Sequence variation and conservation in virulence-related genes of *Bordetella pertussis* isolates from the UK

Erica R. Packard,[1,2] Roger Parton,[1] John G. Coote[1] and Norman K. Fry[2]

[1]Division of Infection and Immunity, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, Scotland, UK

[2]Health Protection Agency, Respiratory and Systemic Infection Laboratory, Specialist and Reference Microbiology Division, 61 Colindale Avenue, London NW9 5HT, UK

Correspondence
Norman K. Fry
Norman.Fry@HPA.org.uk

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To determine the value of gene markers for surveillance and to assess the genetic stability of potential acellular pertussis vaccine components, the sequence variation in ten virulence-related genes of *Bordetella pertussis* was investigated in strains isolated in the UK between 1920 and 2002. These genes encode: pertactin (*prnA*); pertussis toxin subunits S1 (*ptxA*) and S3 (*ptxC*); tracheal colonization factor (*tcfA*); bordetella autotransporter protein C (*bapC*); bordetella resistance to killing protein (*brkA*); fimbrial antigen 2 (*fim2*); outer-membrane protein Q (*ompQ*); virulence-activated gene 8 (*vag8*) and adenylate cyclase toxin (*cyaA*). The encoded proteins are either components of current acellular vaccines (ACVs), or potential virulence markers for *B. pertussis*. Three strains used in the pertussis UK whole-cell vaccine (WCV), strain Tohama-I used for production of ACV components and the type strain of *B. pertussis* (18323^{T}) were also analysed. Several novel alleles were found. The UK isolates were assigned multi-locus sequence types (MLSTs) according to a previously described scheme for *B. pertussis* based on three of these genes (*ptxA*, *ptxC* and *tcfA*). Compared with isolates from other countries, the UK clinical strains showed a distinct distribution of MLSTs. Apart from one strain that was MLST-3, all other recent isolates (2000–2002) were identified as MLST-5. These isolates differed from the three WCV strains, which were MLST-2 or MLST-3, the Tohama-I strain (MLST-2) and the type strain of *B. pertussis* (MLST-9). MLST-3 and MLST-5 differ only by a single synonymous mutation, but this method does indicate that currently circulating strains of *B. pertussis* are not identical to the vaccine types, and they may differ in other important characteristics. Two new MLSTs were identified amongst historical UK isolates. Sequence-based typing offers a convenient method of analysing and comparing populations of *B. pertussis* from different time periods and from different countries. The variation exhibited by *prnA* and *fim2* suggests that they could be useful, additional epidemiological markers in such a typing scheme.

INTRODUCTION

The identification of gene polymorphisms in bacteria by nucleotide sequence analysis can be useful in the epidemiological surveillance of bacterial pathogens (Maslow *et al.*, 1993). The advent of identification libraries derived from sequence-based typing systems that utilize non-selective housekeeping genes, such as the multilocus sequence typing (MLST) schemes (Maiden *et al.*, 1998; Spratt, 1999) (http://www.mlst.net), has facilitated the ability to identify and track particular strains over time. Other markers that may be under selective evolutionary pressure, including those encoding antibiotic resistance (Oliveira *et al.*, 2001) and surface proteins (Maiden *et al.*, 1991; van Loo *et al.*, 2002; Gaia *et al.*, 2003), can also be used, particularly in clonal organisms such as *Bordetella pertussis*, where the selection of useful markers can be problematic.

Nucleotide polymorphisms in genes encoding two virulence factors of *B. pertussis*, pertactin (*prnA*) and pertussis toxin S1 subunit (*ptxA*), were first demonstrated in Dutch isolates by Mooi *et al.* (1998) following a large outbreak of pertussis in The Netherlands in 1996. One possible explanation for this resurgence was that the circulating *B. pertussis* population consisted of strains that were antigenically distinct from the vaccine strains (Mooi *et al.*, 1998). Subsequent studies have reported allelic variation in these two markers in Finland, the UK, Poland, the USA and Canada (Mooi *et al.*, 1999; Cassiday *et al.*, 2000; Fry *et al.*, 2001; Gzyl *et al.*, 2002;

Abbreviations: ACV, acellular vaccine; MLST, multilocus sequence type; SNP, single nucleotide polymorphism; WCV, whole-cell vaccine.

Peppler *et al.*, 2003). Additional targets under presumed selective pressure were examined for nucleotide sequence variation by van Loo *et al.* (2002), including other genes encoding proteins used as acellular vaccine (ACV) components and other surface-associated proteins. Three of these, encoding tracheal colonization factor (*tcfA*) and pertussis toxin subunits S1 and S3 (*ptxA* and *ptxC*), showed sufficient polymorphisms to differentiate *B. pertussis* isolates from The Netherlands, Finland, Italy, the USA and Japan into eight designated MLSTs.

The genes chosen for the present study included eight of the genes investigated by van Loo *et al.* (2002), namely, *prnA*, *ptxA*, *ptxC*, *tcfA*, *fim2* (fimbrial antigen 2), *ompQ* (outer-membrane protein OmpQ), *brkA* (bordetella resistance to killing protein) and *vag8* (virulence-activated gene 8), and two additional genes, *cyaA* (adenylate cyclase toxin) and *bapC* (bordetella autotransporter protein C). The latter gene sequence was originally submitted to GenBank as a putative autotransporter protein gene (accession no. AF081494) and named *bap5* (Blackburn, 2000), but later submitted independently as *bapC* (AJ277634). The protein BapC, like pertactin, Vag8, BrkA and TcfA, is a member of the bordetella autotransporter family (Henderson & Nataro, 2001), and is therefore a potential virulence factor and protective antigen. In addition to its possible use as a component of future ACVs, adenylate cyclase toxin (CyaA), is being investigated for use in multipurpose vaccines, by exploiting its ability to deliver foreign epitopes to antigen-presenting cells (Ladant & Ullmann, 1999). Before using CyaA or BapC as vaccine components, their properties should be fully characterized, including their potential for antigenic variation.

The aims of the present study were (i) to determine the level of sequence variation or conservation in ten genes in historical and recent clinical isolates of *B. pertussis* and to compare these with UK vaccine strains, and (ii) to identify further potential genetic markers for the epidemiological surveillance of *B. pertussis*.

METHODS

Bacterial strains and culture conditions. All isolates were obtained from the Health Protection Agency, Respiratory and Systemic Infection Laboratory, London, and were cultured on charcoal agar with 10 % horse blood (Media Services, CPHL) at 37 °C for up to 5 days with 5 % CO_2. The origin of 285 UK isolates has been described previously (Fry *et al.*, 2001). Numbers of isolates and years of isolation for additional UK isolates included in this study were as follows: 1977, 2; 1983, 1; 2000, 5; 2002, 42. The total number of isolates and years of isolation are shown in Table 1. All isolates in this study were assumed to be epidemiologically unrelated. The three strains (CN2992, CN5476 and CN3099) used in the preparation of the current UK pertussis whole-cell vaccine (Medeva Pharma), the Tohama-I strain (components of which are used in acellular pertussis vaccines in the UK and other countries) and the type strain of *B. pertussis* (18323^T = NCTC 10739^T), were also characterized.

Study design. Sequence polymorphism was investigated in regions of ten genes using various subsets of the 335 *B. pertussis* isolates. Not all isolates were examined for all targets, and data from a previous study (Fry *et al.*, 2001) were used to aid the selection of subsets of isolates containing representatives of all known UK pertactin gene alleles, *prnA*(1–3), and pertussis toxin S1 gene alleles, *ptxA*(1, 2). The number of isolates from each year analysed for each gene is shown in Table 1. Sequences of all ten gene targets were determined for the four vaccine strains and the type strain (18323^T). Pertactin and pertussis toxin S1 gene type and serotype were determined for the 50 isolates from 1997–2002. Only the major polymorphic region designated region 1 of the *prnA* gene was sequenced for these 50 isolates. Serotype and sequence data of the *ptxA* and *prnA* genes from the other UK clinical isolates and the three UK WCV strains were taken from a previous study (Fry *et al.*, 2001).

Extraction of DNA. Genomic DNA was extracted from *B. pertussis* cells grown on charcoal blood agar using the Nucleon BACC2 genomic DNA extraction kit (Amersham Biosciences) including an RNase A step. DNA concentration was determined using a GeneQuant II spectrophotometer (Amersham Biosciences) at A_{260}.

Gene nomenclature and numbering. The description of the pertactin and pertussis toxin S1 gene variants, first described by Mooi *et al.* (1998, 1999, 2000), is as defined by Fry *et al.* (2001). Other gene variants described by van Loo *et al.* (2002) or novel variants found in this study follow the format of Fry *et al.* (2001), i.e. gene designation followed by allele number in parentheses. Throughout the text, the location of numbered positions on the genes is with respect to the GenBank or EMBL reference sequence indicated.

Serotyping. Serotyping was performed with polyclonal antisera to agglutinogens 1, 2 and 3, namely *B. pertussis* anti-agglutinogen 1 (no. 89/596), *B. pertussis* anti-agglutinogen 2 (no. 89/598) and *B. pertussis* anti-agglutinogen 3 (no. 89/600) (National Institute for Biological Standards and Control, UK), in a slide-agglutination assay.

PCR amplification and sequencing. PCR amplification and sequencing of *prnA* and *ptxA* was performed essentially as described by Mooi *et al.* (1998, 2000), with minor modifications (Fry *et al.*, 2001). Amplification and sequencing of *brkA*, *ptxC*, *tcfA* and *vag8* was as described by van Loo *et al.* (2002), with minor modifications using Montage PCR_{96} filter plates (Millipore), the MultiScreen vacuum manifold (Millipore) and the CEQ 8000 genetic analysis system (Beckman Coulter). Nucleotide sequence was determined for the complete open reading frame of *brkA* (3033 bp) and the polymorphic sites of *ptxC*, *vag8*, *tcfA*, *fim2* and *ompQ* reported by van Loo *et al.* (2002). Amplification and sequencing primers designed for this study are described in Table 2. Additional primers used were taken from previous studies (Mooi *et al.*, 1998, 2000; Boursaux-Eude *et al.*, 1999; van Loo *et al.*, 2002). Primer sequences and PCR parameters for amplification and dideoxynucleotide sequencing of *brkA*, *tcfA*, *vag8* and *ptxC* were kindly provided by F. Mooi, National Institute of Public Health and the Environment, Bilthoven, The Netherlands. The *cyaA* and *bapC* genes were also examined for variation by dideoxy sequencing. Oligonucleotide primers were designed from available sequences for *cyaA* (Y00545) (Glaser *et al.*, 1988) and *bapC* (AF081494). For *cyaA*, five primer pairs were used to amplify approx. 330–620 bp products targeting bases 3586–6098 (i.e. 2512 bp in total), with respect to Y00545 (Table 2; Fig. 1). For *bapC*, five primer sets were used to amplify approx. 418–677 bp products, targeting bases 219–2548 (i.e. 2280 bp in total) (AF081494). For three genes, *ptxC*, *fim2* and *ompQ*, the single nucleotide polymorphisms (SNPs) were taken to define these alleles (van Loo *et al.*, 2002), and were determined by a real-time pyrophosphate DNA sequencing method named pyrosequencing (Ronaghi *et al.*, 1998). For five isolates, the *ptxC* sequence type was determined initially by conventional dideoxy sequencing prior to pyrosequencing. The allele type designations by both methods were in complete agreement. Assignment of SNP alleles was made by comparison to those sequences previously described (van Loo *et al.*, 2002).

All PCR mixtures contained 1·5 mM $MgCl_2$ and 200 μM of each

Table 1. UK isolates of *B. pertussis* characterized by gene sequencing

Year of isolation	Isolates per year (*n*)	Isolates analysed for each gene (*n*)									
		prnA	*ptxA*	*ptxC*	*tcfA*	*fim2*	*cyaA*	*ompQ*	*bapC*	*brkA*	*vag8*
1920	1	1	1	1	1	1	1	1	1	1	1
1941	10	10	10	4	4	1	1	1	1	1	1
1942	9	9	9	2	2	1	1	1			
1943	3	3	3	1	1	1	1	1			
1944	7	7	7	2	2	1	1	1			
1946	1	1	1	1	1	1	1	1			
1947	1	1	1	1	1	1	1	1			
1948	6	6	6	2	2	1	1	1			
1949	13	13	13	2	2	1	1	1			
1950	8	8	8	2	2	1	1	1	1	1	1
1954	3	3	3	2	2	1	1	1			
1956	6	6	6	3	3	1	1	1			
1963	8	8	8	2	2	1	1	1			
1964	2	2	2	1	1	1	1	1	1	1	1
1966	2	2	2	1	1	1	1	1			
1967	1	1	1	1	1	1	1	1			
1977	10	10	10	4	4	1	1	1			
1978	3	3	3	2	2	1	1	1	1	1	1
1979	2	2	2	2	2	1	1	1			
1982	32	32	32	6	6	1	1	1			
1983	37	37	37	7	7	2	2	2	1	1	1
1984	5	5	5	2	2	1	1	1			
1985	13	13	13	2	2	1	1	1			
1998	37	37	37	21	21	18	6	6	1	1	1
1999	68	68	68	30	30	26	8	8	2	2	2
2000	5	5	5								
2002	42	37	31	34	34	12	4	4			
Total isolates	335	330	324	138	138	80	42	42	9	9	9

deoxynucleotide in a final volume of 50 µl. In addition, mixtures designed to amplify *cyaA* and *tcfA* contained 10 mM Tris/HCl (pH 8·3), 50 mM KCl, 1·0 µM each primer (except for cyaA-2F and cyaA-2R, 0·1 µM), 5 % (v/v) DMSO (Sigma-Aldrich) and 1 U *Taq* DNA polymerase (Invitrogen). Other target reaction mixtures contained HotStarTaq DNA polymerase with Q-solution (Qiagen) and 1·0 µM each primer (Invitrogen), except for bapC-5F and bapC-5R (0·1 µM). Template DNA (approx. 100 ng) was added and reaction mixtures containing no added DNA served as negative controls. Amplification was performed using a DNA Engine (MJ Research) with the following conditions: initial denaturation for 10 min at 94 °C, then 30 cycles of denaturation for 2 min at 94 °C, annealing for 2 min at 50–60 °C, extension for 1 min at 72 °C and a final extension step of 10 min at 72 °C. The annealing temperatures were as follows: *ptxC*, 50 °C; *ompQ*, 55 °C; *fim2*, 58 °C; and *brkA*, *vag8*, *tcfA*, *cyaA* and *bapC*, 60 °C. Amplified products were purified using Montage PCR_{96} filter plates (Millipore) and sequenced with the primers used for amplification. Nucleotide sequences determined by the dideoxynucleotide method used the dye terminator cycle sequencing quick start kit (Beckman Coulter) and the products were analysed on a CEQ 8000 genetic analysis system (Beckman Coulter).

Pyrosequencing was performed following the manufacturer's instructions (Pyrosequencing). Typically, 20 µl biotinylated PCR product from a 50 µl PCR was immobilized onto streptavidin-coated beads and converted to single-stranded DNA template by denaturation. After washing, removal of the unmodified strand and neutralization, the sequencing primer was annealed to the template. Samples were incubated at 80 °C for 2 min, allowed to cool to room temperature and analysed using the PSQ 96MA system (Pyrosequencing).

Sequence analysis. Sequence analyses were performed using software packages BioNumerics and Kodon (Applied Maths). For dideoxynucleotide analyses, sequence data from forward and reverse primers were combined and aligned manually. New sequence data were aligned with the available sequences for *B. pertussis* from GenBank, *cyaA*, Y00545; *tcfA*(1), U16754; *tcfA*(2), AJ009785; *tcfA*(3), AJ420991; *tcfA*(4), AJ507643; *tcfA*(5), AJ420992; *brkA*, U12776; *vag8*(1), U90124; *bapC*(1), AF081494, and The Sanger Centre, Cambridge, UK (http://www.sanger.ac.uk/Projects/B_pertussis/). Novel alleles found in this study were designated by the authors.

Nucleotide sequence accession numbers. The sequence comprising the complete open reading frame of the tracheal colonization factor gene (*tcfA*) from *B. pertussis* strain DCH154 isolated in 1983 was deposited in GenBank, under the accession number AY375533.

Table 2. Oligonucleotides designed for this study for amplification and sequencing of the target genes of *B. pertussis*

The position of the primer is according to the numbering of the sequences of the following GenBank accession numbers: *cyaA*, Y00545; *tcfA*, U16754; *vag8*, U90124; *ptxC*, M13223; *brkA*, U12276; *bapC*, AF081494; *ompQ*, U16266; and *fim2*, Y00527.

Primer name	Sequence (5′–3′)	Gene	Position
cyaA-1F	TCGTGGGCAAGCAGGACCGC	*cyaA*	3586–3605
cyaA-1R	GCCGCCAACCAGGGTGTCGT	*cyaA*	4136–4117
cyaA-2F	ACGACACCCTGGTTGGCGGC	*cyaA*	4117–4136
cyaA-2R	CCTGGATGGATCATGGCGGA	*cyaA*	4636–4617
cyaA-3F	TCCGCCATGATCCATCCAGG	*cyaA*	4617–4636
cyaA-3R	CACCACGTTCTCGATACCGG	*cyaA*	5186–5167
cyaA-4F	CCGGTATCGAGAACGTGGTG	*cyaA*	5167–5186
cyaA-4R	TGCCCAAGCTCAGGAATACG	*cyaA*	5496–5477
cyaA-5F	CGTATTCCTGAGCTTGGGCA	*cyaA*	5477–5496
cyaA-5R	GCGCCAGTTGACAGCCAGGG	*cyaA*	6098–6079
ptxC-F	CAGCCCGTATGAAGGCAGGT	*ptxC*	3433–3452
ptxC-3270F	TTTATCGCGAAACTTTCT	*ptxC*	3270–3287
ptxC-3658R	biotin-TGGACAGGCGAACAG	*ptxC*	3658–3644
ptxC-3610FS	GGCAGCGTCGATATG	*ptxC*	3610–3624
fim2-632F	biotin-TGGGTGCGAACGAGGCGA	*fim2*	632–649
fim2-900R	CCGGCCGGGCTCCTTGAG	*fim2*	900–885
fim2-720RS	GTAGCGCATCGTGAC	*fim2*	720–706
ompQ-1377F	GCCTATGTCGTGACGCTG	*ompQ*	1378–1394
ompQ-1533R	biotin-AGAAGCGCTGGGTCA	*ompQ*	1533–1519
ompQ-1444FS	GGTATATGAAAGGCTACGA	*ompQ*	1444–1462
tcfA-F1	ACGCCAGCCTGCCAAGACG	*tcfA*	4–22
tcfA-R1	GGCTGCGCTTGAAATCCTCC	*tcfA*	529–510
tcfA-F4	CTCCGGTTGCGAAGCCAGGT	*tcfA*	578–597
tcfA-R5	GATTCAAGCCTCCAGCCGAC	*tcfA*	1032–1014
tcfA-R6	TACCAGGCGTAGCGATAGC	*tcfA*	2345–2327
bapC-1F	GATTCGGTCCTGCGTGGTTC	*bapC*	219–238
bapC-1R	ACGGCCCCATCCTCTACGCT	*bapC*	748–729
bapC-2F	TCAATGGCGAGGCGAACATC	*bapC*	694–713
bapC-2R	TCATCTCGAACAGGCCGCTT	*bapC*	1371–1352
bapC-3F	TGCCGAAAGCGACGGAGAAT	*bapC*	1301–1320
bapC-3R	GTCGAGCTGCTGCTTCTGGG	*bapC*	1790–1771
bapC-4F	TGGTATGCGGAAGGCAATGC	*bapC*	1683–1702
bapC-4R	AGGTGCCGCCTTCGTCCTTG	*bapC*	2244–2225
bapC-5F	ACGTTGCACGACGGCTGGT	*bapC*	2130–2149
bapC-5R	ATGCGCCGCTACCAGGTG	*bapC*	2548–2531
brkA-R5	ATCGGCTCCATCCCTTCCCC	*brkA*	1709–1690
brkA-R4	TCGACACCGTGGTTCCGTCC	*brkA*	2221–2202
brkA-F7	ACAGTCAGCGTGCAGGGCGA	*brkA*	2113–2132
brkA-R3	ATCCTCCGCCAGGCTGTAGC	*brkA*	3291–3272
brkA-R1	GTGTCGAGATAGTAGCCGCCAT	*brkA*	3710–3689
brkA-F6	CCTGCTCGGCTACACCTATGC	*brkA*	3591–3611
brkA-R6	CCTGGCGGGGTTTTTCATTG	*brkA*	4270–4251

RESULTS AND DISCUSSION

Variation in the genes encoding two or more *B. pertussis* components, including the ACV components (*prnA*, *ptxA-E*, *fhaB*, *fim2* and *fim3*), and other surface-associated proteins (*tcfA*, *brkA*, *vag8* and *ompQ*), has been reported in isolates from The Netherlands, Finland, Italy, Japan, Poland and the USA (Mooi *et al*. 1998, 1999; Cassiday *et al.*, 2000; van Loo *et al.*, 2002; Gzyl *et al.*, 2002). Genotypic variation in historical and recent UK isolates in two of these genes, *ptxA* and *prnA*,

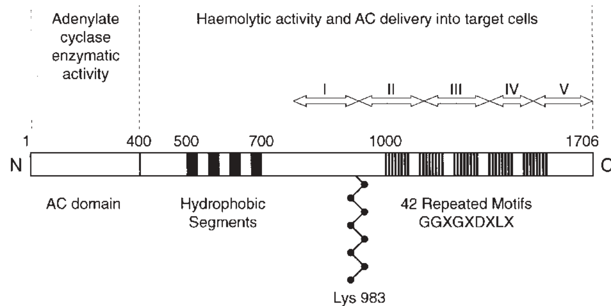

Fig. 1. Schematic of the *B. pertussis* adenylate cyclase toxin (CyaA), showing the enzymic (AC) domain (amino acids 1–400), the hydrophobic membrane-spanning domain, the site of post-translational modification and the 42 calcium-binding nonapeptide repeats. Numbering represents amino acid residues. Arrows indicate the positions on the *cyaA* gene amplified by the five primer pairs (I–V) used to target the immunodominant region. Adapted from Ladant & Ullmann (1999) with permission.

has been reported previously (Fry *et al.*, 2001). In the present study, sequence variation was determined in regions of ten virulence-associated genes (*prnA*, *ptxA*, *ptxC*, *tcfA*, *fim2*, *brkA*, *vag8*, *ompQ*, *bapC* and *cyaA*) in a selection of historical and recent UK isolates of *B. pertussis*, together with the three strains used to produce the UK whole-cell pertussis vaccine, the strain used to produce components for the acellular pertussis vaccine (Tohama-I) and the type strain (18323^T) of *B. pertussis* (Tables 3 and 4). The vaccine and type strains were included as reference strains to help identify vaccine and non-vaccine allele types and to increase the probability of identifying polymorphic sites, as the type strain has been shown to be genotypically distinct from other *B. pertussis* strains (Musser *et al.*, 1986; Gerlach *et al.*, 2001). In *B. pertussis* isolates from the UK for the period 1920–2002, polymorphism was found in five genes, *prnA*, *ptxA*, *ptxC*, *tcfA* and *fim2*. In a selection of these isolates, no polymorphism was found in the *brkA*, *bapC*, *cyaA*, *vag8* and *ompQ* genes, but differences were observed between the type strain and all other strains examined in all genes except *brkA* (Table 3).

Pertussis toxin S1 and S3 genes

Pertussis toxin S1 subunit gene (*ptxA*) types are defined by the number and type (synonymous and non-synonymous) of point mutations in defined regions of the gene. To date, six alleles have been described, *ptxA*(1–4) (Mooi *et al.*, 2000) and *ptxA*(5–6) (AJ506994, AJ506995). Polymorphism within *ptxA* has been described in five major regions within an approximately 600 bp region (position 478–1062, AJ006155). The data for UK isolates for *ptxA* (Table 3) were mainly taken from a previous study (Fry *et al.*, 2001) using primers described by Mooi *et al.* (1998). Fifty additional isolates were included in the present study, but no new alleles were found. Thus, all 324 UK isolates were *ptxA*(1) or *ptxA*(2). Temporal analysis of isolates revealed that *ptxA*(2) variants have not been seen in the UK since 1985 and all subsequent isolates have been *ptxA*(1). Two of the three whole-cell pertussis vaccine strains were *ptxA*(1), the other was *ptxA*(2); the ACV strain (Tohama-I) was *ptxA*(2), and the type strain was *ptxA*(4).

The pertussis toxin S3 subunit gene (*ptxC*) alleles are defined by an SNP at position 3625 (M13223, van Loo *et al.*, 2002): *ptxC*(1) (TG$\underline{C}$, Cys) and *ptxC*(2) (TG$\underline{T}$, Cys). Both allele types were seen in the 138 UK isolates analysed (Table 3). However, there has been an apparent shift in the predominant allele of *ptxC*. From 1920 until 1998, all isolates ($n = 53$) were *ptxC*(1). In 1998, eight of 21 isolates were *ptxC*(2) and, in 1999, 14 of 30 isolates were *ptxC*(2). Of 34 isolates from 2000 to 2002, 33 were *ptxC*(2) and one was *ptxC*(1). The vaccine strains and the type strain were *ptxC*(1).

Tracheal colonization factor gene

Tracheal colonization factor is unique to *B. pertussis*, and has been reported to be important for colonization of the trachea in a mouse model of infection (Finn & Stevens, 1995). The nucleic acid sequence of the *tcfA* gene (between bases 688–1339, with respect to U16754) was determined for 138 UK clinical isolates, the four vaccine strains and the type strain (18323^T). The *tcfA* gene exhibited the greatest allelic variation of the ten genes investigated in this study, with five different alleles, and all of the changes were non-synonymous. Four previously described types, *tcfA*(2), *tcfA*(3), *tcfA*(4) and *tcfA*(5), were found amongst the UK *B. pertussis* population (Table 3; Fig. 2). A novel *tcfA* type, here designated *tcfA*(6), was found in two isolates from 1983, and contained a 15 bp deletion not seen in the other alleles (Fig. 2). To date, *tcfA*(1), which contains a 75 bp segment, has been described only in the type strain (18323^T) (Table 4; Fig. 2). In the alleles *tcfA*(2–4 and 6), this segment is missing and these alleles differ from each other in both nucleotide and amino acid sequence.

Of 138 UK clinical isolates from 1920–2002 investigated for *tcfA* polymorphism, 124 (89·9 %) were *tcfA*(2), two (1·4 %) *tcfA*(3), nine (6·5 %) *tcfA*(4), one (0·7 %) *tcfA*(5) and two (1·4 %) *tcfA*(6). The isolates of type *tcfA*(3) were from a pertussis case in paediatric intensive care isolated in 1999 (Crowcroft *et al.*, 2003) and from an unvaccinated individual in 1999. Allele *tcfA*(4) was found in isolates from 1970–1999 and *tcfA*(5) in an isolate from 1942. The two strains with the novel *tcfA* type, *tcfA*(6), were isolated in 1983. All of the isolates ($n = 34$) from the most recent period (2000–2002) were *tcfA*(2). All four vaccine strains were *tcfA*(2) and the type strain was confirmed as *tcfA*(1).

The predominant *tcfA* allele type in this study, *tcfA*(2), has been reported in isolates from The Netherlands, Finland, Italy, Japan and the USA, and this allele was predominant in all countries except Italy (van Loo *et al.*, 2002). Of ten Italian *B. pertussis* isolates studied, six were *tcfA*(3) and four were *tcfA*(2). Type *tcfA*(3) was not present in the American or Japanese *B. pertussis* isolates. In The Netherlands, Finland and Italy, the allele *tcfA*(3) has been reported in isolates from

Table 3. Frequency of gene polymorphisms in UK isolates of *B. pertussis*

Gene	Allele	Isolates (*n*)	Frequency of gene type (%)	GenBank	
				Accession no.	*B. pertussis* strain
prnA		330			
	prnA(1)	213	64·6	AJ011091	B391
	prnA(2)	111	33·6	AJ011092	B345
	prnA(3)	6	1·8	AJ011093	B343
ptxA		324			
	ptxA(1)	288	88·9	AJ006155	287
	ptxA(2)	36	11·1	AJ006157	Tohama
ptxC		138			
	ptxC(1)	83	60·1	M13223	3779
	ptxC(2)	55	39·9	AJ420987	NK
cyaA		42			
	cyaA(1)	0	0	Y00545	18323^T
	cyaA(2)*	42	100	BX470248	Tohama-I
tcfA		138			
	tcfA(1)	0	0	U16754	18323^T
	tcfA(2)	124	89·8	AJ009785	B596
	tcfA(3)	2	1·4	AJ420991	NK
	tcfA(4)	9	7·1	AJ507643	NK
	tcfA(5)	1	0·7	AJ420592	NK
	tcfA(6)*	2	1·4	AY375533	DCH154
fim2		80			
	fim2(1)	60	75	Y00527	Wellcome 28
	fim2(2)	20	25	AJ420988	NK
ompQ		42			
	ompQ(1)	0	0	U16266	18323^T
	ompQ(2)	42	100	AJ420990	NK
vag8		9			
	vag8(1)	0	0	U90124	18323^T
	vag8(2)	9	100	AJ420993	NK
brkA		9			
	brkA	9	100	U12276	Tohama
bapC		9			
	bapC(1)	9	100	AF081494	Taberman
	bapC(2)*	0	0		18323^T

*Alleles identified in this study.

NK, Not known.

the period 1990–1999 only. This allele is apparently also more dominant in The Netherlands than in the UK, in that 27 of 85 (31·8 %) Dutch *B. pertussis* isolates from 1990–1999 were *tcfA*(3), compared with two of 51 (4 %) UK *B. pertussis* isolates from the same period. Eight of nine UK *tcfA*(4) isolates were from 1970–1989, and the other was from 1999. The allele *tcfA*(4) has not been reported in *B. pertussis* isolates from The Netherlands, Finland, Italy, Japan or the USA, but has been seen in at least one isolate from Australia (AJ507643). The UK isolate that was *tcfA*(5) was unusual as this allele has previously been reported only in isolates from the USA from 1990–1999 (van Loo *et al.*, 2002). It may be significant that the unusual alleles, *tcfA*(4) and *tcfA*(6), except for one isolate, were present in UK isolates from the late 1970s to the mid 1980s, following a drop in vaccine uptake that resulted in two epidemic peaks of pertussis in the UK around 1979 and 1983.

Temporal analysis of MLSTs of *B. pertussis* in the UK

Nine MLSTs (MLST-1 to -9), based on point mutations and the presence of insertions and deletions in the three genes *ptxA*, *ptxC*, and *tcfA* have been described by van Loo *et al.*

Table 4. Genotype and serotype of the *B. pertussis* type strain and strains used in the UK whole-cell and acellular vaccines

Strain	Vaccine	*prnA*	*ptxA*	*ptxC*	*tcfA*	*bapC*	*cyaA*	*fim2*	*brkA**	*ompQ*	*vag8*	Serotype
CN2992	WCV	*prnA*(1)	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	*bap5*(1)	*cyaA*(2)	*fim2*(1)	*brkA*	*ompQ*(2)	*vag8*(2)	1,2,3
CN3099	WCV	*prnA*(1)	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	*bap5*(1)	*cyaA*(2)	*fim2*(1)	*brkA*	*ompQ*(2)	*vag8*(2)	1,2
CN5476	WCV	*prnA*(1)	*ptxA*(1)	*ptxC*(1)	*tcfA*(2)	*bap5*(1)	*cyaA*(2)	*fim2*(1)	*brkA*	*ompQ*(2)	*vag8*(2)	1,3
Tohama-I	ACV	*prnA*(1)	*ptxA*(2)	*ptxC*(1)	*tcfA*(2)	*bap5*(1)	*cyaA*(2)	*fim2*(1)	*brkA*	*ompQ*(2)	*vag8*(2)	1,2
18323^T		*prnA*(6)	*ptxA*(4)	*ptxC*(1)	*tcfA*(1)	*bap5*(2)	*cyaA*(1)	*fim2*(1)	*brkA*	*ompQ*(1)	*vag8*(1)	1

*No variation has been reported in *brkA*.

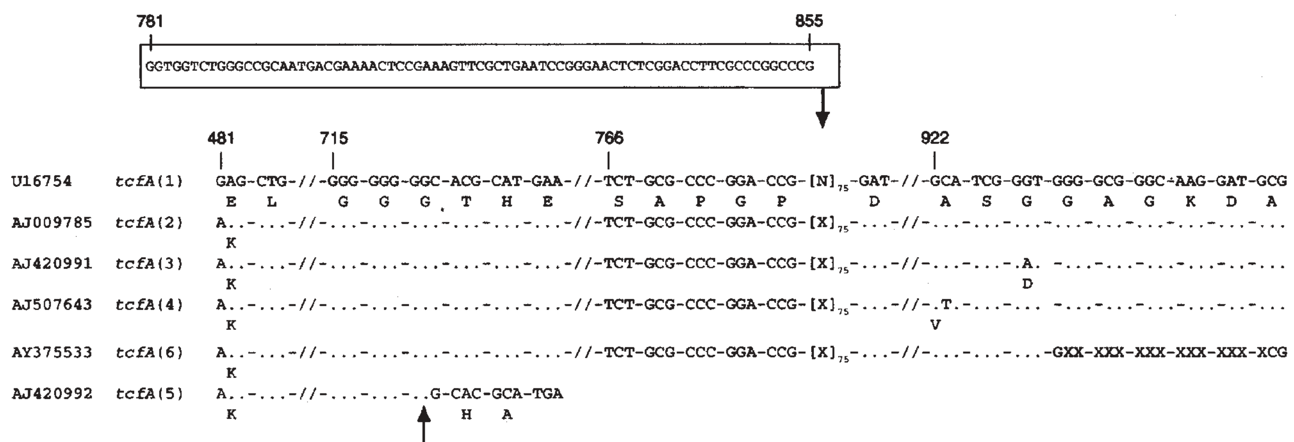

Fig. 2. Primary structure of the *B. pertussis* tracheal colonization factor gene (*tcfA*) showing polymorphism in the six types described to date, designated *tcfA*(1) to *tcfA*(5) (van Loo *et al.*, 2002) and *tcfA*(6) (this study). Numbering is with respect to *tcfA*(1), U16754. Nucleotide polymorphisms are shown and associated amino acid changes are indicated beneath the relevant codon. Dots indicate identity, and codons are separated by dashes. In *tcfA*(1), $[N]_{75}$ indicates a region of 75 bp that is absent from the remaining alleles. Deletions are indicated by X. The arrow below *tcfA*(5) indicates an inserted G, which changes the reading frame and results in premature translational termination two codons downstream. The novel allele *tcfA*(6) reported in this study contains a 15 bp deletion not found in *tcfA*(1–5).

Table 5. MLSTs of *B. pertussis* defined by alleles of genes encoding pertussis toxin subunit 1 (*ptxA*), subunit 3 (*ptxC*) and tracheal colonization factor (*tcfA*)

MLST	Alleles
MLST-1	*ptxA*(3), *ptxC*(1), *tcfA*(2)
MLST-2	*ptxA*(2), *ptxC*(1), *tcfA*(2)
MLST-3	*ptxA*(1), *ptxC*(1), *tcfA*(2)
MLST-4	*ptxA*(1), *ptxC*(1), *tcfA*(3)
MLST-5	*ptxA*(1), *ptxC*(2), *tcfA*(2)
MLST-6	*ptxA*(1), *ptxC*(1), *tcfA*(4)
MLST-7	*ptxA*(1), *ptxC*(1), *tcfA*(5)
MLST-8	*ptxA*(2), *ptxC*(2), *tcfA*(2)
MLST-9	*ptxA*(1), *ptxC*(1), *tcfA*(1)
MLST-10	*ptxA*(1), *ptxC*(1), *tcfA*(6)
MLST-11	*ptxA*(2), *ptxC*(1), *tcfA*(5)

(2002) (Table 5). These combined allelic profiles were used to analyse temporal trends in MLST frequencies in the UK (Fig. 3). As strains were not available from all years, time periods corresponding to six, approximately 10-year periods from 1920–1999, together with recent isolates, from 2000–2002, were used.

Seven distinct MLSTs were identified in 132 isolates selected from each of the different time periods. The earliest available isolate from 1920 was MLST-2, i.e. *ptxA*(2), *ptxC*(1), *tcfA*(2). Two MLSTs, MLST-2 and MLST-3 [*ptxA*(1), *ptxC*(1), *tcfA*(2)], appear to have been predominant prior to the widespread introduction of pertussis vaccination in the UK in the 1960s. Of the 23 isolates from the pre-vaccination era, 10 were MLST-2 and 12 were MLST-3. However, MLST-2 strains were not found after 1989 (Fig. 3). MLST-3 was found in all time periods studied. Nine isolates were MLST-6, found only from 1970–1999. Two isolates with novel MLSTs were

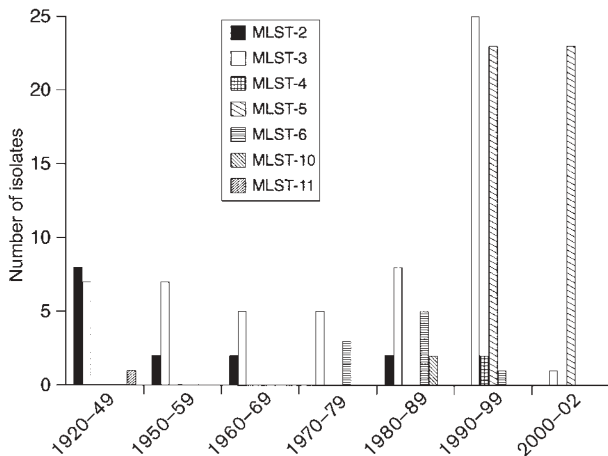

Fig. 3. Temporal trends in the frequency of MLST types in the UK *B. pertussis* population.

identified, MLST-10, defined as *ptxA*(1), *ptxC*(1), *tcfA*(6), from 1983, and MLST-11, defined as *ptxA*(2), *ptxC*(1), *tcfA*(5), from 1942. MLST-4 [*ptxA*(1), *ptxC*(1), *tcfA*(3)], was seen only in two isolates from 1999. In 24 of the most recent isolates, from 2000–2002, only two MLSTs were found, MLST-5 ($n = 23$), detected only from 1990 onwards, and MLST-3 ($n = 1$). It is important to note that two of the three UK WCV strains (CN5476 and CN3099) were MLST-3 and the other (CN2992) was MLST-2. The Tohama-I strain (components of which are used in ACVs) was MLST-2. The type strain of *B. pertussis* was MLST-9.

Using a combination of defined alleles to describe isolates allows the ready comparison of datasets of different geographical origins and from different time periods. Analysis of 132 UK isolates revealed that there has been an apparent shift of sequence types from the pre-vaccination era to recent isolates (Fig. 3). For example, MLST-2 was prevalent in the earliest time period but has not been detected since 1989. MLST-3 was the predominant sequence type from 1920 until 1999, but this appears to have been replaced by MLST-5, although the difference between these MLSTs is conferred by a single silent nucleotide difference in the *ptxC* gene (van Loo *et al.*, 2002). In the American and Dutch *B. pertussis* populations, the trends appear to have been different (van Loo *et al.*, 2002). MLST-1 and MLST-2 isolates were present in the pre-vaccination era, with MLST-2 predominating. In addition, in the USA, MLST-9 was present in the pre-vaccination era, but neither MLST-9 nor MLST-1 has been identified amongst recent (1990–1999) isolates, although MLST-2 was present (10 % of isolates). In recent (1990–1999) Dutch isolates, MLST-1 and MLST-2 were present at low frequencies (1 and 4 %, respectively). In 1990–1999, MLST-3 was present in all three countries, and comprised 52 % of UK isolates, 10 % of American isolates and 25 % of Dutch isolates. MLST-4 was present in the UK and Dutch *B. pertussis* populations from 1990 until 1999 but was not found amongst American isolates from the same time period. The frequency of MLST-4 differed between UK and Dutch populations, being 4 and 32 %, respectively. MLST-5 was the predominant type in recent (1990–1999) *B. pertussis* populations from the USA, The Netherlands and the UK. MLST-7 and MLST-8 were found only in recent isolates from the USA, and at very low frequencies. Van Loo *et al.* (2002) noted that the resurgence of pertussis in The Netherlands since 1996 coincided with the appearance of the new MLSTs, MLST-4 and MLST-5. In the UK, these two types were detected from 1999 onwards, although MLST-5 was the predominant type in the most recent time period. MLST-5 was also the predominant type in The Netherlands in the most recent time period studied, 1996–1999, comprising 40 % of the isolates. In contrast to The Netherlands, however, there has been no resurgence of pertussis in the UK and therefore no evidence that vaccine efficacy has been compromised by change in the antigenic make-up of circulating strains.

MLST and pertactin gene types

A number of *prnA* alleles have been described, *prnA*(1–8) (Mooi *et al.*, 2000), *prnA*(9) (AF218785) and *prnA*(11) (AJ507642). They differ by the number ($n = 4$ to 7) and the composition of the repeat units (GGxxP) found in the major polymorphic region (823–897, AJ011091), designated region 1, in another repeat region $(PQP)_{4–5}$ (1762–1827, AJ011091), designated region 2, or, in one type, *prnA*(7), a point mutation approx. 150 bases upstream of region 2. The *prnA* data presented in Table 3 were mainly taken from a previous study (Fry *et al.*, 2001). Fifty additional isolates were included in the present study, but no new alleles were found. All 330 UK isolates of *B. pertussis* analysed were either *prnA*(1), *prnA*(2) or *prnA*(3). Of the 35 most recent (2000–2002) isolates, 31 were *prnA*(2), three were *prnA*(1) and one was *prnA*(3). The four pertussis vaccine strains were *prnA*(1) and the type strain was *prnA*(6).

A comparison of the MLSTs of UK isolates and their *prnA* types is shown in Fig. 4. MLST-2, -6, -10 and -11 isolates were all exclusively *prnA*(1). The MLST-3 isolates were *prnA*(1) prior to 1980 but, after 1980, were associated with three *prnA* types, *prnA*(1–3). Of the MLST-4 isolates, one was *prnA*(2) and the other was *prnA*(3). MLST-5 isolates, which predominate in recent years, were mainly *prnA*(2) (22 of 23 isolates), with one *prnA*(3). Two of the three UK pertussis WCV strains were MLST-3, *prnA*(1), and the other was MLST-2, *prnA*(1). The ACV strain was MLST-2, *prnA*(1), and the type strain was MLST-9, *prnA*(6).

Polymorphism in the *fim2* gene and relationship to serotype

Eighty UK clinical isolates were investigated for the SNP defining the *fim2* alleles. Sixty (75 %) were *fim2*(1) and 20 (25 %) were *fim2*(2) (Table 3). All four vaccine strains and 18323^T were *fim2*(1) (Table 4). The allele *fim2*(2) was not found in isolates prior to 1998. This allele showed association with serotype 1,2 such that all *fim2*(2) isolates identified

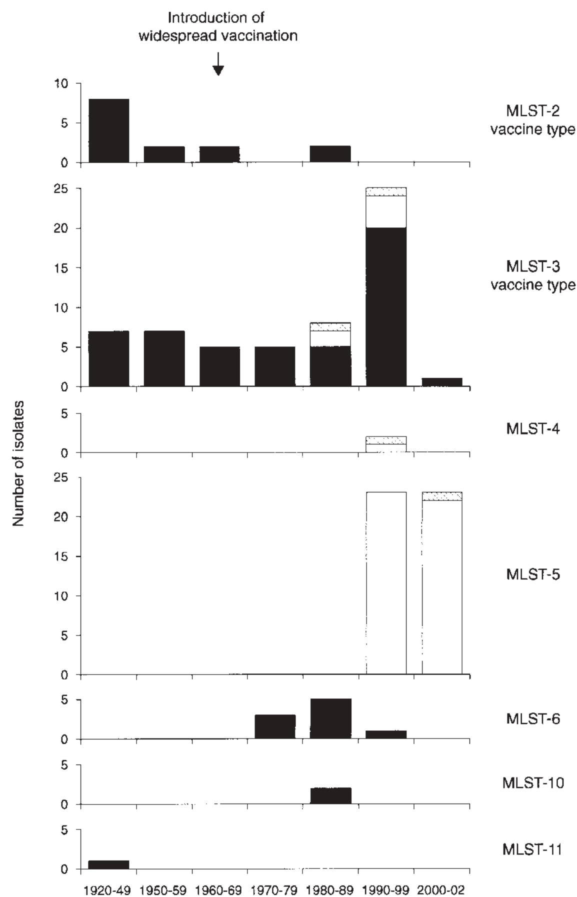

Fig. 4. *B. pertussis* MLST frequencies in the UK from 1920 to 2002 showing distribution of pertactin gene type; *prnA*(1) (filled bars), *prnA*(2) (open bars), *prnA*(3) (hatched bars). The number of isolates of each MLST is indicated.

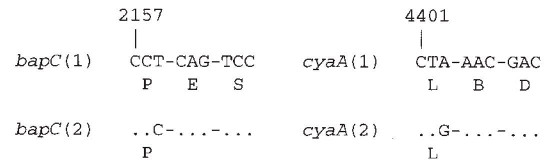

Fig. 5. Alleles of genes encoding bordetella autotransporter protein C (*bapC*) and adenylate cyclase toxin gene (*cyaA*). Numbering is relative to the start of the GenBank accession numbers AF081494 (*bapC*) and Y00545 (*cyaA*). Dots indicate identity.

($n = 20$) were serotype 1,2, although not all serotype 1,2 strains were *fim2*(2). Using a larger sample, it would be possible to investigate whether *fim2*(2) is a potential marker for virulence due to its association with serotype 1,2. Isolates of *B. pertussis* of serotype 1,2 have been linked to more severe cases of pertussis (van Buynder *et al.*, 1999).

Adenylate cyclase toxin gene

Forty-two clinical UK isolates of *B. pertussis*, the four vaccine strains and 18323^{T} were investigated for polymorphism in the region of the *cyaA* gene encoding the immunodominant moiety (approx. 2·5 kb). Only two allelic types were detected. The allelic type of *cyaA* in strain 18323^{T} sequenced in this study was designated *cyaA*(1) (Fig. 5) and that of all the other isolates was designated *cyaA*(2). The two allele types differ by a single nucleotide at position 4403 (Y00545) corresponding to a synonymous change. The *cyaA*(2) sequence, which is also present in the genome sequence of Tohama-I strain (Parkhill *et al.*, 2003), should perhaps be regarded as the prototype sequence. Comparison of the *cyaA* sequence from the type strain ($18323^{T} = \text{NCTC } 10739^{T}$) determined in this study with that of the same strain obtained from GenBank (Y00545) (Glaser *et al.*, 1988) showed one nucleotide difference: C (this study) compared with G at position 3981 with respect to Y00545. In our study, sequencing from forward and reverse primers covering this region was performed in triplicate with concordant results and, therefore, the most likely explanation is that there is a sequencing error at position 3981 in the previously deposited sequence, Y00545.

The lack of polymorphism in the region encoding the immunodominant moiety of *B. pertussis* CyaA is in contrast to strains of *Bordetella bronchiseptica*, where variation was reported in this region (Betsou *et al.*, 1995b). There are also nucleotide differences between the *cyaA* sequences of *Bordetella parapertussis* and of *B. pertussis*, but the two available *B. parapertussis cyaA* sequences (strain 63.2, AJ249835; NCTC 13253, BX470249) are identical. The immunodominant region of the *B. pertussis cyaA* gene is extremely stable which suggests that antigenic variation within *cyaA* would be predicted to be extremely rare. This lack of variation in *cyaA* could perhaps be attributed to the toxin not being subjected to the same immune pressure as the other virulence factors, such as pertussis toxin S1 subunit (*ptxA*), pertactin (*prnA*) and tracheal colonization factor (*tcfA*). Adenylate cyclase toxin (CyaA) has been shown to be protective in mouse studies (Betsou *et al.*, 1995a; Hormozi *et al.*, 1999) and, in view of the stability of its immunodominant region, it may be suitable as a component in new generation ACVs.

Outer-membrane protein Q, virulence-activated gene 8, bordetella resistance to killing protein and bordetella autotransporter protein C

Polymorphism in *ompQ* was investigated in 42 UK isolates chosen from different time periods, in the four vaccine strains and in the type strain. The alleles of *ompQ* are defined by an SNP at position 1465 (U16266), *ompQ*(1) (C<u>T</u>G, Leu), found only in the type strain, and *ompQ*(2) (C<u>C</u>G, Pro), found to be present in all 42 UK isolates and the four vaccine strains (Tables 3 and 4). Nine isolates selected from each of the different time periods, the four vaccine strains and the type strain were investigated for variation in all, or part of, *vag8*, *brkA* and *bapC*. There was no sequence variation in

vag8 between the UK isolates and the UK vaccine strains, but there were three single nucleotide differences in this gene between the type strain and the other strains. These differences occurred at positions 510, 782 and 2697 (U90124), *vag8*(1) (CGT, Arg; GTC, Val; CGC, Arg), in the type strain, and *vag8*(2) (CGC, Arg; GCC, Ala; CGT, Arg) in all other strains. In *bapC*, there was a single nucleotide difference at position 2159 (AF081494), *bapC*(1) (CCT, Pro) between all isolates, including the vaccine strains, and *bapC*(2) (CCC, Pro) in the type strain (Tables 3 and 4; Fig. 5). Similarly, no sequence variation was found in *brkA* between the UK isolates, the vaccine strains or the type strain and no variation was found in *brkA* in any of the strains examined by van Loo *et al*. (2002). As strain 18323^{T} is, in many respects, an atypical strain of *B. pertussis*, the *ompQ*(2) and *vag8*(2) sequences, which are also present in the whole-genome sequence of the strain Tohama-I (Parkhill *et al.*, 2003), should perhaps be regarded as prototype sequences. With *bapC*, the sequence found in all isolates except the type strain and the sequence deposited in GenBank has already been designated allele *bapC*(1).

Vaccination and epidemiology

WCVs for pertussis have been in use since widespread vaccination began in the UK in the 1960s (Therre & Baron, 2000). However, the reactogenicity of these vaccines stimulated research into the various virulence factors expressed by *B. pertussis* and the protection that these proteins could provide if included in ACVs. Some countries have replaced WCVs with ACVs or included them as part of their vaccination programme as booster vaccines for adolescents and adults (Campins-Martí *et al.*, 2001). The impact of the use of ACVs with their limited antigenic repertoire on the antigenic make-up of the circulating *B. pertussis* population is unknown at the present time. However, as use of ACVs becomes more widespread, it will be important to monitor the genotypic and phenotypic characteristics of *B. pertussis* isolates for significant changes.

Characterization of strains by nucleotide sequence analysis has allowed epidemiological tracking of shifts in the circulating populations of *B. pertussis* of several countries. The choice of targets for tracking clonal organisms such as *B. pertussis* remains a difficult one, but *prnA*, *ptxC*, *tcfA* and *fim2* appear to offer useful data. In the UK, the absence of historical isolates of *B. pertussis* for which patient information (such as age, vaccination status, etc.) is available has hindered this choice. However, prospective studies to identify useful markers including those described here will focus on a combination of matched isolate and patient data.

Regarding the current *B. pertussis* MLST scheme, there is no guarantee that, for example, an MLST-5 isolate in the UK is genotypically or phenotypically the same as an MLST-5 isolate from another country. There may be differences in other gene sequences and/or gene expression. The MLST scheme used here has thus been limited by the number of genes chosen. Of the allele types described to date, there are a possible 72 MLSTs. To help define the genotype and presumptive phenotype of isolates, it would be more informative to include all other targets that have demonstrated sequence variation, such as *prnA* and *fim2*. The addition of the *prnA* and *fim2* alleles described would increase the number of possible *B. pertussis* sequence types to 1440. The other genes that were investigated for sequence variation revealed no polymorphism in UK isolates, which precludes them from use as epidemiological markers.

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