Title: Prevalence and molecular characterization of pertactin-deficient *Bordetella pertussis* in the US

Running title (limit 54 characters/spaces): Pertactin-deficient *B. pertussis* in US


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Abstract (limit 250 words)

Pertussis has made a striking resurgence in the US, returning to record numbers of reported cases last observed in the 1950s. *Bordetella pertussis* isolates lacking pertactin, a key antigen component of the acellular pertussis vaccine, have been observed, suggesting that *B. pertussis* is losing pertactin in response to vaccine immunity. Screening of 1300 isolates from outbreak and surveillance studies (historical isolates collected from 1935 up to 2009, the 2010 California pertussis outbreak, US isolates from routine surveillance between 2010-2012, and the 2012 Washington pertussis outbreak) by conventional PCR and later by Western blot and *prn* sequencing analyses ultimately identified 306 pertactin-deficient isolates. Of these pertactin-deficient strains, 276 were identified as having an IS481 in the *prn* gene (*prn*IS481-positive). The first *prn*IS481-positive isolate was found in 1994, with the next *prn*IS481-positive isolates not detected until 2010. Pertactin-deficient isolates increased substantially to over 50% in 2012. Sequence analysis of pertactin-deficient isolates revealed various types of mutations in the *prn* gene, including two deletions, single nucleotide substitutions resulting in a stop codon, an inversion in the promoter, and a single nucleotide insertion resulting in a frame shift mutation. All but one mutation type were found in *prn*2 alleles. CDC013 was a predominant PFGE profile in the pertactin-positive isolates (203/994), but was found in only 5% (16/306) of the pertactin-deficient isolates. Interestingly, PFGE profiles CDC002 and CDC237 represented 55% (167/306) of the identified pertactin-deficient isolates. These results indicate that there has been a recent, dramatic increase in pertactin-deficient *B. pertussis* isolates throughout the US.

Introduction

Reported pertussis cases in the United States (US) have increased in the last ten years with recent peaks in both 2010 and 2012 despite a successful vaccination program (1, 2). Record numbers of over 48,000 cases were reported in 2012 (1). Many factors have been attributed to this resurgence,
such as improved surveillance capacity, increased awareness among clinicians and the public, more sensitive and specific laboratory diagnostics, and waning protection from the acellular vaccines (3, 4). Increased pertussis case counts have been seen in multiple countries with varied vaccination programs (5-7). In the US, the whole cell pertussis vaccine was introduced for children in the 1950s and later replaced by an acellular vaccine in the 1990s. The purified protein components of the two major childhood acellular pertussis vaccines licensed in the US both contain pertussis toxin, pertactin, and filamentous hemagglutinin [GlaxoSmithKline (Rixensart, Belgium) and Sanofi Pasteur (Lyon, France)]. One vaccine also contains fimbrial proteins 2/3 (Sanofi Pasteur).

The genomes of currently circulating *Bordetella pertussis* isolates differ from the isolates used in the manufacture of vaccine components (8-12). Allelic variations in several of the acellular vaccine components, such as *ptxP3*, *prn2*, and *fim3B*, have appeared and the predominant profile observed no longer completely matches the strains used to make the acellular vaccine components (12-15). Another genomic change has been the deletion of pertactin, a key antigen component of pertussis vaccines. *B. pertussis* isolates that lack the presence of pertactin protein, here called “pertactin-deficient isolates” have been identified in France, Italy, Japan, and Finland (13, 16-19). Prevalence has ranged between countries, from 2.6% in Finland (16) to 27% in Japan (19). In the US, 11 pertactin-deficient isolates were recently reported in a collection of 12 contemporary isolates from a Philadelphia hospital (20). Overall, the mutations causing the pertactin deficiency are widely varied, including IS481 insertions, deletion of the 5′ signal sequence, premature stop codons, and a large deletion of the promoter and 5′ coding region (13, 16-20). To our knowledge, no longitudinal studies have been described to interpret the progression of this deficiency over time.

To better understand the timing in the emergence and prevalence of this phenomenon in the US, we analyzed 1300 isolates from the Centers for Disease Control and Prevention (CDC) collection that
represent four different origins and time periods: historical isolates collected from 1935 up to 2009 (12); the 2010 California (CA) pertussis outbreak (21); US isolates collected during routine surveillance between 2010-2012 from various state public health laboratories, including the Enhanced Pertussis Surveillance (EPS) system of the Emerging Infections Program Network (EIP); and the 2012 Washington (WA) pertussis outbreak (2). We found the emergence of pertactin-deficient isolates to be a very recent occurrence, their prevalence to be wide-reaching, and the mutations creating the pertactin-deficiency to be highly variable. This is the first report of the recent and rapid expansion of pertactin-deficient isolates in the US.

**Materials and Methods**

**Isolate Collection.** The CDC collection bank included 1300 *B. pertussis* isolates divided in four groups: 1) 666 historical isolates collected from 1935 up to 2009 (12); 2) 33 from the 2010 California (CA) pertussis outbreak (21); 3) 385 surveillance isolates collected between 2010-2012; and 4) 216 from the 2012 Washington (WA) pertussis outbreak (2). Historical isolates were received by the public health laboratories of 46 states between 1935 and 2009. They were selected for analysis using random sampling stratified by geography and time to ensure as much equal geographic and temporal representation as possible (12). Very little to no clinical information is available for the majority of the historical isolates. The CA 2010 outbreak isolates were collected between February 2010 and November 2010. The routine surveillance isolates were all isolates collected between January 2010-November 2012 from six states participating in Enhanced Pertussis Surveillance (Colorado, Connecticut, Minnesota, New Mexico, New York, and Oregon), as well as other state public health laboratories. The Enhanced Pertussis Surveillance is supported by the CDC Emerging Infection Program network to conduct enhanced pertussis surveillance. The WA 2012 isolates comprised all isolates received from the WA...
Public Health Department and Seattle Children’s Hospital, collected between May 2011 and October 2012.

**PCR Screening.** Conventional polymerase chain reaction (PCR) screening for the IS481 insertion within the *prn* was performed at CDC with the PF and PR primers from Mooi, et al. (22), following methods previously described (12) (Table 1). These primers are just internal to the 5'/3’ ends of the *prn* gene and produce a 2.5 Kb amplicon. Further combinations of primers were also utilized, such as AF, AR, BF, and BR from Mooi, et al. (22), Prn-F and Prn-R from Otsuka, et al. (19), and newly constructed primers to encompass the entire gene and promoter region (Table 1). At CDC, PCR was run as follows: in a 50µl reaction, 2.7U of Roche Diagnostics Expand High Fidelity Enzyme (Indianapolis, IN) were used with final concentrations of 1.5mM MgCl$_2$ buffer, 2µM forward/reverse primers, 0.2µM deoxyribonucleotide triphosphates (dNTPs), and 10% dimethyl sulfoxide (DMSO). Cycling conditions were 95°C for 15 min, 20 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 2.5 min, and 10 cycles of 94°C for 45 sec, 50°C for 45 sec, and 72°C for 2.5 min. Isolates were identified as either “*prn*IS481-negative” for producing the expected 2.5 Kb amplicon or as “*prn*IS481-positive” for producing a 3.5 Kb amplicon that contains the IS481 insertion.

**Western Blot Analysis.** After PCR screening, 100 isolates were selected for characterization by Western blot analysis. Because isolates harboring the *prn*IS481 insertion have been shown to no longer express pertactin (13, 16, 19), more emphasis was placed on selecting the *prn*IS481-negative isolates for detection of pertactin deficiency due to other types of possible mutations. The selection of isolates was randomized following certain criteria to ensure an equal representation between the four groups of isolates, as was possible: historical isolates 1994-2009 (the first *prn*IS481-positive isolate was identified in 1994), CA 2010, US 2010-2012, and WA 2012. The selection criteria was: 1) 25 isolates from each group; 2) 91 isolates were *prn*IS481-negative; 3) 9 isolates were *prn*IS481-positive to confirm lack of...
protein expression; and 4) random selection of both the prnIS481-positive and prnIS481-negative isolates from each of the four groups (8 sub-groups total) except for the sole 1 and 2 prnIS481-positive isolates found in the historical and CA 2010 group, respectively (Figure 1).

For Western blot analysis, cultures were grown overnight at 35°C in Stainer-Scholte medium. Lysates were made by processing harvested cells in saline using a FastPrep homogenizer. Approximately 5µg of protein was run on 12% NuPAGE gels in MOPS buffer (Life Sciences, Carlsbad CA) and transferred to PVDF membranes using an iBlot apparatus. Pertactin was detected on Western blots using the α-goat Western Breeze Kit (Life Sciences, Carlsbad CA) with NIBSC anti-69K antiserum 97/558 at 1:1000 dilution. The WHO strain 18323 served as the pertactin-positive control.

**Sequencing Analysis.** The prn alleles of a total of 93 isolates were sequenced (Figure 1). This included the 18 pertactin-deficient isolates found through the Western blot analysis. These 18 prn alleles were sequenced by ACGT (Wheeling, IL) using primers that covered the entire coding region (23). The remaining 75 isolates were from the WA 2012 outbreak group. Of these 75 isolates, 10 were prnIS481-positive isolates to confirm the PCR screening results and the presence of the insertion. The other 65 isolates were the remaining prnIS481-negative isolates from the WA 2012 outbreak that were not selected for Western blot analysis. The 75 isolates were sequenced at CDC using Applied Biosystems 3130xl Analyzers (Foster City, CA) with a previously described method (24) and analyzed using DNASTAR Lasergene 9 (Madison, WI).

Sequence identity was based on Tohama I sequence (Genbank accession number NC_002929.2) and alignment with IS481 was performed with Genbank accession number M22031 (25). Five novel sequences were identified and submitted to GenBank: KF804023 for the prnIS481 insertion at nt 2735; KF804024 for the STOP mutation at nt 760; KF804025 for the nucleotide G insertion at nt 1180; KF804026 for the STOP mutation at nt 760; KF804027 for the nucleotide G insertion at nt 1180;
Pulsed-field Gel Electrophoresis. Pulsed-field gel electrophoresis (PFGE) was performed on all 1300 isolates at CDC with a method previously described (26). Briefly, DNA in agarose plugs was digested with restriction enzyme XbaI. Restricted plugs were placed in 1% agarose gels. Electrophoresis was conducted in 0.5X Tris-Borate-EDTA buffer at 6V/cm for 18h at 14°C with a ramped switch time of 2.2-35 seconds. Gels were stained with ethidium bromide and DNA bands were visualized using UV light. A digital image in the TIFF format was captured for each gel. TIFF images were analyzed using BioNumerics software version 5.01 (Applied Maths, Inc., Austin, TX). Profiles consisted of bands in the range of 125-525 Kb.

Results

Of the 1300 isolates screened by prn PCR and/or later identified by Western blot and sequence analyses, 276 (21%) were identified as prnIS481-positive (Figures 1 and 2). Strikingly, only one (0.2%) prnIS481-positive isolate was found in the historic collection that dated from 1935 to 2009 (Figure 2A). This isolate was collected in 1994 from North Carolina, while the next prnIS481-positive isolates were observed in 2010 in both the CA outbreak and in the US 2010-2012 collection (Figure 2A). In the CA 2010 outbreak, only 2/33 (6%) prnIS481-positive isolates were identified. In the WA 2012 outbreak, over half (63%) of the isolates collected were prnIS481-positive (137/216 isolates). This observation does not appear to be a regional or isolated incidence, as the US isolates captured from 2010-2012, when stratified by year, also show a similar trend, with 14%, 40%, and 53% of the isolates being prnIS481-positive for years 2010, 2011, and 2012, respectively (Figure 2B).

Western blot analysis of 100 selected isolates confirmed that the nine prnIS481-positive isolates did not express pertactin and of the 91 prnIS481-negative isolates, 18 were deficient for pertactin
protein expression (Figure 1). Sequence analysis of these 18 isolates revealed seven different mutations in prn (Figure 1). Furthermore, sequencing of the remaining 65 prnIS481-negative isolates from the WA 2012 outbreak revealed an additional 21 mutants. In total, 306 pertactin-deficient isolates were detected. The geographic spectrum of these 306 pertactin-deficient isolates was far-reaching, with mutations found in AZ, CA, CO, CT, FL, GA, MA, MN, MO, NC, NM, NY, OR, PA, VT, and WA. The highest percentage (12.1%) was in WA, likely due to having the largest, most recent collection of isolates submitted and all prnIS481-negative isolates were sequenced. The next highest proportions were observed by OR (4.4%), MN and NY (1.5%), and MA (1%). From the 306 pertactin-deficient isolates, ten different types of mutations in the pertactin coding region were identified, including two deletions, single nucleotide substitutions resulting in a stop codon, and single nucleotide insertions resulting in frame shift mutations (Table 2, Figure 3). The majority of the mutant types (9/10) revealed prn2 alleles. Sequencing analysis of the prnIS481 insertion revealed the insertion to be located in various locations throughout the gene (Table 2). Two of these insertion mutations were previously identified in the US (GenBank accession numbers KC445198 and KC445197), with the insertion of the IS481 in either direction (20). A third mutation, a truncated pertactin protein with the stop codon at AA 425, was also identified from the US (GenBank accession number KC445199) (20). Only three isolates were identified as having the prn1 alleles and they contained a 5\textsuperscript{Δ} signal sequence deletion, a mutation that was previously identified in Japan (GenBank accession number AB670735) (19). Interestingly, nine prnIS481-positive isolates had the IS481 insertion at the 3\textsuperscript{′} end of the gene (nt 2735), which was outside of the coding region amplified in the initial PCR screen (Figure 1). Of note, the predicted protein resulting from this IS481 insertion is nearly full-length in size, yet this mutation resulted in a lack of detectable pertactin expression by Western blot analysis. PCR and sequencing of the region upstream of the coding sequence revealed a large deletion that
encompassed the promoter and 5\textsuperscript{\textprime} region (Table 2, Type VIII) and a genomic rearrangement that disrupted the pertactin promoter (Table 2, Type IX). Since the Type VIII mutation was found only through the sequencing of the WA 2012 \textit{prn}\textit{IS}\textit{481}-negative isolates, it was later confirmed to lack protein expression through Western blot analysis. Finally, one isolate had a wild-type promoter and coding region, suggesting that it contains a mutation in the regulatory system controlling pertactin expression (Table 2, Type X).

Predominant PFGE profiles of the 994 isolates that did not have a mutation identified ("pertactin-present") and the 306 isolates that were identified as lacking pertactin protein ("pertactin-deficient") are shown in Figure 4. CDC013, a profile that has predominated in US since 1999, peaked at 48\% in 2001, and has gradually been decreasing since the peak (27), was a predominant profile in the pertactin-present isolates of all four time periods, representing 20\% (203/994) of the pertactin-present isolates but only 5\% (16/306) of the pertactin-deficient isolates. Two profiles that have recently emerged, CDC237 and CDC253, predominated in only the pertactin-deficient groups. Finally, unlike the pertactin-present isolates, two profiles, CDC002 and CDC237, represented 55\% (167/306) of the identified pertactin-deficient isolates from 2010 to present. Interestingly, while CDC237 is a much more recently identified profile, CDC002 was identified in the 1980s. CDC002 accounted for only 4\% of U.S. isolates collected between 2000 and 2009 and increased to 9\%, 24\%, and 28\% of isolates from 2010, 2011, and 2012, respectively (26, 28).

\textbf{Discussion}

This work marks the first documentation of the frequency and geographic distribution of pertactin-deficient isolates in the US. Based upon data from PCR, Western blot analysis, and sequencing, pertactin-deficient isolates have been found to be nation-wide. While the first pertactin-deficient isolate identified here was from 1994, the high frequency appears to be more recent with...
expansion of pertactin-deficient isolates first observed in 2010. It must be noted that this work was based on an initial screening method that did not encompass all the possible mechanisms for losing pertactin protein expression. Likewise, it cannot be discounted that pertactin molecules may exist that can be expressed but are not functional. Therefore, while the subset of isolates analyzed by Western blot was a good representation of each of the four time period groups, the current numbers of each type of mutation may be underrepresented. Finally, because one wild-type prn (Table 2), pertactin-deficient isolate was identified, sequencing of the coding region may not be sufficient to determine lack of protein expression.

The loss of pertactin expression has been reported from several other countries (16-19). Despite the emergence of pertactin-deficient isolates occurring over a decade ago, the expansion of these isolates has been a recent phenomenon in the US. Our first prnIS481-positive isolate identified in 1994 is similar to discoveries from other countries, namely in Italy that identified a mutant from isolates collected between 1993 and 1995 (18) and in Japan, where the earliest isolate identified from a collection between 1990-2009 was in 1997 (19). In other countries, pertactin-deficient isolates were analyzed in more recent collections, such as 2000 to 2011 for France and 2006-2011 in Finland (13, 17, 19). Not only has the expansion been recent in the US, but the proportion of pertactin-deficient isolates in the circulating population is also noteworthy, with over 50% of the isolates received by the CDC in 2012 being pertactin-deficient isolates. In France, the highest prevalence was 13% in 2011 and in Finland, only two of 76 isolates were found to be pertactin-deficient (13, 17, 19). The variety of mutations has also differed between countries, with only two mutation types identified, the IS481 insertion in one location and the 5\(^\text{\textsuperscript{\textcircled{b}}}\) signal sequence deletion, in the Japanese collection to several more in the French collection, including a large deletion of the promoter and 5\(^\text{\textcircled{b}}}\) region (different from our large deletion mutants), IS481 insertions, and stop codon mutations (13, 17).
Indeed, the fact that *B. pertussis* has eliminated pertactin expression using a variety of mutational events indicates a strong selection for loss of pertactin. Clonal expansion of specific mutations does appear to be occurring in multiple states, regardless of whether it was from an outbreak population or surveillance site (Table 2). The frequency of two PFGE types, CDC002 and CDC237, also appears to predominate among the recent pertactin-deficient isolates (Figure 4). However, the lack of co-association of the mutations causing pertactin-deficiency with specific PFGE patterns suggest these mutations are perhaps independent of pertussis clonal selection and redistribution or at least fall under different selective pressures as those of the whole genome (Figure 3). Additionally, it must be noted that not all the prnIS481-positive isolates were sequenced and these mutation types make up the majority of the total population of mutants, so the clonal nature of these mutations remains unclear.

The presence of genetic mutations in one of the vaccine antigens reveals the importance of remaining vigilant in monitoring the emergence of mutations in other vaccine antigens or those targets used for molecular diagnostic testing. Indeed, the deletion of the pertussis toxin gene was once observed in France in 2009 (17). However, *prn* is not a target commonly used for pertussis diagnosis through PCR. Additionally, the CDC Pertussis and Diphtheria Laboratory currently monitors multiple PCR targets in our isolate and specimen collections and, to date, no mutations in these diagnostic targets (29) have been observed.

Research is shedding more light on the function of pertactin in virulence and pathogenesis, revealing multiple and/or potentially conflicting roles. One pertactin-deficient isolate showed improved entry into human monocyte-driven dendritic cells, suggesting pertactin may play a role in preventing internalization (30). Earlier opsonophagocytic work supports this suggestion, finding that anti-pertactin antibodies were crucial for *B. pertussis* phagocytosis (31). However, cytotoxicity remains high despite the loss of pertactin, suggesting some functional redundancy may be involved (17); indeed, the
identification of numerous autotransporters, with as of yet undefined roles, has been suggested to be
the reason for the comparable virulence in pertactin-deficient isolates (32). From a clinical perspective,
the loss of pertactin does not appear to alter disease severity, as a recent study of hospitalized infants
<6 months old showed that pertussis disease, measured by the presence of classical symptoms, such as
apnea, vomiting, paroxysmal cough, and whoop, caused by pertactin-positive or pertactin-deficient
isolates had a similar clinical course (33). The mechanism of the selective advantage leading to the
expansion of pertactin-deficient variants still remains unclear.

Previous hypotheses suggest that the acellular vaccine helped drive the expansion of these
particular clones, as the bacterial genome was already well adapted to the environment and only
needed small mutations to thrive in current vaccinated populations (13, 15). However, vaccination with
the acellular vaccine may not be the sole cause of this recent phenomenon in the US as the first
pertactin-deficient isolate discovered was from the time of early implementation of the acellular vaccine
primary series, suggesting that these isolates may have already been in circulation. It is interesting to
note that the phenomenon coincides with the time when the US is experiencing large outbreaks in a
population receiving primarily acellular vaccine (2, 3); however, pertactin-deficient isolates have
expanded only within the last few years despite over a decade of acellular vaccine usage.

Regardless of the reasons for this recent increase of pertactin-deficient isolates in the US,
clinical information, in vivo functional assays, animal modeling, and vaccine effectiveness studies are
crucial for not only understanding the clinical implications of these changes, but to also help develop
and test new vaccine formulations (34). Vigilant monitoring of cellular and molecular changes of not
only B. pertussis, but also other Bordetella spp., via bacterial isolation and molecular methods, is critical
for determining the next steps to protecting the public health from these highly contagious pathogens.

Acknowledgements
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AQ, AL, and WS are employees of Johnson & Johnson and own Johnson & Johnson stock.


Figure Legends

FIGURE 1. Flow chart of the progression of pertactin-deficiency identification, from the conventional PCR screening of the IS481 insertion in the prn gene to Western blot analysis and sequencing. IS481-positive = IS481 insertion in prn; IS481-negative = IS481 insertion in prn not detected; WB+ = pertactin protein present by Western blot; WB- = pertactin protein absent by Western blot; WT = wild-type; STOP = stop codon.


FIGURE 3. Location of mutations in the prn gene, identified through PCR, Western Blot, and sequencing analyses. Mutations included 3 IS481 insertions, 2 premature stop codons (star), a nucleotide insertion (G), a signal sequence deletion (ΔSS), a large deletion in 5' region and upstream (Δ2.6Kb), and a large inversion of the promoter region (Inv). IS481 insertions were found in both directions, where indicated, according to alignment with GenBank accession number M22031. Mutations marked with an asterisk (*) have been previously identified. AA= amino acid; VR= variable region.

FIGURE 4. PFGE profiles of the 1300 B. pertussis isolates analyzed for pertactin-deficiency, stratified by origin and time period. The top three predominant profiles are indicated per group; all remaining profiles are identified as “All others”. PRN+= B. pertussis isolates that do not contain genetic mutations.
and/or express pertactin; PRN = *B. pertussis* isolates that are pertactin-deficient as tested by PCR screening, Western blot analysis, and *prn* sequencing; n = Number of isolates
TABLE 1. Primers used for PCR screening and sequence analysis.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>Position on Tohama I relative to prn start codon*</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PF</td>
<td>TGTCTCTGTCACGCATTGTC</td>
<td>8 to 27</td>
<td>(22)</td>
</tr>
<tr>
<td>PR</td>
<td>ATGCCGTTGGTGTGACCGT</td>
<td>2551 to 2570</td>
<td>(22)</td>
</tr>
<tr>
<td>AF</td>
<td>GCCAATGTCACGGTCCAA</td>
<td>505 to 522</td>
<td>(22)</td>
</tr>
<tr>
<td>AR</td>
<td>GCAAGGTGATCGACAGGG</td>
<td>1073 to 1090</td>
<td>(22)</td>
</tr>
<tr>
<td>BF</td>
<td>AGCTGGGCGGTTCAAGGT</td>
<td>1398 to 1415</td>
<td>(22)</td>
</tr>
<tr>
<td>BR</td>
<td>CGGATTCAGGCGCAACTC</td>
<td>1915 to 1932</td>
<td>(22)</td>
</tr>
<tr>
<td>PRN-F</td>
<td>CGTACTTTTGCTGCCCAT</td>
<td>-79 to -60</td>
<td>(19)</td>
</tr>
<tr>
<td>PRN-R</td>
<td>CCAAGCTCCAGGAAAACCTC</td>
<td>2761 to 2780</td>
<td>(19)</td>
</tr>
<tr>
<td>PRNUP2354</td>
<td>GAGAGCCATTACTGGAGATT</td>
<td>-2353 to -2333</td>
<td>New</td>
</tr>
<tr>
<td>PRN-P-F</td>
<td>TGCCAAGACGGTATCTGT</td>
<td>-331 to -314</td>
<td>New</td>
</tr>
<tr>
<td>PRN-P-R</td>
<td>GACTGTTGTTCCAGTCG</td>
<td>102 to 119</td>
<td>New</td>
</tr>
<tr>
<td>PRN342R</td>
<td>GGTGACGGTGCCCAGAAGC</td>
<td>323 to 342</td>
<td>New</td>
</tr>
<tr>
<td>PRNA2F</td>
<td>AGGGTGACGGTGTGGGCC</td>
<td>982 to 999</td>
<td>New</td>
</tr>
<tr>
<td>PRN1627R</td>
<td>TATCGACCTTGGCTCCTT</td>
<td>1627 to 1645</td>
<td>New</td>
</tr>
<tr>
<td>PRNB2F</td>
<td>CAGCAGCTGGACAACCCG</td>
<td>1972 to 1989</td>
<td>New</td>
</tr>
<tr>
<td>PRNP2R</td>
<td>CTTGCCCTTGACCGCGT</td>
<td>2258 to 2274</td>
<td>New</td>
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<td>PRN2258F</td>
<td>CGGTCAAGGGCAGTACC</td>
<td>2261 to 2278</td>
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</tr>
<tr>
<td>PRN2357F</td>
<td>CCGAGCTGGCGGTATTC</td>
<td>2357 to 2373</td>
<td>New</td>
</tr>
</tbody>
</table>

*prn lies in region nt 1098091 to 1100823 of the Tohama I complete genome, Genbank Accession number NC_002929.2.
TABLE 2. Characterization of pertactin-deficient isolates by sequencing analyses. MLST = multi-locus sequence typing, IS = insertion sequence, PFGE = pulsed-field gel electrophoresis, STOP = stop codon, AA = amino acid.

<table>
<thead>
<tr>
<th>Mutation Type+</th>
<th>Pertactin mutation, nucleotide #</th>
<th>Pertactin MLST allele</th>
<th>Predicted pertactin protein*</th>
<th>State (#)</th>
<th>Year (#)</th>
<th>PFGE (#)</th>
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<tbody>
<tr>
<td>I.</td>
<td>IS481, 246† prn2</td>
<td></td>
<td>Stop at AA 87</td>
<td>WA (2)</td>
<td>2011 (1)</td>
<td>CDC253 (2)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>2012 (1)</td>
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</tr>
<tr>
<td>II.</td>
<td>IS481, 1613† prn2</td>
<td></td>
<td>Stop at AA 543</td>
<td>WA (8)</td>
<td>2012 (8)</td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>III.</td>
<td>IS481, 2735† prn2</td>
<td></td>
<td>Stop at AA 913</td>
<td>WA (8)</td>
<td>2012 (7)</td>
<td>CDC322 (6)</td>
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<tr>
<td>IV.</td>
<td>STOP, 760 prn2</td>
<td></td>
<td>Stop at AA 254</td>
<td>NY (4)</td>
<td>2011 (1)</td>
<td>CDC242 (4)</td>
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<td></td>
<td></td>
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<td>2012 (3)</td>
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<td>V.</td>
<td>STOP, 1273 prn2</td>
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<td>Stop at AA 425</td>
<td>VT (1)</td>
<td>2011 (1)</td>
<td>CDC002 (1)</td>
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<td>VI.</td>
<td>Insert G, 1185 prn2</td>
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<td>Frame shift at AA 395</td>
<td>MN (2)</td>
<td>2012 (2)</td>
<td>CDC002 (2)</td>
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<td>VII.</td>
<td>Deletion, 26-109 prn1</td>
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<td>Deletion of 28 AA</td>
<td>CA (1)</td>
<td>2010 (1)</td>
<td>CDC268 (1)</td>
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<td>WA (2)</td>
<td>2012 (2)</td>
<td>CDC260 (2)</td>
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<td>VIII.</td>
<td>Deletion, -2090-478 prn2</td>
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<td>Deletion of 2.6 Kb</td>
<td>WA (2)</td>
<td>2012 (2)</td>
<td>CDC013 (1)</td>
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<td>IX.</td>
<td>Inversion, -74 prn2</td>
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<td>Inversion of ~22 Kb in promoter</td>
<td>WA (16)</td>
<td>2011 (2)</td>
<td>CDC010 (2)</td>
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* Predicted pertactin protein: Stop at AA 87, Stop at AA 543, Stop at AA 913.
<table>
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<tr>
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<th>X. Wild-type</th>
<th>prn2</th>
<th>Full length 915 AA</th>
<th>CA (1)</th>
<th>2010 (1)</th>
<th>CDC013 (1)</th>
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* At least one isolate of each mutation type has been confirmed to lack protein expression by Western blot analysis.

* prn lies in region between nucleotides 1098091 to 1100823 of the Tohama I complete genome, Genbank Accession number NC_002929.2.

† Shows forward IS481 insertion as example, according to GenBank accession number M22031.
FIG 1.

PCR screening to identify prnIS481+ or prnIS481-

Historical 1935-2009 (666 isolates)

CA 2010 (33 isolates)

US 2010-2012 (385 isolates)

WA 2012 (216 isolates)

1 prnIS481+ confirmed
24 WB+

1 prnIS481+/24 prnIS481-
analyzed by WB

2 prnIS481+ confirmed
21 WB+/2 WB-

2 WB+ sequenced

1 deletion, nt 26-109
1 WT prn

Total 4 prn mutants

Total 1 prn mutant

Total 157 prn mutants

65 prnIS481- sequenced

1 prnIS481+/22 prnIS481-
analyzed by WB

3 prnIS481+ confirmed
13 WB+/9 WB-

9 WB- sequenced

1 prnIS481+, nt 2735
4 STOP, nt 760
2 STOP, nt 1273
2 insert G, nt 1180

Total 4 prn mutants

Total 144 prn mutants

4 prnIS481+, nt 2735
2 deletion, nt 26-109
2 deletion, nt -2090-478
3 inversion, nt -74

Total 15 prn mutants

3 prnIS481+ confirmed
15 WB+/7 WB-

7 WB- sequenced

4 prnIS481+, nt 2735
3 inversion, nt -74

3 prnIS481+ confirmed
10 sequenced
87 prnIS481-

129 prnIS481+, (10 sequenced)
665 prnIS481-

1 prnIS481+/24 prnIS481-
analyzed by WB

3 prnIS481+/22 prnIS481-
analyzed by WB

1 prnIS481+/23 prnIS481-
analyzed by WB

3 prnIS481+ confirmed
13 WB+/9 WB-

9 WB- sequenced

1 prnIS481+, nt 2735
4 STOP, nt 760
2 STOP, nt 1273
2 insert G, nt 1180

Total 4 prn mutants

Total 144 prn mutants

4 prnIS481+, nt 2735
2 deletion, nt 26-109
2 deletion, nt -2090-478
3 inversion, nt -74

Total 15 prn mutants

3 prnIS481+ confirmed
10 sequenced
87 prnIS481-

129 prnIS481+, (10 sequenced)
665 prnIS481-

1 prnIS481+/24 prnIS481-
analyzed by WB

3 prnIS481+/22 prnIS481-
analyzed by WB

1 prnIS481+/23 prnIS481-
analyzed by WB

3 prnIS481+ confirmed
13 WB+/9 WB-

9 WB- sequenced

1 prnIS481+, nt 2735
4 STOP, nt 760
2 STOP, nt 1273
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Total 4 prn mutants

Total 144 prn mutants

4 prnIS481+, nt 2735
2 deletion, nt 26-109
2 deletion, nt -2090-478
3 inversion, nt -74

Total 15 prn mutants
FIG 2.

A.  [Bar chart showing the percentage distribution of prmS481-positive and prmS481-negative across different categories (Historical 1935-2009, CA 2010, US 2010-12, WA 2012).]

B.  [Bar chart showing the percentage distribution of prmS481-positive and prmS481-negative for the years 2010, 2011, and 2012.]
FIG 3.
FIG 4.