

Pertactin-Negative and Filamentous Hemagglutinin-Negative *Bordetella pertussis*, Australia, 2013–2017

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During the 2008–2012 pertussis epidemic in Australia, pertactin (Prn)–negative *Bordetella pertussis* emerged. We analyzed 78 isolates from the 2013–2017 epidemic and documented continued expansion of Prn-negative *ptxP3* *B. pertussis* strains. We also detected a filamentous hemagglutinin-negative and Prn-negative *B. pertussis* isolate.

Despite high vaccination coverage, pertussis remains a major public health concern. In many industrialized countries, including Australia, whole-cell vaccine was replaced by the less reactogenic acellular vaccine (ACV). In Australia, the 3-component ACV (containing pertactin [Prn], pertussis toxin [Ptx], and filamentous hemagglutinin [Fha]) has been more widely used than the 5-component ACV (which also contains fimbrial antigen: Fim2 and Fim3).

Since 1991, when notifications began, pertussis has reemerged in Australia, and epidemics occur every 3–5 years. The largest epidemic occurred in 2008–2012; 39,000 cases were recorded at its peak in 2011 (1,2). Most *Bordetella pertussis* isolates from that epidemic belonged to 1 genetic group, referred to as single-nucleotide polymorphism (SNP) cluster I (1–3). SNP cluster I had *prn2* allele of the *prn* gene and *ptxP3* allele of the Ptx promoter (3).

In a study of the 2008–2012 epidemic, Lam et al. (1) reported a rapid increase in the number of isolates not expressing the ACV antigen Prn (Prn-negative), from 5.13% in 2008 to 77.78% in 2012. Sequencing of 22 isolates revealed 5 epidemic lineages (ELs) (EL1–EL5) and independent

origins of Prn-negative strains in different ELs (4). A smaller epidemic occurred during 2013–2017, peaking at 22,000 cases in 2015 (Figure 1, panel A). We investigated the genotypic and phenotypic characteristics of 78 *B. pertussis* isolates from 2013–2017 to determine the epidemic trends of pertussis in Australia.

The Study

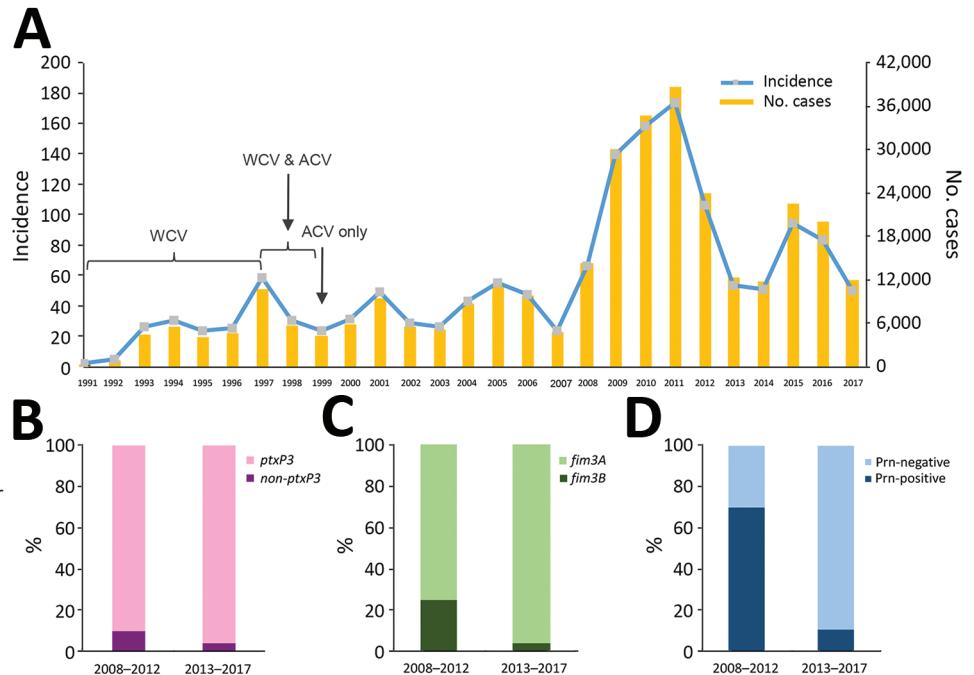
We sequenced 78 *B. pertussis* isolates (Appendix 1 Table 1) from New South Wales (NSW) (17/78 [21.8%]) and Western Australia (WA) (61/78 [78.2%]) that were collected during the 2013–2017 epidemic. We conducted SNP detection (Appendix 1 Table 2) and examined variation in ACV antigen genes (*prn*, *ptxA*, *ptxP*, and the 2 fimbrial genes *fim2* and *fim3*). Using the SNP-based classification scheme by Octavia et al. (3), we typed the 78 isolates into 2 SNP profiles (SPs): SP13 (SNP cluster I, *ptxP3*, 75/78 [96.2%]) and SP18 (noncluster I, *ptxP1*, 3/78 [3.8%]). All isolates harbored the *ptxA1* allele. Most (75/78 [96.2%]) of the SP13 isolates had the *prn2* and *fim3A* alleles. The 3 noncluster I SP18 isolates had a *fim3A** allele that differs from *fim3A* by a synonymous mutation (3) with genotype *ptxP1-fim3A*-prn1*. The frequency of *ptxP3* and *fim3A* alleles was higher than during the 2008–2012 epidemic (Figure 1, panels B, C). All but 1 isolate carried the *fim2-1* allele. One isolate (L2263 [SP18]) contained a *fim2* allele with a new 3-nucleotide insertion (AGA) at position 506, resulting in the insertion of a lysine in the epitope (F2.9) region of Fim2 (5). PROVEAN analysis (6) suggests that the insertion does not affect protein structure and thus might or might not affect immune recognition. We designated this allele as *fim2-3* (GenBank accession no. MG824989). Western immunoblotting showed that all isolates expressed Ptx, and all but 1 (L2228) expressed Fha. For Prn, 89.7% (70/78) isolates were Prn-negative (Figure 1, panel D), suggesting continued expansion of Prn-negative strains.

We found multiple mechanisms of *prn* inactivation in the isolates, all but 1 of which were reported previously (1,7–9). For most (66/70) isolates, inactivation was caused by insertion sequences (IS), including 45 IS481F insertions (F/R denotes insertion orientation relative to *prn*), 17 IS481R insertions, and 4 IS1002R insertions (Table). We found an IS481F insertion, which has been reported in *prn1*

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Figure 1. Pertussis trends and temporal changes of allele frequencies of vaccine antigens genes, Australia. A) Pertussis notifications in Australia, 1991–2017. Source: National Notifiable Diseases Surveillance System (<http://www9.health.gov.au/cda/source/cda-index.cfm>). Incidence is number of cases per 100,000 population. Reporting system was established in 1991. ACV was first introduced in Australia in 1997, and WCV was replaced with ACV in 1999. A large pertussis epidemic occurred during 2008–2012, and another epidemic occurred during 2013–2017. B) Percentage of *Bordetella pertussis* isolates carrying *ptxP3* allele in the 2 epidemic periods. C) Percentage of *B. pertussis* isolates carrying *fim3A* and *fim3B* allele in the 2 epidemic periods. D) Percentage of pertactin-expressing (Prn-positive) and pertactin-deficient (Prn-negative) *B. pertussis* isolates in the 2 epidemic periods. ACV, acellular vaccine; Prn, pertactin; WCV, whole-cell vaccine.



and *prn2* isolates only (1,8), in 3 of the *prn3* isolates. One Prn-negative isolate contained a SNP (C→T) in position 223, resulting in a stop codon, a mutation found previously in US isolates only (10). Two isolates had a deletion (position -297, 1325 [relative to the initiation codon ATG]) between the promoter and 5' end of *prn* that was replaced

with a fragment of IS1663, which might have mediated the deletion (Table). A similar but slightly different deletion (position -292, 1340) was reported in US isolates (7). We identified a new inactivation by a 4-bp deletion, from position 2020 to 2023 in *prn*, in 1 isolate (L2210) (Appendix 1 Table 1).

Table. Mechanisms of pertactin deficiency and characteristics of *Bordetella pertussis* isolates from pertussis epidemics, Australia, 2013–2017*

Prn deficiency mechanism	Position in <i>prn</i> †	<i>prn</i> allele type	State (no. of isolates)	Year (no. isolates)	References
IS481F	1613	<i>prn2</i>	Western Australia (32) New South Wales (10)	2013 (13) 2014 (5) 2015 (11) 2016 (9) 2017 (4)	(1)
IS481R	1613	<i>prn2</i>	Western Australia (12) New South Wales (5)	2013 (6) 2014 (5) 2015 (4)	(1)
IS481F	1598	<i>prn3</i>	Western Australia (3)	2013 (1) 2014 (2)	This study
IS1002R	1613	<i>prn2</i>	Western Australia (4)	2013 (2) 2016 (1) 2017 (1)	(1)
Deletion	-297 to 1325	Not determined‡	Western Australia (2)	2014 (1) 2015 (1)	(8), newly found in Australia
Stop codon	223	<i>prn2</i>	Western Australia (1)	2014 (1)	(10), newly found in Australia
Deletion	2020–2023	<i>prn2</i>	Western Australia (1)	2013 (1)	This study

*F/R denotes IS insertion orientation relative to *prn*. F, forward; IS, insertion sequence; Prn, pertactin; R, reverse.

†The nucleotide positions are relative to the initiation codon (ATG) of the *prn* in Tohama I.

‡*prn* allele type was not determinable because the repeat regions that define *prn* allele type were deleted in this mechanism.

One Prn-negative isolate (L2228) was also Fha-negative (i.e., Prn⁻, Fha⁻) by Western immunoblotting. The Fha inactivation probably resulted from changes within the homopolymeric G tract (site: 1078–1087) from 10 Gs to 11 Gs in *fhaB*, resulting in a downstream stop codon that produces a truncated FhaB protein (11). Both Illumina and Sanger sequencing (Appendix 2) showed a mixture of 10 Gs and 11 Gs. The bacterial population most likely contained predominantly 11 Gs with a lower proportion of 10 Gs. Proteomic analysis using liquid chromatography tandem mass spectrometry (12) found that, in the whole cell of L2228, only 2.3% of the FhaB protein was detected as peptides and derived mainly from the first 350 aa of the FhaB protein. In contrast, in the Fha-positive isolate (L2248), 30.7% of the FhaB protein was detected as peptides and derived from the entire protein. However, in the supernatant of L2228, we detected peptides across the entire FhaB protein and at a higher coverage of 22.7% than for whole-cell FhaB. For the Fha-positive isolate, we detected 52.0% of the FhaB across the entire protein. Western immunoblotting could not detect any FhaB in supernatant or whole-cell proteins of the Fha-negative isolate.

Together with the 27 *B. pertussis* isolates from Australia previously sequenced, we analyzed a total of 105 *B. pertussis* isolates to determine their genomic relationships (Figure 2). Five preepidemic SP13 isolates from 1997–2002 were ancestral to the SP13 epidemic clade as expected; 3 noncluster I (*ptxP1*) isolates grouped together as a separate

clade outside SNP cluster I. Most (68/75) isolates grouped into 4 previously defined ELs (EL1–EL4) (4). However, no isolates from the new epidemic fell into the 2008–2012 EL5. Four isolates (L2233, L2234, L2261, and L2262) did not cluster with any of the ELs.

Prn-positive isolates from the 2008–2012 and 2013–2017 epidemics were distributed among different lineages. Prn-negative isolates were largely grouped by mechanism of inactivation in different ELs. Prn-negative isolates in EL1 and EL4 were caused by IS48/R insertion. All but 1 Prn-negative isolate in EL2 was caused by IS1002 insertion; the exception was an IS48/R insertion. Prn-negative isolates in EL3 were caused by IS48/F insertion. Three Prn-negative isolates with new inactivation mechanisms found in Australia were distributed in EL4 (*prn::del* [-297, 1325]; note that the *prn* allele was indeterminate) and non-ELs (*prn2::stop* [C233T]).

EL1 contained isolates from NSW (6/20) and WA (14/20). EL2 was a small lineage (8 isolates), but these isolates were from both periods and both states. EL3 was predominantly a WA lineage; 30/33 isolates from WA and nearly half of the WA isolates (30/61) from 2013–2017 were EL3. EL4 was largely an NSW lineage (14/23 isolates).

Conclusions

The 2013–2017 pertussis epidemic in Australia was predominantly caused by Prn-negative strains, with local and

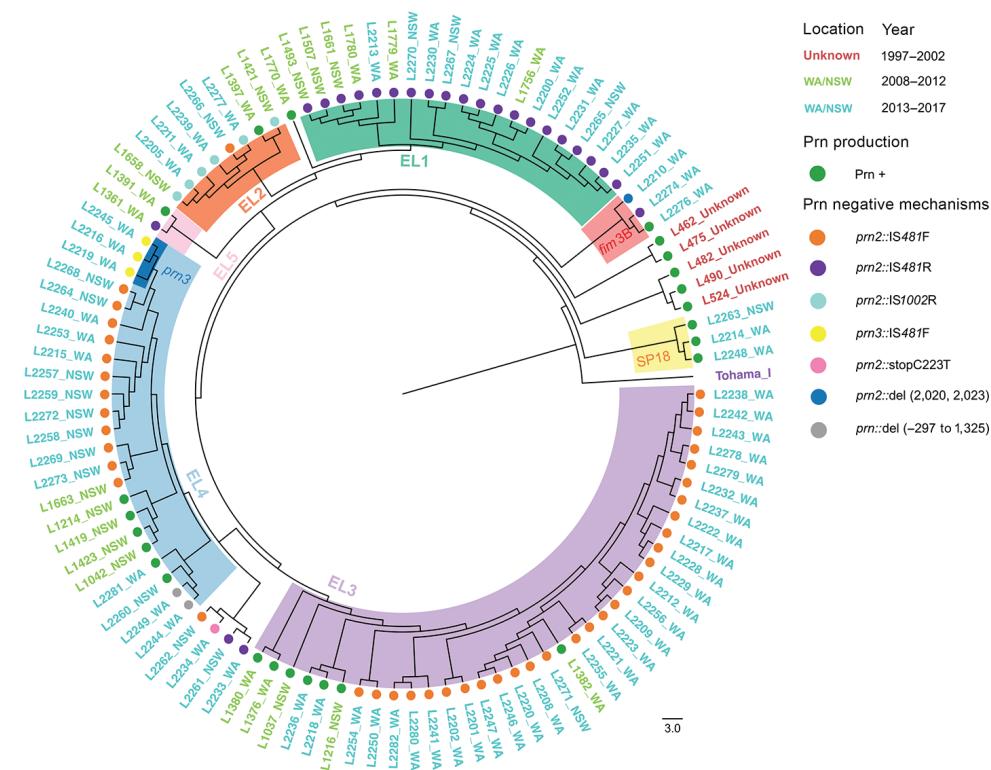


Figure 2. Phylogenomic relationship of 105 *Bordetella pertussis* epidemic isolates from Australia. The minimum-evolution tree was constructed using 705 SNPs. The isolates are labeled by name (L numbers), followed by states with color to indicate years of isolates. Shaded areas inside circle indicate ELs (EL1–EL5) and branches with isolates belonging to SP18 and those carrying *fim3B* and *prn3* alleles. Prn-positive isolates and different mechanisms of Prn-negative isolates are marked by circles with different colors. Scale bar represents 3 SNPs. EL, epidemic lineage; NSW, New South Wales; Prn, pertactin; SNP, single-nucleotide polymorphism; SP, SNP profile; WA, Western Australia.

interstate expansion of 4 epidemic lineages. The ongoing expansion of Prn-negative strains is most likely due to continued vaccine selection pressure because Australia has been using ACVs that contain Prn since their introduction. This observation contrasts with the declining circulation of Prn-negative strains in Japan, where changes in the vaccine probably caused the decrease because 2 of the 3 vaccines used after 2012 did not contain Prn (13). The emergence of an Fha-negative and Prn-negative *B. pertussis* in Australia may offer higher potential to escape ACV-induced immunity.

Our results provide further evidence of *B. pertussis* evolution under vaccine selection. Continued surveillance of *B. pertussis* will provide a better understanding of the effect of vaccination on the evolution of the pathogen and optimize strategies to reduce the occurrence of pertussis.

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About the Author

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Appendix 2

Materials and Methods

Bacterial Strains and Genomic DNA Preparation

Bordetella pertussis were sampled from Princess Margaret Hospital for Children, Subiaco WA, and Westmead Hospital, Sydney, NSW, from 2013 to 2017. A total of 78 isolates (details of the strains used in this study are given in Appendix 2 Table 1, <https://wwwnc.cdc.gov/EID/article/25/6/18-0240-App1.xlsx>) were acquired from cryovials stored in -80°C and inoculated on Bordet Gengou agar (Becton Dickinson, Sparks, MD, USA, supplemented with 7% horse blood). All samples were incubated at 37°C for 4–5 days. Genomic DNA were extracted and purified by the phenol-chloroform method.

Fewer isolates were collected in NSW than WA, possibly due to different numbers of cases coming into the collecting hospital and different uptake of direct PCR as the default method of laboratory diagnosis. The number of isolates did not reflect the population size of the state nor the local incidence of pertussis.

Genome Sequencing and Assembly

Whole-genome sequencing (WGS) of isolates was performed by Illumina MiSeq/NextSeq (Illumina, Scoresby, VIC, Australia). DNA libraries were constructed using Nextera XT Sample preparation kit (Illumina Inc., San Diego, CA, USA). The fragment size distribution of the tagmented DNA was analyzed using a High Sensitivity DNA assay kit (Caliper Life Science, Hopkinton, MA, USA). Genome sequencing was done in a multiplex of 60 samples. Libraries were sequenced either using the MiSeq Personal Sequencer (Illumina Inc) or NextSeq sequencer (Illumina Inc.). Raw reads were submitted to GenBank under the BioProject PRJAN432286). De novo assembly was performed for all sequencing data using

SPAdes (version 3.7.0). Sanger sequencing (ABI 3730 Capillary Sequencer, Ramaciotti Center for Genomics, UNSW Sydney, Kensington, NSW, Australia) was performed to confirm the genotypes of *prn*, *fim2*, and *fhaB*. *prn* was amplified by PCR and sequenced using the published primers (PRN1157 5'-CACCGCACGGCAATGTCAT-3', PRNBR 5'-CGGATTCAGGCGCAACTC-3') (*1*), while newly designed primers (fhaBF 5'-TCAAGTTGGGCACTGGAGAC-3', fhaBR 5'-CTGGGCTATTTTCGACGTGGT-3') were used for *fhaB* and (fim2F 5'-ACGGCATTGGCAGTGGTGGGA-3', fim2R 5'-CACACAACTTGATGGGCGA-3') for *fim2*.

Bioinformatics Analysis

SNP detection used a combination of mapping by Burrow-Wheeler Alignment (BWA) tool (version 0.7.12), SAMtools (version 0.1.19), and alignment by progressiveMauve (version snapshot_2015_02–25). Phylogenetic trees were constructed using MEGA (version 5.2.1), the Minimum Evolution tree was applied based on the Close-Neighbor-Interchange (CNI) method and the bootstrap analysis was based on 1,000 replicates (SNPs files shown in Appendix 2 Table 2). Short insertion/deletions (indels) (<100 bp) were identified by SAMtools.

Western Immunoblotting

Western immunoblotting was used to detect the expression of Pertussis toxin (Ptx), Pertactin (Prn) and Filamentous hemagglutinin (Fha). Bacterial suspensions were mixed with Laemmli buffer (5% β -mercaptoethanol were added before use) and boiled for 10 min. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto a polyvinylidene difluoride membrane at 100 V for 1.5 hr. Membranes were washed 3 times in washing buffer (50 mM Tris-HCl pH7.6, 150 mM NaCl, 0.05% Tween-20) before incubated, first with blocking buffer (washing buffer, 5% [wt/vol] skim milk powder] at room temperature (RT) for 2 hr, and then with mouse polyclonal antibodies against Ptx, Fha, and Prn (diluted in 1:1,000, 1:1,000, and 1:3,000, respectively with blocking buffer) at 4°C, overnight. After being washed 3 times with washing buffer, membranes were incubated with sheep antimouse (IgG) antibodies conjugated to horse radish peroxidase (Abcam) diluted in 1:20,000 with blocking buffer at RT for 1 hr and 15 min.

Sample Preparation for LC-MS/MS

Liquid chromatography–mass spectrometry (LC-MS/MS) was performed on 2 samples (L2228 and L2248) to confirm the expression of Fha. Each sample had 3 biological repeats. For trypsin digestion, 10 mM Dithiothreitol (DTT) was added to samples (10 µg/sample) for the reduction of disulfide bonds and incubated at 37°C for 30 min. Following incubation, samples were alkylated through the addition of 20 mM Iodoacetamide (IA) and incubated for an additional 30 min at 37°C in the dark. Samples were then incubated at 37°C overnight after Trypsin was added at a ratio of 1:100 enzymes to protein. Strong cationic exchange (SCX) was performed using 200 µL SCX stage tips (Thermo Fisher Scientific) on each sample for clean-up. After SCX, samples were then dried using a Speedivac (Thermo Fisher Scientific) and resuspended in 10 µL of 0.1% formic acid.

LC-MS/MS

Samples were analyzed in the Bioanalytical Mass Spectrometry Facility (BMSF) at the University of New South Wales using LC-MS/MS. Protein identifications were searched against the NCBI database using the Mascot server (v.2.51) (Matrix Science).

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