The Differentiation of *Bordetella parapertussis* and *Bordetella bronchiseptica* from Humans and Animals as Determined by DNA Polymorphism Mediated by Two Different Insertion Sequence Elements Suggests Their Phylogenetic Relationship

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We describe a novel insertion sequence (IS) element, IS1002, which was found to be closely related to IS481, which is found only in *Bordetella pertussis*; we found that these two IS elements have a level of sequence identity of 61.5% and also have almost identical terminal inverted repeats. IS1002 was present in both *B. pertussis* and *Bordetella parapertussis* strains isolated from humans. In contrast, IS1002 was absent from *B. parapertussis* strains isolated from sheep. A DNA fingerprint analysis performed with another IS element, IS1001, which is present in *B. parapertussis* and *Bordetella bronchiseptica*, revealed that *B. parapertussis* isolates obtained from sheep are distinct from human isolates. Thus, human and ovine *B. parapertussis* strains comprise two distinct populations, indicating that little or no transmission occurs between sheep and humans. An IS-associated restriction fragment length polymorphism analysis revealed that *B. parapertussis* strains isolated from sheep are genetically more polymorphic than the human *B. parapertussis* population, which is genetically very homogeneous. This suggests that human *B. parapertussis* strains diverged from a single clone only recently. IS1001 is present in a subset of *B. bronchiseptica* strains that were derived mainly from pigs and rabbits, suggesting that these strains had a common ancestry. On the basis of the results of a comparison of IS1001 band patterns and IS1001 sequences, ovine and human *B. parapertussis* strains appear to have evolved independently from *B. bronchiseptica* strains and to have adapted to different hosts (sheep and humans). Once in the human host, *B. parapertussis* probably acquired IS1002 from *B. pertussis*. In contrast to human *B. parapertussis* isolates, *B. pertussis* strains produced polymorphic IS1002-related DNA fingerprint patterns.

Bordetella bronchiseptica, *Bordetella parapertussis*, and *Bordetella pertussis* are pathogens of respiratory tracts and have adapted to a variety of mammalian hosts (6, 25). *B. bronchiseptica* is found in a wide range of animals, but only rarely in humans. Conversely, *B. pertussis* is an obligate human pathogen, causing whooping cough or pertussis. Although it has been assumed that *B. parapertussis* is also a strictly human pathogen (12), this organism has recently been isolated from both healthy sheep and sheep affected with chronic nonprogressive pneumonia (5, 8, 24).

It has been estimated that between 5 and 30% of all pertussis cases are caused by *B. parapertussis* (16, 20). However, the incidence of *B. parapertussis* infections may be underestimated because this bacterium causes less severe symptoms than *B. pertussis* and infections may therefore go unnoticed (16). Indeed, serological surveys have shown that between 40 and 90% of adults have antibodies against *B. parapertussis*, indicating that most humans are infected by *B. parapertussis* during their lifetimes (10, 21). Although *B. parapertussis* generally causes a milder disease than *B. pertussis*, there have been numerous examples of severe, life-threatening infections caused by *B. parapertussis* (11, 30).

Multilocus enzyme electrophoresis (MEE) analysis has revealed that the genetic diversity of human *B. parapertussis* and *B. pertussis* strains is very limited, suggesting that these organisms descended from a common ancestor recently (23). *B.*

bronchiseptica strains are more polymorphic than human *B. parapertussis* and *B. pertussis* strains, indicating that *B. bronchiseptica* represents an older lineage. Notwithstanding these differences, *B. bronchiseptica*, *B. parapertussis*, and *B. pertussis* are very closely related and may be considered members of a single species (23). It has been suggested that human *B. parapertussis* and *B. pertussis* are merely clones of *B. bronchiseptica* which have become adapted to human hosts (23).

Given the observation that *B. parapertussis* is found both in sheep and in humans, one may raise the question whether sheep constitute a reservoir from which transmission of *B. parapertussis* to humans (or vice versa) takes place. If no such transmission occurs, one may expect the human and sheep strains to be distinct. Preliminary support for such a distinction was found by Porter et al. (24), who found that *B. parapertussis* strains isolated from sheep and humans can be differentiated by biochemical characteristics and MEE data.

To characterize the putative differences between *B. parapertussis* strains isolated from sheep and humans in more detail and to determine the phylogenetic relationships among the three *Bordetella* species, we studied the DNA polymorphism associated with repetitive DNA sequences present in the *Bordetella* species.

The following three repetitive DNA elements have been found in the genus *Bordetella* previously: two insertion sequence (IS) elements, IS481 (19) and IS1001 (27), and one uncharacterized element (19). IS481 is found exclusively in *B. pertussis*, and *B. pertussis* strains contain approximately 100 copies per genome. van der Zee et al. (27) found IS1001 in *B. parapertussis* and in some, but not all, *B. bronchiseptica* strains and did not find IS1001 in *B. pertussis*. In the study of van der Zee et al. only Dutch *B. parapertussis* strains isolated from

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humans were investigated, and no IS1001-associated restriction fragment length polymorphism (RFLP) was observed. This is in contrast to *B. bronchiseptica*, in which different RFLP patterns were found (27).

The uncharacterized repetitive element was originally identified in *B. pertussis*, and this element was found to be located adjacent to a copy of IS481 (19). It was also found to be present in *B. paraptussis* strains isolated from humans (19). In order to use this repetitive DNA element for strain differentiation and phylogenetic analysis, we characterized it and showed that it is an IS element. Furthermore, in this paper we describe the distribution of and RFLP associated with this IS element and with IS1001 in *B. paraptussis* strains obtained from both humans and sheep and in *B. bronchiseptica* strains isolated from humans and a variety of different animals. The phylogenetic relationships among the three *Bordetella* species are discussed below.

MATERIALS AND METHODS

Bacterial strains and media. The origins of the isolates used in this study are shown in Table 1. We used 103 *B. pertussis* strains and 31 *B. paraptussis* strains isolated from humans with clinical symptoms of whooping cough. *B. paraptussis* strains were kindly provided by J. Mertsola (Turku, Finland) (five strains), C. H. Wirsing von König (Krefeld, Germany) (three strains), P. Short (Porirua, New Zealand) (three strains), J. Porter, (Edinburgh, United Kingdom) (two strains), and T. Aoyama (Kawasaki, Japan) (four strains). A total of 17 *B. paraptussis* strains isolated from sheep were provided by J. Porter, and 5 *B. paraptussis* strains isolated from sheep were provided by B. W. Manktelow (Palmerston North, New Zealand). The 41 *B. bronchiseptica* strains which we studied were isolated from various animals having different geographic origins, and 30 of these strains were provided by W. Gastra, (Utrecht, The Netherlands). All of the *Bordetella* strains were grown on Bordet-Gengou agar plates.

DNA techniques. Preparation of chromosomal DNA and isolation of plasmid DNA were carried out as described previously (3). Restriction endonucleases and T4 ligase were used as recommended by the manufacturer. Cloning procedures were performed by using pEMBL19 and *Escherichia coli* DH5 α (9).

Southern blotting. For the IS1001- and IS1002-based fingerprint analyses, chromosomal DNAs were digested with *Sph*I and *Sma*I, respectively. DNA fragments were separated by agarose gel electrophoresis on 0.8% agarose gels in TAE buffer at 4 V/cm for 5 h and blotted onto Hybond N+ membranes (Amersham, Buckinghamshire, United Kingdom). Southern blotting was performed as described by Maniatis et al. (18). The IS1001 probe that was used in this study was the 498-bp PCR fragment generated by primer A (5'-CGCGCTTGATGACCTTGATA-3') and primer Z (5'-CACCGCCTACGAGTTGGAGAT-3'), as described previously (27). This internal IS1001 fragment does not contain restriction recognition sites for *Sph*I or *Sma*I. The IS1002 probe (Fig. 1, probe 2) was 293 bp long and was generated by PCR by using primers HG1 (5'-GCCGATGCGTTCCATATA-3') and HG2 (5'-AGCCCTTCTGTGAACAGGG-3'), corresponding to positions 424 to 406 and 131 to 151 on the sequence of IS1002a, respectively (Fig. 2). PCR fragments were isolated from gels by using GeneClean (Bio 101, La Jolla, Calif.) and were labeled with peroxidase by using the method recommended by the manufacturer (ECL, Amersham, United Kingdom). The hybridization conditions used when IS1001 was the probe were the conditions recommended in the ECL kit. Hybridization of *B. pertussis* strains with IS1002 was performed under higher-stringency conditions by lowering the NaCl concentration in the hybridization buffer from 0.5 to 0.1 M. This was done to minimize hybridization to IS481, whose sequence is similar to the IS1002 sequence.

Cloning of IS1002 from *B. paraptussis* and *B. pertussis*. IS1002 was isolated from *B. paraptussis* B24 (a human strain isolated in The Netherlands) by randomly cloning *Pst*I fragments. Recombinant clones were screened with the *Hpa*I-*Cl*aI fragment on cosmid p11-11 (Fig. 1, probe 1), which contained repetitive DNA derived from *B. pertussis*. One hybridizing plasmid, designated pRPP30, was selected, and this plasmid contained a 1.8-kb *Pst*I fragment. Inverse PCR with primers H (5'-GGCCTGCCTCATCGTCCAGTT-3') and C (5'-ATCGGCCCCAGAACCGCAA-3'), corresponding to positions 187 to 166 and 556 to 575 on the IS1002 sequence, respectively, was performed with *Bam*HI- and *Sph*I-digested and religated *B. paraptussis* chromosomal DNA to determine the termini of the element. Inverse PCR was performed as described previously (27). To sequence the IS1002 homolog of *B. pertussis*, which was harbored on cosmid p11-11 (19), the appropriate *Sal*I fragments and the *Hpa*I-*Cl*aI fragment were subcloned.

PCR detection of IS elements. The *Bordetella* strains were screened for the presence of IS elements by PCR, using primers based on the DNA sequences of IS1001 and IS1002a. The following primers were used: for IS1001, primers A and Z, which are described above; and for IS1002, primers P3 (5'-TCCCAACTC CACGCACACCG-3') and H2 (5'-AACAACCATAAGCATGCGCG-3'), cor-

responding to positions 797 to 777 and 93 to 113 on the sequence of IS1002a, respectively (Fig. 2). To isolate DNA, bacterial cells were scraped from agar plates and suspended in a physiological salt solution supplemented with 1 mM EDTA. The preparations were diluted to an optical density at 600 nm of 2×10^{-5} and boiled for 10 min to release the DNA, and 5 μ l of each suspension was used in a PCR. The PCR conditions used have been described previously (26). The reaction mixtures were preheated at 95°C for 3 min. A total of 33 amplification cycles consisting of 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C were performed with a DNA thermocycler (Perkin-Elmer Cetus); this was followed by incubation at 5 min at 72°C to complete the final polymerase reaction.

DNA sequence analysis. The sequences of 420-bp DNA fragments of IS1001 amplified by primers A and Z were compared by using PCR products derived from the following strains: one Dutch *B. paraptussis* isolate obtained from a human, two *B. paraptussis* ovine isolates (from Scotland and New Zealand), three *B. bronchiseptica* isolates obtained from pigs (from The Netherlands, the United States, and Australia), and two isolates obtained from rabbits (from Denmark and Switzerland). The sequences of the PCR fragments were determined as described by Craxton (7) by using with dye-labeled dideoxynucleotides, primers A and Z, and a model 370 automated sequencer (Applied Biosystems).

The sequences of plasmid clones containing IS1002 sequences were determined in both directions by using *Taq* polymerase (Promega), dye-labeled M13 primers or dye-labeled dideoxynucleotides, and primers P3, HG1, H, HG2, C, and H2 as described above. To investigate whether IS481 had been inserted into IS1002, the sequence of the downstream flanking region of IS481 was determined by using primer S23 (5'-GGCGCGCTGTACCATCTCC-3'), corresponding to positions 989 to 1009 on the IS481 sequence (19). Searches for sequence similarity were carried out with the BLAST algorithm (1).

Nucleotide sequence accession number. The IS1002 sequence has been deposited in the EMBL databank under accession number Z54268.

RESULTS

IS1001-associated RFLP analysis of *B. paraptussis* and *B. bronchiseptica* strains. In a previous study (27) we found that 12 human *B. paraptussis* strains isolated in The Netherlands produced a single IS1001 RFLP pattern, pattern X1 (Fig. 3A, lane 1). In this study we investigated 19 human *B. paraptussis* isolates obtained from various geographic areas (Finland, Germany, New Zealand, United Kingdom, Japan, and the United States). Again, most of the isolates produced the same fingerprint pattern, pattern X1 (Table 1 and Fig. 3A). Two slightly different fingerprint patterns were produced by a Finnish isolate and an American isolate (pattern X2) (Fig. 3A, lane 2) and by a German isolate (pattern X3) (Fig. 3A, lane 3).

To determine the genetic relationship of *B. paraptussis* isolates obtained from sheep with isolates obtained from humans, we subjected 22 *B. paraptussis* strains obtained from sheep to Southern blotting and hybridization with IS1001 as the probe. The IS element was found in all of these strains. Although the number of IS1001 copies found in the sheep strains was similar to the number of copies found in the strains obtained from humans (20 to 23 copies), only a limited number of common hybridizing *Sph*I bands were found in the human and sheep isolates of *B. paraptussis* (approximately 5 of 20 bands). The IS1001 fingerprints of the sheep isolates were clearly distinct from those of the human isolates. Furthermore, the patterns of the sheep strains were more variable than the patterns of the human strains. The *B. paraptussis* strains isolated from sheep in Scotland produced three IS1001 band patterns (patterns Y2 to Y4) (Fig. 3A, lanes 5 to 7), while the strains from New Zealand produced only one pattern (pattern Y1) (Fig. 3A, lane 4). The latter pattern was very similar but not identical to the fingerprint pattern observed in the majority of the sheep strains from Scotland (pattern Y2).

A total of 41 *B. bronchiseptica* strains were analyzed for the presence of IS1001. IS1001 was detected in 26 of these strains. The number of IS copies varied from one to seven, values which were much lower than the values found in *B. paraptussis* strains. Interestingly, there was an association between the presence of IS1001 and the host from which the *B. bronchiseptica* strains originated (Table 1). IS1001 was found in 15 of 16 pig strains and in 6 of 8 rabbit strains. However, IS1001

TABLE 1. *Bordetella* strains used in this study

Species	No. of strains	Host	IS1001 RFLP pattern ^a	IS1002 RFLP pattern(s) ^b	Geographic origin
<i>B. pertussis</i>	100	Human	—	A4-A36	The Netherlands
	1	Human	—	A1	Japan ^c
	1	Human	—	A2	United Kingdom ^d
	1	Human	—	A3	United States ^e
<i>B. parapertussis</i>	12	Human	X1	B1 (1) or ND (11)	The Netherlands
	4	Human	X1	B1 (2) or ND (2)	Finland
	2	Human	X1	B1	Germany
	3	Human	X1	B1 (2) or ND (1)	New Zealand
	2	Human	X1	B1 or ND	United Kingdom
	4	Human	X1	ND	Japan
	1	Human	X1	B1	United States
	1	Human	X2	B1	Finland
	1	Human	X2	B1	United States
	1	Human	X3	B1	Germany
	5	Sheep	Y1	—	New Zealand
	15	Sheep	Y2	—	Scotland
	1	Sheep	Y3	—	Scotland
	1	Sheep	Y4	—	Scotland
<i>B. bronchiseptica</i>	1	Pig	—	—	United Kingdom
	1	Pig	R1	—	The Netherlands
	1	Pig	R3	—	Australia
	1	Pig	R4	—	South Africa
	1	Pig	R5	—	USSR
	1	Pig	R7	—	Australia
	2	Pig	R10	—	The Netherlands
	1	Pig	R12	—	United States
	1	Pig	R13	—	The Netherlands
	1	Pig	R14	—	The Netherlands
	1	Pig	R15	—	The Netherlands
	1	Pig	R16	—	The Netherlands
	2	Pig	R17	—	South Africa
	1	Pig	R18	—	USSR
	4	Dog	—	—	The Netherlands
	1	Dog	—	—	South Africa
	1	Dog	R9	—	The Netherlands
	2	Cat	—	—	The Netherlands
	1	Cat	R2	—	The Netherlands
	1	Rabbit	—	—	The Netherlands
	1	Rabbit	—	—	United States
	1	Rabbit	R11	—	Switzerland
	2	Rabbit	R17	—	South Africa
	1	Rabbit	R19	—	Denmark
	1	Rabbit	R20	—	The Netherlands
	1	Rabbit	R21	—	United States
	1	Horse	—	— ^f	Denmark
	1	Cow	R6	—	Ireland
	1	Mouse	R8	—	United States
	1	Unknown	R17	—	South Africa
	1	Koala	—	+	Australia
	2	Human	—	—	The Netherlands
	1	Human	—	—	Germany

^a —, strain(s) do(es) not contain IS1001.^b —, strain(s) do(es) not contain IS1002; +, strain contains IS1002; ND, not determined. The numbers in parentheses are numbers of strains.^c Data from reference 13.^d Data from reference 17.^e Data from reference 14.^f This strain contains IS481.

was present in only 2 of 9 strains obtained from dogs and cats. The IS1001 fingerprints of *B. bronchiseptica* strains were clearly distinct from the *B. parapertussis* patterns, and more variation was observed among the *B. bronchiseptica* strains than among the *B. parapertussis* strains. A total of 21 different fingerprint patterns were observed among the strains analyzed, a selection of which is shown in Fig. 3A. The human and ovine *B. parapertussis* strains shared a number of hybridizing *Sph*I

bands with a *B. bronchiseptica* strain obtained from a pig (Fig. 3A, lane 8, 13-, 7.4-, 5.2-, 4.3-, and 2.5-kb bands), suggesting that common sites for IS1001 insertion were present. Most of these bands were also observed in other *B. bronchiseptica* strains which we analyzed (Fig. 3A). When chromosomal DNA was digested with *Sma*I to generate IS1001 fingerprints, 4.8-, 3.5-, 3.3-, and 2.6-kb bands were found in both the pig *B. bronchiseptica* strain and *B. parapertussis* strains isolated from

FIG. 1. Genetic and physical maps of the chromosomal regions of *B. pertussis* and *B. parapertussis* harboring IS481 and/or IS1002 and probes and primers used. The locations of the putative transposase genes (*tnpA*) are indicated by large arrows. Small arrows c and h indicate the positions of the primers used to clone the IS1002-flanking regions of *B. parapertussis* by inverse PCR. Probes 1 and 2 were generated by PCR and used to isolate IS1002 from *B. parapertussis* and for DNA fingerprinting, respectively. Abbreviations: C, *Cla*I; S, *Sal*I; H, *Hpa*I; P, *Pst*I.

sheep and humans (Fig. 3B). In addition, a 1.4-kb band was found in only the *B. bronchiseptica* strain and human *B. parapertussis* strains (Fig. 3B). The occurrence of shared bands suggests that the three (sub)species descended from an ancestor which already contained IS1001. The distinct RFLP patterns of the human and ovine *B. parapertussis* strains indicate that these organisms constitute two separate populations. Furthermore, the human *B. parapertussis* population appears to be very homogeneous, whereas the ovine *B. parapertussis* strains are genetically more heterogeneous. The *B. bronchiseptica* strains exhibited the highest degree of IS1001-associated DNA polymorphism.

Comparison of IS1001 sequences of different *B. parapertussis* and *B. bronchiseptica* strains. To investigate the divergence of IS1001 element sequences in different *Bordetella* species, we determined the DNA sequences of internal 420-bp PCR fragments from three *B. parapertussis* strains (one human strain and two ovine strains) and five *B. bronchiseptica* strains (three strains from pigs and two strains from rabbits). The results of this analysis are shown in Figure 4. The maximum level of sequence divergence was 1.5%. The DNA sequence of the 420-bp IS1001 fragment of the human *B. parapertussis* strain was identical to the sequence of one of the *B. bronchiseptica* pig strains and differed from the sequences of the two other pig strains in only one residue. The DNA sequences of the two ovine *B. parapertussis* strains were identical and differed from the sequences of the human strain in four residues. The IS1001 sequence of one of the two rabbit *B. bronchiseptica* strains was the most divergent (Fig. 4).

Characterization of a novel IS element designated IS1002. As mentioned above, human *B. parapertussis* strains harbor a second, uncharacterized repetitive DNA element, which originally was discovered in *B. pertussis* (19). This element was detected previously on a cosmid (p11-11) derived from *B. pertussis*, and it was found to be located adjacent to the IS481 sequence on this cosmid (19). To characterize this repetitive DNA element, we cloned a homologous sequence from *B. parapertussis*. To do this, a probe containing the *B. pertussis* repetitive DNA (Fig. 1, probe 1) was derived from p11-11 and used to select its homologue by hybridization with a pool of cloned *B. parapertussis* *Pst*I fragments. One of the hybridizing plasmids obtained was designated pRPP30, and this plasmid was found to contain a 1.8-kb *Pst*I fragment. This fragment was sequenced. To identify the termini of the repetitive sequence, the flanking DNA of this repeat was cloned by inverse PCR with primers h and c (Fig. 1) on restricted, religated *B. parapertussis* chromosomal DNA. The sequences of the PCR frag-

ments containing different ends of the repeat allowed us to delineate the DNA element.

The repetitive DNA sequence had all of the characteristic features of an IS element (see below). We designated this IS element IS1002. The copies of IS1002 derived from *B. parapertussis* and *B. pertussis* are referred to in this paper as IS1002a and IS1002b, respectively. IS1002a was 1,040 bp long and had a G+C content of 63.9 mol% and 29-bp inverted terminal repeats (Fig. 2). The junctions at the site of insertion showed no target duplication.

We also determined the sequence of IS1002b, which is located on cosmid p11-11. This IS element was truncated compared with IS1002a. The 5' 127 bp were missing in IS1002b, and because IS481 sequences directly bordered the truncated sequence (Fig. 1 and 2), it is conceivable that the truncation was the result of transposition of IS481 into this copy of IS1002. The sequence downstream of IS481, however, exhibited no homology to IS1002 (data not shown).

The *B. pertussis* and *B. parapertussis* copies of IS1002 exhibited a high level of DNA sequence identity (93%). A search of the GenBank and EMBL databases revealed that the most similar sequence was the IS481 sequence (level of sequence identity, 61.5%). The inverted terminal repeats of IS1002 and IS481 were almost identical. The major open reading frame (ORF) of IS1002a, comprising 214 amino acids, exhibited a level of sequence identity of 29.4% with the 129 N-terminal amino acids of the putative transposase encoded by IS481. The results of a comparison of the ORFs and DNA sequences of IS1002a and IS1002b suggested that a deletion of a single base had occurred in IS1002a at position 474 (Fig. 2), resulting in a frameshift. When the frameshift was corrected, an ORF consisting of 316 amino acid residues was obtained in IS1002a, and the level of amino acid sequence identity with the putative transposase encoded by IS481 was 48%. Significant levels of sequence identity were also found with IS elements found in *E. coli*, *Xanthomonas campestris*, and *Mycobacterium intracellulare* (data not shown).

Host range of IS1002 in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* strains. The host range of IS1002 was investigated by PCR by using IS1002-specific primers. IS1002 was detected in all 100 *B. pertussis* strains investigated and all 31 *B. parapertussis* human strains investigated. However, it was absent from all 22 *B. parapertussis* ovine strains tested and was found in only 1 of 100 *B. bronchiseptica* strains tested (Table 1).

IS1002-associated RFLP analysis of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* strains. Southern blot analyses of *Sma*I-restricted chromosomal DNAs of different *Bordetella*

IS1002a	<u>CTAGGTGTGATGTCTCAATAGGTTGTTCA</u> TCGAGGCCGACCTGGGATGC	50
IS1002a	TGTGGTTACCAACGCCATCGCACTCAGGCGAGCAGCTGGATGAACAACC	100
TnpA	M H N	3
IS481/IS1002b	acacacattatgaattcttcacacctag.....	150
IS1002a	ATAAGCATGCGGATGACCTCTCAGGTCGAGCCCTCTCTGTTAACAGG	20
TnpA (Bpp)	H K H A R L T L L G R A L L V N R	20
IS1002b	A..C..A.....A.....	200
IS1002a	GTGATGCAACAGAACTGACGATGAGCGAGGCCAGCGGCGGAGGGT	37
TnpA (Bpp)	V M Q Q N W T H R Q A S Q A G V	37
TnpA (Bp)	P N.....E.....	
IS1002b	C.A.....A.....A.....	250
IS1002a	GAGCCTGCGCAGGTTTACAGTGGCTTGCCTCCGAGCGAAGGTC	53
TnpA (Bpp)	S L R T G Y K W L A R F R S E G	
TnpA (Bp)	Q.....R.....	
IS1002b	A.....A.....A.....A.....	300
IS1002a	LDGACGGCTGCTTGACCGCGCTTCGACCGCGAGCGGCGGAGGCC	70
TnpA (Bpp)	L D G L L D R S S R P H R S P K A	
TnpA (Bp)	E.....	
IS1002b	C.....	350
IS1002a	TGTGCGCGCGGATGACGCTCTCAGGTCGAGCCCTCTCTGTTAACAGG	87
TnpA (Bpp)	C A P E Q V E H F A Q Q R R Q R L	
TnpA (Bp)		
IS1002b		400
IS1002a	GCCGCTGCGCAGGTTTACAGTGGCTTGCCTCCGAGCGAAGGTC	103
TnpA (Bpp)	P L W R I A R E A G R S L A T V	
TnpA (Bp)		
IS1002b	T.....	450
IS1002a	CGCGGTATATGGAACGATCGGCTCTCAGGTCGAGCCCTCTCTGTTAACAGG	120
TnpA (Bpp)	A R Y M E R I G L S R L A S L E P	
TnpA (Bp)		
IS1002b	A.....	499
IS1002a	CGCGCTGCGCAGGTTTACAGTGGCTTGCCTCCGAGCGAAGGTC	137
TnpA (Bpp)	P A P V R R Y X R A S P G E L L H	
TnpA (Bp)	R.....	
IS1002b	C.....	549
IS1002a	CATCGACGAGCGGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	153
TnpA (Bpp)	I D S K R L G R I R G V G H R I	
TnpA (Bp)	T.....	
IS1002b		599
IS1002a	CGCGGTATATGGAACGATCGGCTCTCAGGTCGAGCCCTCTCTGTTAACAGG	170
TnpA (Bpp)	T G D R A Q N R N R G I G W D A V	
TnpA (Bp)		
IS1002b	A.....	649
IS1002a	CACGCTGCGCAGGTTTACAGTGGCTTGCCTCCGAGCGAAGGTC	187
TnpA (Bpp)	H L A I D D P S R V S F A R I L D	
TnpA (Bp)	S.....	
IS1002b	A.....G.....	699
IS1002a	CGATGAGGCGGAGCGGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	203
TnpA (Bpp)	D E G G D Q C A E F L H Q A T A	
TnpA (Bp)	R.....	
IS1002b		749
IS1002a	ACTACGCGCTGCTGAGGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	220
TnpA (Bpp)	Y Y A S L G V R I D R V M T D N G	
TnpA (Bp)		
IS1002b	G.....	799
IS1002a	AGGGGTATACGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	237
TnpA (Bpp)	R G Y V S R T F R A V C V E L G I	
TnpA (Bp)		
IS1002b	T.....C.....G.....G.....	849
IS1002a	CGGACGATCGGAGCGGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	253
TnpA (Bpp)	R H I R T R P Y T S K T N V K A	
TnpA (Bp)	R.....G.....	
IS1002b	C.....	899
IS1002a	AGCGCTGCTGAGGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	270
TnpA (Bpp)	E R L V K T C L R E W A Y A R P Y	
TnpA (Bp)	Q.....	
IS1002b	C.....	949
IS1002a	ACGACGATCGGAGCGGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	287
TnpA (Bpp)	T S S D R Q A A L Q P F I D R V	
TnpA (Bp)	A.....	
IS1002b	CA...A.GC...CAA.GCA...G.CGCGTGT...T.CA	999
IS1002a	CAACTGCTGCGGCGACACTCGGCTTACAGTGGCTTGCCTCCGAGCGAAGGTC	303
TnpA (Bpp)	N W C R P H S A L G H Q P P I T	
TnpA (Bp)	H.....R.....A.....V.....S.....	
IS1002b	..AC..AA..T.G.C.AAG.C.....C.G.TC...G.....	1040
IS1002a	GTATC--CCAG--ATGTGAATACCTATGAGAAATCGACACCTAG	316
TnpA (Bpp)	R I P D V N N L L R I D T	
TnpA (Bp)	..L N L..E D...T V H S-	

FIG. 2. DNA sequences of IS1002a from *B. paraptentussis* and IS1002b from *B. pertussis*. The inverted repeats are underlined. The derived amino acid sequence of the putative transposase is also shown. The dash at amino acid residue 128 indicates a gap introduced to restore the ORF. All other dashes in the DNA sequence were introduced to maximize alignment of the sequences. Bases of IS1002b identical to IS1002a bases and amino acid residues identical to IS1002a-encoded ORF residues are indicated by dots above and below the sequence of IS1002a, respectively. The left inverted repeat of IS481, interrupting IS1002b, is shown in lowercase letters. The numbers of bases and amino acid residues are indicated on the right. Abbreviations: Bpp., *B. paraptentussis*; Bp., *B. pertussis*.

strains were performed by using an internal fragment of IS1002a as the probe (Fig. 1, probe 2). Four to seven hybridizing *Sma*I bands were observed in the patterns of the three *B. pertussis* strains investigated, and all three patterns were dif-

ferent. The Southern blot analysis of 13 human *B. paraptentussis* strains revealed nine hybridizing fragments, and all of the patterns were identical (Table 1 and Fig. 5). The single *B. bronchiseptica* strain containing IS1002 produced one hybridizing band. None of the 22 *B. paraptentussis* strains from sheep hybridized with IS1002 DNA (data not shown), confirming the PCR results described above.

DISCUSSION

In this study we exploited DNA polymorphism associated with repetitive DNA to study the population structure of the genus *Bordetella*. Previously, similar studies have been performed successfully with other bacteria, such as *Mycobacterium tuberculosis* (28). For a long time it was assumed that *B. paraptentussis* is an obligate human pathogen that generally causes a milder form of whooping cough than *B. pertussis*. This view changed in 1987 when *B. paraptentussis* was isolated from sheep (8). To investigate whether *B. paraptentussis* may be transmitted from this animal reservoir and infect humans, we studied the population structure of *B. paraptentussis* by using repetitive DNA.

We characterized a novel IS element, which was designated IS1002. IS1002 appeared to be closely related to another IS element, IS481, which is found in *B. pertussis* but not in *B. paraptentussis* and in only 1 of the 41 strains of *B. bronchiseptica* investigated. These two elements exhibit 61.5% sequence identity and have almost identical terminal inverted repeats. IS1002 was found in all *B. pertussis* strains and all *B. paraptentussis* strains isolated from humans. In contrast, none of the *B. paraptentussis* strains from sheep investigated contained this element. The distribution of IS1002 suggests that the *B. paraptentussis* strains isolated from sheep and humans represent two separate populations which have adapted to different hosts.

Another IS element, IS1001, has been found previously in human *B. paraptentussis* isolates and in some *B. bronchiseptica* isolates (27). In this study, IS1001 was also found in all *B. paraptentussis* isolates obtained from sheep. The IS1001-related DNA fingerprints of human and ovine strains were clearly distinct, providing additional evidence that the human and ovine *B. paraptentussis* strains make up distinct populations. This is consistent with the observation of Porter et al. (24) that ovine *B. paraptentussis* strains can be differentiated from human strains on the basis of biochemical characterization and MEE data. Therefore, we concluded that the reservoirs of *B. paraptentussis* in sheep and humans constitute two different bacterial populations and that little or no transmission takes place between these two reservoirs.

We found that most human *B. paraptentussis* strains produce a single IS1001 fingerprint pattern, pattern X1. Only three of the human strains investigated produced a slightly different pattern, which differed in the position of only one or two bands. When IS1002 was used to generate DNA fingerprints, only one fingerprint pattern was obtained for human *B. paraptentussis* strains, whereas various patterns were obtained for *B. pertussis* strains. The results of these analyses indicate that the human *B. paraptentussis* population is genetically very homogeneous and thus probably descended relatively recently from a single clone. This is consistent with the preliminary data of other workers. Pulsed-field gel electrophoresis of 10 human *B. paraptentussis* strains isolated at different times or from various locations revealed three electrophoretic types (15), and an MEE analysis of 21 human *B. paraptentussis* strains revealed only one electrophoretic type (23). Thus, the results obtained with two other approaches to differentiate strains also indicate that the human *B. paraptentussis* population is highly clonal.

FIG. 3. IS1001-generated fingerprints of *B. paraptussis* and *B. bronchiseptica* strains. Chromosomal DNA was restricted with *SphI* (A) or *SmaI* (B), and an internal fragment from IS1001 was used as the probe. (A) Lanes 1 through 7, *B. paraptussis* strains (lane 1, human strain from The Netherlands; lane 2, human strain from Finland; lane 3, human strain from Germany; lane 4, sheep strain from New Zealand; lanes 5 through 7, sheep strains from Scotland); lanes 8 through 19, *B. bronchiseptica* strains (lane 8, pig strain from The Netherlands; lane 9, cat strain from The Netherlands; lane 10, pig strain from Australia; lane 11, pig strain from South Africa; lane 12, pig strain from the USSR; lane 13, cow strain from Ireland; lane 14, pig strain from Australia; lane 15, mouse strain from the United States; lane 16, dog strain from The Netherlands; lane 17, pig strain from The Netherlands; lane 18, rabbit strain from Switzerland; lane 19, pig strain from the United States). The difference in the fingerprint patterns in lanes 1 and 2 consists of the slightly lower mobility of the fifth band (from the top) in lane 2. (B) Lanes 1 through 3, sheep strains from Scotland; lane 4, sheep strain from New Zealand; lane 5, human strain from The Netherlands; lane 6, human strain from the United States; lane 7, *B. bronchiseptica* strain from a pig in The Netherlands. All *B. paraptussis* strains analyzed in this study produced hybridization patterns identical to one of the patterns shown, and the RFLP patterns are shown in Table 1. The designations of the RFLP patterns are indicated at the bottom. The sizes of 1-kb marker DNA fragments are indicated on the left.

Populations of pathogenic bacteria are generally genetically more diverse (29) than the human *B. paraptussis* isolates observed in this study. Two alternative explanations could account for the genetic homogeneity of human *B. paraptussis* strains. First, a *B. paraptussis* clone may have escaped from the animal reservoir and become adapted to human hosts only recently. Consequently, not enough time has passed for divergence to have occurred. Second, the association of *B. paraptussis* with humans may be quite ancient, but a single clone may have expanded worldwide relatively recently; perhaps this clone acquired IS1002.

As *B. pertussis* is found only in humans, the most parsimonious explanation for the observed distribution of IS1002 seems to be that relatively recently a strain of *B. paraptussis* in a human acquired IS1002 from a coinfecting *B. pertussis* strain. IS1002 was found in only one *B. bronchiseptica* strain isolated from a koala. This strain also may have acquired IS1002 by horizontal transfer.

In contrast to the IS1002 RFLP patterns of *B. paraptussis* strains from humans, the IS1002 band patterns of *B. pertussis* strains were quite polymorphic. This suggests that IS1002 may be a useful tool for studying the epidemiology of whooping cough. At this time, highly discriminative tools for tracing the transmission routes of *B. pertussis* in addition to pulsed-field gel electrophoresis are very limited. We are now using IS1002 to study the epidemiology of *B. pertussis* by performing experiments similar to experiments done with *M. tuberculosis* (28).

On the basis of the phylogenetic relationships in the genus

FIG. 4. Comparison of the DNA sequences of PCR products internal to IS1001 derived from *B. paraptussis* human and ovine isolates and *B. bronchiseptica* pig and rabbit isolates. The PCR fragment was 420 bp long, ranging from position 764 to position 1184 on the sequence of IS1001 as described previously (27). The positions at which base substitutions were found are indicated above the sequence, and bases that differed from IS1001 bases are enclosed in boxes. The host strains are indicated on the left. The origins of the strains tested are indicated on the right. The dendrogram was generated by comparing the 420-bp sequences by neighbor joining. Abbreviations: Bpp, *B. paraptussis*; Bb, *B. bronchiseptica*.

Bordetella, as established by using MEE, it has been suggested that *B. paraptussis* strains can be considered derivatives of *B. bronchiseptica* that have adapted to the human host (23). In this context it is interesting that IS1001 was found to be absent from *B. pertussis*, but present in all human and ovine *B. paraptussis* strains and in most *B. bronchiseptica* strains used in this study. Most of the *B. bronchiseptica* strains which contained IS1001 were obtained from pigs or rabbits, suggesting that these organisms represent distinct groups that became specialized and infect particular animals. This suggestion is supported by the results of previous MEE analyses of the *B. bronchiseptica* pig strains, most of which have been shown to belong to a single electrophoretic type (22). The IS1001 band patterns of *B. bronchiseptica* strains were more diverse than the *B. paraptussis* patterns. This finding supports the hypothesis that *B. bronchiseptica* represents the oldest phylogenetic branch in the genus *Bordetella* (23). The ovine *B. paraptussis* strains exhibited more variation in their IS1001 band patterns than the human strains. The greater polymorphism in the ovine

strains suggests that the human *B. paraptussis* strains evolved more recently than the ovine strains.

Arber (2) and Blot (4) have reviewed the putative role of IS elements in the evolution of microbial species and their adaptation to changing environments. It may well be that a specific combination of insertions of IS elements leads to a phenotype that is beneficial in a particular host. Thus, IS1001-mediated DNA rearrangements may have played a role in the adaptation of *B. paraptussis* to either sheep or humans. The strains isolated from these two hosts were found to be distinct, but closely related in terms of copy number and a limited number of common sites of insertion of IS1001, suggesting that these organisms descended from a common ancestor, possibly a *B. bronchiseptica* strain in the animal reservoir, and that once they were in the ovine and human hosts, selection for strains with a higher IS copy number may have occurred. The results of our comparison of DNA sequences internal to IS1001 in *B. paraptussis* and *B. bronchiseptica* strains from humans and animals showed that the overall level of homology is very high and that the human *B. paraptussis* strains are more closely related to *B. bronchiseptica* strains from pigs than the ovine *B. paraptussis* strains are. The dendrogram based on IS1001 sequences suggests that the human *B. paraptussis* strains share a more recent ancestor with *B. bronchiseptica* strains than the ovine *B. paraptussis* strains. Thus, the results of both comparisons of IS1001-associated RFLP and comparisons of IS1001 sequences suggest that the ovine and human *B. paraptussis* strains evolved independently from *B. bronchiseptica* strains.

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FIG. 5. IS1002-generated fingerprints of *B. pertussis*, human *B. paraptussis*, and *B. bronchiseptica* strains. Chromosomal DNAs were restricted with *Sma*I, and an internal fragment derived from IS1002 was used as the probe (Fig. 1, probe 2). Lane 1, *B. pertussis* Tohama (24); lane 2, *B. pertussis* Wellcome 28 (25); lane 3, *B. pertussis* 18323 (28); lane 4, *B. paraptussis* human strain from The Netherlands; lane 5, *B. paraptussis* human strain from Finland; lane 6, *B. paraptussis* human strain from Germany; lane 7, *B. bronchiseptica* koala strain from Australia. The sizes of 1-kb marker DNA fragments are indicated on the left.

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