

Polymorphism in the Pertussis Toxin Promoter Region Affecting the DNA-Based Diagnosis of *Bordetella* Infection

MALIN NYGREN,¹ ELISABET REIZENSTEIN,² MOSTAFA RONAGHI,¹ AND JOAKIM LUNDEBERG^{1*}

Department of Biotechnology, KTH, Royal Institute of Technology,¹ and Department of Bacteriology, Swedish Institute for Infectious Disease Control,² Stockholm, Sweden

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The pertussis toxin (PT) promoter region is a frequently used target for DNA-based diagnosis of pertussis and parapertussis infections. The reported polymorphism in this region has also allowed discrimination of species in mixtures with several *Bordetella* species by their specific PCR amplicon restriction patterns. In the present study, we investigated the degree of polymorphism in order to confirm the reliability of the assay. Five different sequence types of the amplified 239- or 249-bp region were found among the 33 *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* American Type Culture Collection reference strains and patient isolates analyzed. According to the sequences that were obtained and according to the PT promoter sequences already available in the databases, restriction enzyme analysis with *TaqI*, *BglI*, and *HaeII*, which gave four different patterns, can be performed to reliably identify *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*.

In pertussis, an early diagnosis is of vital importance both for the antibiotic treatment to effectively reduce symptoms and to avoid the spread of this highly contagious disease (9, 40). Both isolation of *Bordetella pertussis* from nasopharyngeal secretions by culture and serological diagnosis are highly specific methods, but the sensitivity of culture is low (32). Since culture takes 3 to 12 days to complete (24) and paired serum specimens collected 1 month apart are preferred for definitive diagnosis (21), more rapid methods are needed. In several studies, detection of *B. pertussis* by PCR has been compared to conventional methods, i.e., culture, serology, and clinical diagnosis (7, 10, 19, 27, 35, 37, 44). Different regions in *Bordetella* sp. DNA are used as targets in diagnostic PCR, such as insertion sequences (8, 19, 22, 26, 33, 45), the adenylate cyclase gene (6), the porin gene (25), and the pertussis toxin (PT) promoter region (22, 36, 41, 42). PT is an important virulence factor for *B. pertussis*. Both the PT promoter and the structural genes are also present in *B. parapertussis* and *B. bronchiseptica* (3). Due to a number of mutations in the PT promoter regions of these two species, PT is not expressed, resulting in milder symptoms with a lower frequency of spasmodic cough compared to the frequency in patients with classical pertussis (15, 30). An advantage to using the PT promoter in pertussis diagnostics is the possibility of amplifying *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* DNAs in a single PCR with generic primers, with subsequent differentiation of the three species by restriction analysis of the PCR amplicons (36, 37, 41). This enables both a rapid diagnosis and epidemiological studies of the occurrence of the three species when they coexist in the same sample (37). A drawback to using the PT promoter region for diagnosis could be the high degree of polymorphism in this region. In recent years, studies involving genetic and functional analyses of the PT promoter and its regulation (2, 4, 12, 18, 29, 43, 46) have also resulted in publication of several different PT promoter sequences that differ from the *B. pertussis* sequence first described by Loch and Keith (28) and the *B. parapertussis*

and *B. bronchiseptica* PT promoter sequences reported by Aricò and Rappuoli (3).

The aim of this study was to analyze in detail the PT promoter regions from a number of strains to investigate if this region can be used for reliable discrimination of the three major *Bordetella* species causing human infection. Swedish clinical isolates retrieved from 1975 to 1994 as well as American Type Culture Collection (ATCC) type strains of the three species were used for this purpose. In addition, clinical isolates from samples originally negative by a PT promoter PCR directly with nasopharyngeal aspirates but positive by culture (37) were analyzed. Another objective was to investigate if DNA sequence polymorphism could explain the lower diagnostic sensitivity obtained for patients with *B. parapertussis* infection than for patients with *B. pertussis* infection in a validation of a nested PT promoter PCR in a pertussis vaccine trial (37).

MATERIALS AND METHODS

Bacterial strains. The clinical isolates and ATCC strains used in the study are listed in Table 1. All strains were obtained from the Swedish Institute for Infectious Disease Control, where they were cultured on charcoal medium with 40 mg of cephalixin per liter as described by Regan and Lowe (34). ATCC strains were originally cultured separately, aliquoted after harvest, and stored lyophilized, after which they were recultured for quality control. Patient strains were identified at the time of isolation by slide agglutination, biochemical verification, and serotyping as described elsewhere (14, 16, 17) and were stored at -70°C in a sucrose-phosphate-glutamate medium containing 2.5% bovine serum albumin. For bacterial lysates, approximately 10 μl of colony material was suspended in 0.5 ml of distilled water, and the mixture was incubated at 100°C for 10 min and frozen until amplified.

In vitro amplification. Bacterial lysates were diluted 1:1,000, and 10 μl was used as template in a nested PCR with outer primers STMA10 (5'-CTG CTC AAC CGC CAC ATC AAC GA-3') and STMA11 (5'-CCA GCC ACG TCA GCC AGC CTG TT-3') and inner primers STMA17 (5'-biotin-CAG CCC TCC AAC GCG CCA TCC C-3') and STMA18 (5'-AAT TGT TAT CCG CTC ACA ATT GCC CGA GTG CAA CGC AT-3') as described previously (41). Negative controls were processed in parallel between every fifth sample (37). The risk for cross contamination was minimized by frequent changing of gloves and by using three separate rooms for mixing of reagents, addition of template, and post-PCR work. Alternatively, 10 μl of bacterial lysate (not diluted) was amplified in a single PCR with primers STMA17 and STMA18. These inner primers encompass a 239-bp region of the PT promoter region (nucleotides 268 to 506 according to Loch and Keith [28]), enabling amplification of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*. After amplification, species can be identified by DNA sequencing or restriction enzyme analysis.

* Corresponding author. Mailing address: Department of Biotechnology, KTH, Royal Institute of Technology, Teknikringen 30, 100 44 Stockholm, Sweden. Phone: 46-8-790 87 58. Fax: 46-8-24 54 52. E-mail: joakim.lundeberg@biochem.kth.se.

TABLE 1. Strains used in the study

Strain or patient isolate	Sequence type	Restriction pattern	Source	Year of isolation
<i>B. pertussis</i>				
KP92\2\92	a	I	PVT ^a 92-95	1992
KP92\9\92	a	I	PVT 92-95	1992
KP92\12\92	a	I	PVT 92-95	1992
KP92\42\92	a	I	PVT 92-95	1992
KP92\47\92	a	I	PVT 92-95	1992
KP92\357\93	a	I	PVT 92-95	1993
KP92\757\94	a	I	PVT 92-95	1994
KP92\1145\94	a	I	PVT 92-95	1994
KP92\1162\94	a	I	PVT 92-95	1994
R613257 SBL 77-09-29	a	I	Routine clinical sample	1977
RA776 SBL 75-10-02	a	I	Routine clinical sample	1975
ATCC 9340	b	I	ATCC	
ATCC 9797	b	I	ATCC	
<i>B. paraptussis</i>				
KP92\11\92	c	II	PVT 92-95	1992
KP92\41\92	c	II	PVT 92-95	1992
KP92\83\93	c	II	PVT 92-95	1993
KP92\106\93	c	II	PVT 92-95	1993
KP86\148\86	c	II	PVT 86-87	1986
KP92\214\93	c	II	PVT 92-95	1993
KP92\218\93	c	II	PVT 92-95	1993
KP86\250\86	c	II	PVT 86-87	1986
KP92\283\93	c	II	PVT 92-95	1993
KP92\308\93	c	II	PVT 92-95	1993
KP92\373\93	c	II	PVT 92-95	1993
KP92\377\93	c	II	PVT 92-95	1993
KP92\437\93	c	II	PVT 92-95	1993
KP92\756\94	c	II	PVT 92-95	1994
KP92\1180\94	c	II	PVT 92-95	1994
ATCC 15237	c	II	ATCC	
ATCC 15311	c	II	ATCC	
<i>B. bronchiseptica</i>				
ATCC 786	d	III	ATCC	
AB 1254	d	III	SS ^b	
ATCC 19395	e	IV	ATCC	

^a PVT, pertussis vaccine trials, 1986 to 1987 (PVT 86-87) (1) and 1992 to 1995 (PVT 92-95) (13).

^b SS, Statens Seruminstitut, Copenhagen, Denmark.

Agarose gel electrophoresis. After amplification, 5 μ l of the product obtained by PCR (with primers STMA17 and STMA18) was analyzed on a 3.5% NuSieve agarose gel at 100 V for 5 h in TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA). The gel was stained with ethidium bromide (1 μ g/ml) for 30 min, destained in water for 10 min, and analyzed under UV light. A 50-bp ladder (Amersham Pharmacia Biotech, Uppsala, Sweden) was used as a marker.

DNA sequence analysis. Several strategies were applied to determine the sequence of the PCR-amplified PT promoter region as described by Ronaghi et al. (38): briefly, conventional bidirectional Sanger DNA sequencing by either (i) a solid-phase method (23) with fluorescein isothiocyanate-labeled terminators, primers STMA17 and STMA18, and T7 DNA polymerase, (ii) a cycle sequencing protocol with Cy5-labeled terminators, primers STMA17 and STMA18, and ThermoSequenase (Amersham Pharmacia Biotech), or (iii) a cycle sequencing protocol with Cy5-labeled primers STMA31 (5'-Cy5-CCC-TCC AAC GCG CCA TCC C-3') and STMA32 (5'-Cy5-ATT GCC CGA GTG CAA CGC AT-3') and ThermoSequenase. The generated Sanger fragments were loaded onto an ALF or ALF Express electrophoretic instrument (Amersham Pharmacia Biotech). To resolve a region containing sequence compressions, pyrosequencing (39) was performed. Two internal primers (primers STMA33 [5'-CGT GTT GGC AAC CGC CAA CGC G-3'] and STMA34 [5'-GGA AGG ATT GAG GGC TTT GTG CGA CG-3']) were synthesized and used in bidirectional pyrosequencing as described by Ronaghi et al. (39). Briefly, the template (sequencing primer hybridized to the single-stranded DNA [23]) was added to a four-enzyme mixture containing Klenow DNA polymerase, ATP sulfurylase, luciferase, and apyrase. The pyrophosphate released during the nucleotide incorporation is coupled to

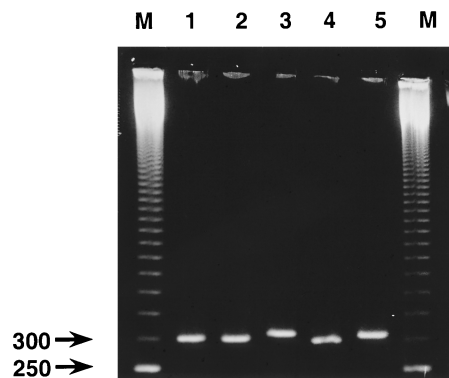


FIG. 1. Results of agarose gel electrophoresis demonstrating the two different fragment sizes obtained with PCR primers STMA17 and STMA18. Lane 1, amplified product of *B. pertussis* patient isolate KP92\2\92; lane 2, *B. pertussis* ATCC 9797; lane 3, *B. paraptussis* ATCC 15311; lane 4, *B. bronchiseptica* ATCC 786; lane 5, *B. bronchiseptica* 19395; lane M, a 50-bp ladder.

the ATP generated by sulfurylase and a proportional amount of light is emitted by luciferase, which is detected with a photon detector device. Unincorporated nucleotides are degraded by apyrase, allowing sequential, iterative addition of the four different nucleotides.

Colorimetric restriction analysis of PCR products. Identification of bacterial species by restriction enzyme analysis was performed as described previously (41). Briefly, biotinylated PCR products containing a *lac* operator sequence, which is introduced as a handle in the nonbiotinylated inner primer (STMA18), was immobilized onto streptavidin-coated super paramagnetic beads (DynaL AS, Oslo, Norway). The magnetic beads with the captured PCR product were divided into three aliquots and were separately treated with *TaqI* (which cleaves only the amplified sequences originating from *B. pertussis* and *B. bronchiseptica*), *BglI* (which cleaves only amplicons originating from *B. paraptussis*), and *HaeII* (which cleaves *B. paraptussis* and *B. bronchiseptica* amplicons). The beads were then incubated with the DNA-binding LacI- β -galactosidase fusion protein, which specifically recognizes the *lac* operator sequence. After washing, substrate was added, the change in absorbance was measured in an enzyme-linked immunosorbent assay plate reader, and species were identified on the basis of the colorimetric restriction pattern; i.e., only noncleaved amplicons yielded a colorimetric response.

Nucleotide sequence accession numbers. The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF157332 to AF157364).

RESULTS

PCR amplification and agarose gel analysis. To evaluate the degree of polymorphism in the PT promoter region, a part of this region encompassing nucleotides 268 to 506 was amplified with primers STMA17 and STMA18 (28). These generic primers amplify the promoter regions from *B. pertussis*, *B. paraptussis*, and *B. bronchiseptica*, which can subsequently be identified by restriction enzyme analyses due to sequence variations in the three species (3, 36, 41).

All strains in the study (Table 1) were amplified by the PCR, including those in the culture-positive nasopharyngeal samples which were originally negative by PCR when amplification directly with clinical nasopharyngeal aspirate samples was used (37). When the PCR products were analyzed by the highly resolving agarose gel electrophoresis, two different fragment lengths of the PCR products were observed; these differed by approximately 10 bp in length (Fig. 1). Amplification of all *B. pertussis* strains resulted in a shorter fragment (approximately 300 bp), which is in accordance with the length of the fragment expected with primers STMA17 and STMA18 by using the sequence published by Loch and Keith (28). Amplification of all *B. paraptussis* strains resulted in slightly longer PCR amplicons. The majority of the published *B. paraptussis* PT promoter sequences are 5 to 10 bp longer than the corresponding *B. pertussis* sequence (3, 18). Surprisingly, fragments of both

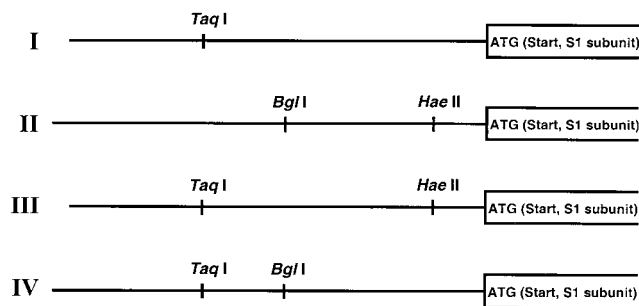


FIG. 3. The four different restriction patterns resulting from the five different sequence types in Fig. 2 obtained with restriction enzymes *TaqI*, *BglI*, and *HaeII*.

Gothenburg, Gothenburg, Sweden, as *B. bronchiseptica* by use of 103 classical biochemical features and numerical analysis (data not shown; E. Falsen, personal communication).

Colorimetric restriction analysis. The polymorphic positions in the PT promoter region of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* has previously been shown to be amenable for species identification on the basis of restriction analysis (36, 41). After alignment of the observed sequences in this study (Fig. 2), we focused on the restriction enzyme recognition sequences used for species differentiation. In both *B. pertussis* sequences, a recognition sequence for *TaqI* is present and there are no *BglI* or *HaeII* restriction sites. The *B. parapertussis* sequence (identical for all strains in the study) contains, as expected, a *BglI* and an *HaeII* recognition sequence but no *TaqI* site. In both *B. bronchiseptica* sequence types, the *TaqI* site is intact. *B. bronchiseptica* ATCC 786 and AB 1254 (sequence d in Fig. 2) also contain the *HaeII* site and lack the *BglI* site, as expected. However, *B. bronchiseptica* ATCC 19395 contains the *BglI* recognition sequence, in addition to the *TaqI* site. The *HaeII* site, present in the other *B. bronchiseptica*

strains, is disturbed by a G-to-A substitution (sequence e in Fig. 2).

Altogether, five different sequence types for the PT promoter region and four different restriction patterns (Fig. 3 and Table 1) were found among the 33 strains analyzed. To confirm the restriction sites found by sequencing, we performed the previously described (41) colorimetric restriction analysis with the PCR products. Briefly, biotinylated PCR products containing a *lac* operator sequence were immobilized onto streptavidin-coated super paramagnetic beads and were separately treated with *TaqI*, *BglI*, and *HaeII*. After washing and binding of a DNA binding fusion protein (*LacI*- β -galactosidase) that specifically recognizes the *lac* operator sequence, substrate was added and the change in absorbance resulting from successful cleavage of the PCR amplicon was measured. Figure 4 shows the results for the five sequence types (sequences a to e in Fig. 2) represented by *B. pertussis* KP92/2/92, a patient isolate, and ATCC 9797, *B. parapertussis* ATCC 15311, and *B. bronchiseptica* ATCC 786 and ATCC 19395. The results demonstrate that for the strains sequenced in this study, discrimination between the three different *Bordetella* spp. is possible by use of *TaqI*, *BglI*, and *HaeII*. In addition, the two different *B. bronchiseptica* sequence types can be separated.

DISCUSSION

Several PT promoter sequences that differ from the sequences originally published (3, 28) have been reported since Aricò et al. (2) demonstrated sequence heterogeneity within the same species. The PT promoter and its genes have been used both to try to solve the classification problems of the *Bordetella* species (11) and to construct phylogenetic trees to elucidate the evolutionary relationships in the genus *Bordetella* (2, 11). Since the sequence encompassing the PT promoter region is also widely used for pertussis diagnosis by performing PCR (22, 36, 41, 42), subsequent detection by hybridization (5,

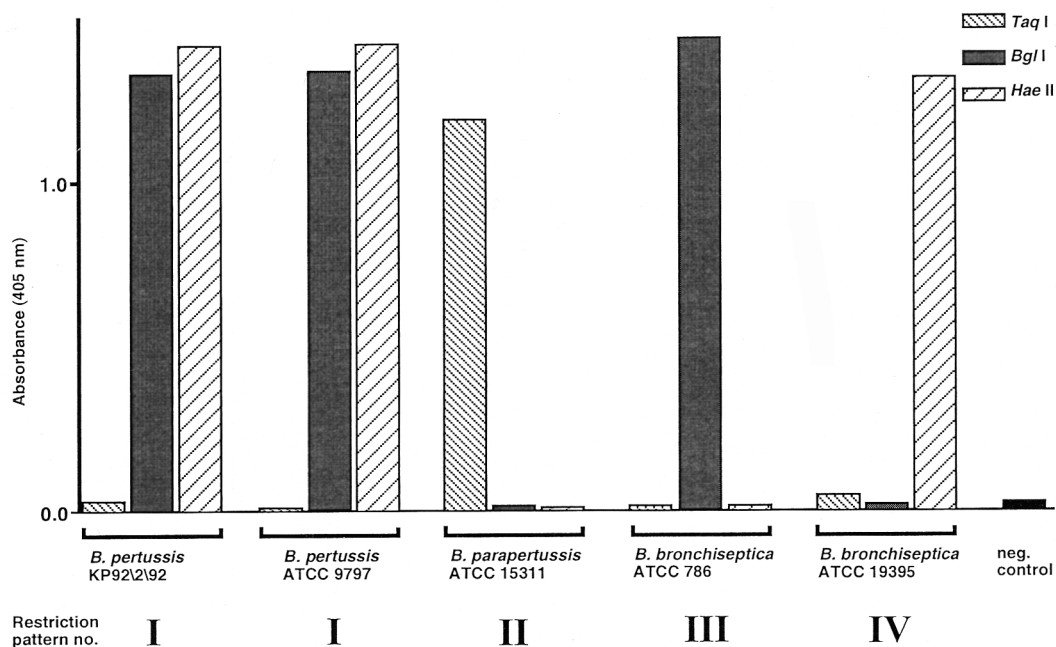


FIG. 4. Results from colorimetric restriction enzyme analysis of *Bordetella* strains representing the five different PT promoter sequence types in Fig. 2 to confirm the four restriction patterns in Fig. 3. The negative control corresponds to a PCR-negative sample that was immobilized onto magnetic beads and mixed with fusion protein and substrate (see text for details).

22), and restriction enzyme analysis (36, 41) of this region, detailed knowledge about sequence polymorphism is of great importance. For example, nucleotide variations in the primer annealing region have been suggested as a source of false-negative PCR results (31).

We decided to undertake sequence analysis of the promoter region used by us to detect and distinguish *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* strains (41) with PCR primers encompassing nucleotides 268 to 506, as described by Locht and Keith (28), to further investigate whether restriction analysis can be used to reliably identify the three species. From the results that we obtained we found that the two different *B. pertussis* sequences generated the same restriction pattern (restriction pattern I in Fig. 3) and that all *B. parapertussis* strains had the same sequence in this region (restriction pattern II). Two different sequences were found among the *B. bronchiseptica* strains analyzed, resulting in restriction patterns III and IV. When the sequence of *B. bronchiseptica* ATCC 19395 (sequence e in Fig. 2 and restriction pattern IV) was compared to the *Bordetella* sequences deposited in public databases, the most similar sequence was, surprisingly, a *B. parapertussis* sequence. However, *B. bronchiseptica* ATCC 19395 was analyzed further by use of 103 classical biochemical features and numerical analysis and was classified as a *B. bronchiseptica* strain.

When analyzing the 33 ATCC type strains and clinical isolates, we experienced technical difficulties in determining the nucleotide sequence in the promoter region (nucleotides 48 to 71) of all 17 *B. parapertussis* strains and of *B. bronchiseptica* ATCC 19395 due to sequence compressions in a palindromic region (38). Consequently, there is uncertainty whether variations in earlier published sequences are due to problems associated with conventional DNA sequencing methodology or reflect a natural nucleotide variation. However, by retrieving the 12 sequences for this region that have been deposited in the databases and that match in whole or in part the region that we sequenced, we found that all sequences contain the expected restriction enzyme recognition sequences. All nine *B. pertussis* sequences in the database contain restriction sites, according to restriction pattern I in Fig. 3. The two retrieved *B. parapertussis* sequences contain restriction sites for *Bgl*I and *Hae*II (restriction pattern II) and the *B. bronchiseptica* sequence contains restriction sites for *Taq*I and *Hae*II (restriction pattern III). Thus, according to the PT promoter sequences available today, restriction analysis of amplicons from the promoter region with *Taq*I, *Bgl*I, and *Hae*II results in identification of the three species. In addition, the results indicate that the two different *B. bronchiseptica* sequence variants can be distinguished. Alternatively, the five different sequence types could be identified by designing a sequencing primer (which hybridizes to nucleotides 113 to 90 in Fig. 2) and performing pyrosequencing to detect at least 32 bases that could distinguish the three species.

In this study, we could not find any sequence differences correlated to year of isolation (1975 to 1994) or to whether the original aspirate sample was positive by culture but negative by PCR. Nor did we find any nucleotide variation in *B. parapertussis* that explains the lower diagnostic sensitivity obtained for *B. parapertussis* in a recent study (37). We suggest that these problems are more associated with processing of the clinical samples prior to PCR than with polymorphisms in the amplified PT region. However, since only the internal regions encompassed by the PCR primers were sequenced, the possibility that nucleotide variations are present in the primer annealing regions cannot be disregarded. Furthermore, this study indicates the internal consensus sequences that can be used to design new generic primers. A high degree of PT promoter

sequence homology among the clinical *B. pertussis* isolates as well as clinical *B. parapertussis* isolates was observed in this study, although the number of sequenced isolates is limited ($n = 11$ and $n = 17$ isolates with identical sequences, respectively). The sequence differences between the two *B. pertussis* ATCC strains and the clinical isolates are in agreement with previous findings which suggested that type strain 18323 (ATCC 9797) might not be a typical *B. pertussis* strain (2).

Recent reports emphasize the need to consider *B. parapertussis* and *B. bronchiseptica* in the diagnosis of *Bordetella* infections. *B. bronchiseptica* might be of increased importance in the diagnosis of disease in humans in the future, since *B. bronchiseptica* was recently identified in two vaccinated children with cough illnesses (43). The sequence of the PT promoter region in these strains is identical to sequence e (Fig. 2) in this study except for the absence of the 10-nucleotide insertion. Several studies demonstrate the importance of *B. parapertussis*, which also causes a whooping cough-like disease in vaccinated populations (15, 30). Hausman et al. (18) have analyzed three *B. parapertussis* strains isolated from unvaccinated children exhibiting antibodies to PT. According to those investigators, the possibility of a mixed infection was investigated, but no such infection was demonstrated. The sequences of those isolates are identical to all ($n = 17$) *B. parapertussis* sequences in the present study (sequence c). In a recent study estimating the prevalence of *B. pertussis* and *B. parapertussis* in a highly immunized population, *B. parapertussis* was found to be the cause of one-third of laboratory-confirmed *Bordetella* infections (20). In pertussis vaccine trials, the use of PCR methods that differentiate pertussis and parapertussis infections is highly recommended (31). Detection and species identification with common PCR primers and subsequent restriction analysis can therefore be used as complements to biochemical and serological methods, which in some cases produce inconclusive results (42). However, further sequence analysis of patient isolates for which biochemical and serological reactions and clinical history contradict is recommended to discover any alternative PT promoter sequences in possible intermediate or variant *Bordetella* strains.

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