Kamachi K, et al. (2010) Safe and effective booster immunization using DTaP in teenagers. Vaccine 28: 7626–7633.
4. Cherry JD (1997) Comparative efficacy of acellular pertussis vaccines: an analysis of recent trials. Pediatr Infect Dis J 16: S90–96.

5. Olin P, Rasmussen F, Gustafsson L, Hallander HO, Heijbel H (1997) Randomised controlled trial of two-component, three-component, and five-component acellular pertussis vaccines compared with whole-cell pertussis vaccine. Lancet 350: 1569 1577.
6. Gustafsson L, Hallander HO, Olin P, Reizenstein E, Storsaeter J (1996) A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N Eng J Med 334: 349 355.
7. Hellwig SM, Rodriguez ME, Berbers GA, van de Winkel JG, Mooi FR (2003) Crucial role of antibodies to pertactin in *Bordetella pertussis* immunity. J Infect Dis 188: 738 742.
8. Henderson IR, Navarro-Garcia F, Nataro JP (1998) The great escape: structure and function of the autotransporter proteins. Trends Microbiol 6: 370 378.
9. Henderson IR, Navarro-Garcia F, Desvaux M, Fernandez RC, Ala'Aldeen D (2004) Type V protein secretion pathway: the autotransporter story. Microbiol Mol Biol Rev 68: 692 744.
10. Junker M, Schuster CC, McDonnell AV, Sorg KA, Finn MC, et al. (2006) Pertactin beta-helix folding mechanism suggests common themes for the secretion and folding of autotransporter proteins. Proc Natl Acad Sci U S A 103: 4918 4923.
11. Hynes RO (1987) Integrins: a family of cell surface receptors. Cell 48: 549 554.
12. Doulatov S, Hodes A, Dai L, Mandhana N, Liu M, et al. (2004) Tropism switching in *Bordetella* bacteriophage defines a family of diversity-generating retroelements. Nature 431: 476 481.
13. Miller JL, Le Coq J, Hodes A, Barbalat R, Miller JF, et al. (2008) Selective ligand recognition by a diversity-generating retroelement variable protein. PLoS Biol 6: e131.
14. Mooi FR, van Oirschot H, Heuvelman K, van der Heide HG, Gaastra W, et al. (1998) Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun 66: 670 675.
15. Kallonen T, He Q (2009) *Bordetella pertussis* strain variation and evolution postvaccination. Expert Rev Vaccines 8: 863 875.
16. Mooi FR (2010) *Bordetella pertussis* and vaccination: the persistence of a genetically monomorphic pathogen. Infect Genet Evol 10: 36 49.
17. Kodama A, Kamachi K, Horiuchi Y, Konda T, Arakawa Y (2004) Antigenic divergence suggested by correlation between antigenic variation and pulsed-field gel electrophoresis profiles of *Bordetella pertussis* isolates in Japan. J Clin Microbiol 42: 5453 5457.
18. van Gent M, van Loo IHM, Heuvelman KJ, de Neeling AJ, Teunis P, et al. (2011) Studies on Prn variation in the mouse model and comparison with epidemiological data. PLoS One 6: e18014.
19. Mastrantonio P, Spigaglia P, van Oirschot H, van der Heide HG, Heuvelman K, et al. (1999) Antigenic variants in *Bordetella pertussis* strains isolated from vaccinated and unvaccinated children. Microbiology 145: 2069 2075.
20. Bouchez V, Brun D, Cantinelli T, Dore G, Njamkepo E, et al. (2009) First report and detailed characterization of *B. pertussis* isolates not expressing pertussis toxin or pertactin. Vaccine 27: 6034 6041.
21. Caro V, Hot D, Guigon G, Hubans C, Arrivé M, et al. (2006) Temporal analysis of French *Bordetella pertussis* isolates by comparative whole-genome hybridization. Microbes Infect 8: 2228 2235.
22. Heikkinen E, Kallonen T, Saarinen L, Sara R, King AJ, et al. (2007) Comparative genomics of *Bordetella pertussis* reveals progressive gene loss in Finnish strains. PLoS One 2: e904.
23. Kurniawan J, Maharjan RP, Chan WF, Reeves PR, Sintchenko V, et al. (2010) *Bordetella pertussis* clones identified by multilocus variable-number tandem-repeat analysis. Emerg Infect Dis 16: 297 300.
24. Stibitz S (1998) IS*481* and IS*1002* of *Bordetella pertussis* create a 6-base-pair duplication upon insertion at a consensus target site. J Bacteriol 180: 4963 4966.
25. Bouchez V, Caro V, Levillain E, Guigon G, Guiso N (2008) Genomic content of *Bordetella pertussis* clinical isolates circulating in areas of intensive children vaccination. PLoS One 3: e2437.
26. Roberts M, Fairweather NF, Leininger E, Pickard D, Hewlett EL, et al. (1991) Construction and characterization of *Bordetella pertussis* mutants lacking the vir-regulated P.69 outer membrane protein. Mol Microbiol 5: 1393 1404.
27. Leininger E, Roberts M, Kenimer JG, Charles IG, Fairweather N, et al. (1991) Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. Proc Natl Acad Sci U S A 88: 345 349.
28. Leininger E, Ewanowich CA, Bhargava A, Peppler MS, Kenimer JG, et al. (1992) Comparative roles of the Arg-Gly-Asp sequence present in the *Bordetella pertussis* adhesins pertactin and filamentous hemagglutinin. Infect Immun 60: 2380 2385.
29. van den Berg BM, Beekhuizen H, Mooi FR, van Furth R (1999) Role of antibodies against *Bordetella pertussis* virulence factors in adherence of *Bordetella pertussis* and *Bordetella parapertussis* to human bronchial epithelial cells. Infect Immun 67: 1050 1055.
30. Stefanelli P, Fazio C, Fedele G, Spensieri F, Ausiello CM, et al. (2009) A natural pertactin deficient strain of *Bordetella pertussis* shows improved entry in human monocyte-derived dendritic cells. New Microbiol 32: 159 166.
31. Inatsuka CS, Xu Q, Vujkovic-Cvijin I, Wong S, Stibitz S, et al. (2010) Pertactin is required for *Bordetella* species to resist neutrophil-mediated clearance. Infect Immun 78: 2901 2909.
32. Nicholson TL, Brockmeier SL, Loving CL (2009) Contribution of *Bordetella bronchiseptica* filamentous hemagglutinin and pertactin to respiratory disease in swine. Infect Immun 77: 2136 2146.
33. Bouchez VC, Brun D, Dore GC, Njamkepo E, Guiso N (2011) *Bordetella parapertussis* isolates not expressing pertactin circulating in France. Clin Microbiol Infect 17: 675 682.
34. Greco D, Salmaso S, Mastrantonio P, Giuliano M, Tozzi AE, et al. (1996) A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. N Engl J Med 334: 341 348.
35. Storsaeter J, Hallander HO, Gustafsson L, Olin P (1998) Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. Vaccine 16: 1907 1916.
36. Mooi FR, van Loo IH, van Gent M, He Q, Bart MJ, et al. (2009) *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis 15: 1206 1213.
37. Han H-J, Kamachi K, Okada K, Toyoizumi-Ajisaka H, Sasaki Y, et al. (2008) Antigenic variation in *Bordetella pertussis* isolates recovered from adults and children in Japan. Vaccine 26: 1530 1534.
38. Aoyama T, Murase Y, Iwata T, Imaizumi A, Suzuki Y, et al. (1998) Comparison of blood-free medium (cyclodextrin solid medium) with Bordet-Gengou medium for clinical isolation of *Bordetella pertussis*. J Clin Microbiol 23: 1046 1048.
39. Mooi FR, Hallander H, Wirsing von Konig CH, Hoet B, Guiso N (2000) Epidemiological typing of *Bordetella pertussis* isolates: recommendations for a standard methodology. Eur J Clin Microbiol Infect Dis 19: 174 181.
40. Bassinet L, Gueirard P, Maitre B, Housset B, Gounon P, et al. (2000) Role of adhesins and toxins in invasion of human tracheal epithelial cells by *Bordetella pertussis*. Infect Immun 68: 1934 1941.
41. Cassiday P, Sanden G, Heuvelman K, Mooi F, Bisgard KM, et al. (2000) Polymorphism in *Bordetella pertussis* pertactin and pertussis toxin virulence factors in the United States, 1935 1999. J Infect Dis 182: 1402 1408.
42. Schouls LM, van der Heide HG, Vauterin L, Vauterin P, Mooi FR (2004) Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. J Bacteriol 186: 5496 5505.
43. Litt DJ, Neal SE, Fry NK (2009) Changes in genetic diversity of the *Bordetella pertussis* population in the United Kingdom between 1920 and 2006 reflect vaccination coverage and emergence of a single dominant clonal type. J Clin Microbiol 47: 680 688.
44. Han H-J, Kuwae A, Abe A, Arakawa Y, Kamachi K (2011) Differential expression of type III effector BteA protein due to IS*481* insertion in *Bordetella pertussis*. PLoS ONE 6: e17797.
45. Sekiya K, Ohishi M, Ogino T, Tamano K, Sasakawa C, et al. (2001) Supermolecular structure of the enteropathogenic *Escherichia coli* type III secretion system and its direct interaction with the EspA-sheath-like structure. Proc Natl Acad Sci U S A 98: 11638 11643.
46. Pradel E, Guiso N, Menozzi FD, Locht C (2000) *Bordetella pertussis* TonB, a Bvg-independent virulence determinant. Infect Immun 68: 1919 1927.