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Bordetella parapertussis and *Bordetella bronchiseptica* Contain Transcriptionally Silent Pertussis Toxin Genes

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Pertussis toxin, the major virulence factor of *Bordetella pertussis*, is not produced by the closely related species *Bordetella parapertussis* and *Bordetella bronchiseptica*. It is shown here that these two species possess but do not express the complete toxin operon. Nucleotide sequencing of an *Eco*RI fragment of 5 kilobases comprising the regions homologous to the pertussis toxin genes shows that in this region, *B. parapertussis* and *B. bronchiseptica* are 98.5% and 96% homologous, respectively, to *B. pertussis*. The changes (mostly base pair substitutions) in many cases are identical in *B. parapertussis* and *B. bronchiseptica*, suggesting that these two species derive from a common ancestor. Many of the mutations common to *B. parapertussis* and *B. bronchiseptica* involve the promoter region, which becomes very inefficient. The S1 subunits of both species, when expressed in *Escherichia coli*, have the same ADP-ribosylating activity as the S1 subunit from *B. pertussis*, indicating that the mutations in the S1 gene described here do not affect its function.

Pertussis toxin (PT) is the main virulence factor produced by *Bordetella pertussis*, which is the human pathogen responsible for whooping cough (20, 26). It has recently been shown that the five genes coding for the pertussis toxin are organized as an operon (11, 18).

Bordetella parapertussis and *Bordetella bronchiseptica* are two species closely related to *B. pertussis*, responsible for mild forms of respiratory diseases in humans and animals (20). The three species colonize the respiratory tract by adhering to the cilia of the mucosa, have common surface antigens, produce toxic substances (heat-labile toxin and extracellular adenyl cyclase), and undergo phase variation (20). DNA sequence homology between the genomes of the three *Bordetella* species has also been shown (7). Within this framework of evolutionary relationships, one feature clearly distinguishes the three bacterial species: PT is not produced by *B. parapertussis* or *B. bronchiseptica* (20). It is not clear whether the absence of the protein is due to mutations of the pertussis toxin genes or to their absence from the genomes of *B. parapertussis* and *B. bronchiseptica*. The availability of the PT genes has allowed us to tackle this problem at the molecular level. A detailed analysis of the structure and location of PT genes of the related species is relevant since a conversion from *B. pertussis* to *B. parapertussis* has been postulated during pertussis epidemics, when the two species are often isolated together (5, 10, 15), sometimes even from the same patient (3, 9). It has also been suggested that such conversion can occur under laboratory conditions after nitrosoguanidine mutagenesis or phage lysogenization (8, 14).

Our results show that the genomes of *B. parapertussis* and *B. bronchiseptica* contain mutated PT genes which are not transcriptionally active within the promotor and coding regions. Nucleotide sequence analysis of the genes reveals that *B. parapertussis* and *B. bronchiseptica* derive from a common ancestor and that *B. parapertussis* cannot easily be converted to *B. pertussis*.

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MATERIALS AND METHODS

Strains and media. The strains of *Bordetella* used in this work are listed in Table 1. *B. pertussis* strains 165 and Tohama were available in our laboratory. *B. pertussis* S86, S861, and SA1 were isolated in Siena, Italy. The strains of *B. pertussis* from England and Argentina, *B. parapertussis* PP1 and 70, *B. bronchiseptica* 7306, 4, and 241, and *Alcaligenes faecalis* 171 were kindly supplied by N. W. Preston of the World Health Organization Pertussis Reference Laboratory (Manchester, England). The strains of *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, *Bordetella avium*, and *Alcaligenes* designated CCUG were kindly supplied by Enevold Falsen from the Culture Collection, University of Göteborg, Sweden. *B. parapertussis* 3715 and 32259 were obtained from J. Hoppe (Tübingen, Federal Republic of Germany). *Escherichia coli* JM101 (27) and K-12 ΔH1 Δ*trp* (21) have already been described. *B. pertussis* strains were grown in Bordet-Gengou plates (20) or Vervey liquid medium containing cyclodextrin (1 mg/ml) (6). Vervey medium contains (per liter) 14 g of Casamino Acids, 0.2 g of KCl, 0.5 g of KH_2PO_4, 0.1 g of $MgCl_2 \cdot 6H_2O$, 20 mg of nicotinic acid, 10 mg of glutathione, and 1 g of starch. *E. coli* strains were grown in LB plates or LB liquid medium (12).

DNA procedures. Manipulations of DNA were performed by standard procedures (12). The chromosomal libraries of *B. parapertussis* and *B. bronchiseptica* were constructed in the EMBL4 phage vector as described by Frischauf et al. (2). Following identification of positive recombinant clones by plaque hybridization (12) with the cloned PT operon as a probe (18), the *Eco*RI fragments containing sequences homologous to the PT operon were subcloned in the plasmid vector PEMBL8$^+$ (1) and sequenced by the method of Sanger et al. (22), with synthetic oligonucleotides as primers. The oligonucleotide primers were designed by the sequence published by Nicosia et al. (18) and synthesized with an Applied Biosystems model 380A synthesizer.

RNA procedures. RNA was prepared from *Bordetella* species as follows. Samples were taken at intervals from a culture growing in liquid medium and centrifuged, and the

TABLE 1. Strains used

Strain	Source and yr of isolation	Serotype	Reference	Hybridization pattern[a]
B. pertussis				
165[b]	Unknown	1, 2, 3	23	C
M/M23/2	England, 1967	1, 2	This work	C
M9	England, 1963	1, 2, 3	This work	C
BN3	England, 1963	1, 3	This work	C
M/06092/70/6	England, 1970 (Isolated from the same child)	1, 2	This work	C
M/06092/70/5	England, 1970 (Isolated from the same child)	1, 2, 3	This work	C
M/04772/70/2	England, 1970 (Isolated from the same child)	1, 3	This work	C
AR1	Argentina, 1971	1, 2	This work	C
AR4	Argentina, 1971	1, 2, 3	This work	C
AR2	Argentina, 1971	1, 3	This work	C
Tohama	Unknown	1, 2, 3	6	C
18323 (type strain, ATCC 9797)	Unknown, before 1947	1, 2, 3	20	C
CCUG 4472	Sweden, 1975	Unknown	This work	C
CCUG 4473	Sweden, 1975	Unknown	This work	C
CCUG 4475	Sweden, 1975	Unknown	This work	C
CCUG 13456	Sweden, 1983	Unknown	This work	C
CCUG 13337	Sweden, 1983	Unknown	This work	C
CCUG 17531	Sweden, 1985	Unknown	This work	C
CCUG 17138	Sweden, 1985	Unknown	This work	C
S86	Italy, 1986	Unknown	This work	C
S861	Italy, 1986	Unknown	This work	C
SA1	Italy, 1987	Unknown	This work	C
B. parapertussis				
CCUG 413	England, 1939		This work	C-D
PP1	England, before 1948		This work	C-D
CCUG 882A	England, 1948		This work	C-D
11107	England, 1966		This work	C-D
CCUG 13437	Sweden, 1983		This work	C-D
32259	Federal Republic of Germany, 1985		5	C-D
3715	Federal Republic of Germany, 1985		5	C-D
P14	United States, 1986		This work	C-D
ATCC 9305	Unknown		This work	C-D
70	Unknown		This work	C-D
B. bronchiseptica				
CCUG 219 (type strain, ATCC 19395)	United States, 1912		20	C-D
CCUG 1422	England, 1950		This work	C-D
7306	Denmark, before 1957		This work	C-D
CCUG 1326	Denmark, 1957		This work	C-D
CCUG 1111	United States, 1970		This work	C-D
CCUG 4878	Sweden, 1976		This work	C-D
CCUG 7865	Sweden, 1979		This work	C-D
ATCC 4617	Unknown		20	C-D
4	Unknown		This work	C-D
241	Unknown		This work	C-D
B. avium				
CCUG 13726	Belgium, 1983		This work	—
CCUG 14270	Belgium, 1983		This work	—
CCUG 14271	Belgium, 1983		This work	—
CCUG 14939	France, 1983		This work	—
Alcaligenes spp.				
A. piechaudii CCUG 366	Denmark, before 1968		This work	—
A. piechaudii CCUG 1273	Sweden, 1971		This work	—
A. xylosoxidans CCUG 407	England, before 1969		This work	—
A. xylosoxidans CCUG 367	Denmark, before 1968		This work	—
A. faecalis 171	Unknown		This work	—

[a] Southern blots of chromosomal DNA digested with *Eco*RI were hybridized with *Eco*RI-C (Fig. 1). The probe hybridized with a band comigrating with fragment C or fragment C-D (Fig. 1) or did not hybridize (shown as C, C-D, and —, respectively).

[b] This strain is used to prepare cellular vaccine by Sclavo S.p.a. and was obtained from the Bureau of Biologics.

pellet was frozen in a dry ice-ethanol bath. The pellets were then suspended in 1 ml of cold 10 mM Tris–1 mM EDTA, pH 7.5, containing 500 μg of proteinase K per ml, vortexed, and mixed with 1 ml of 0.2% sodium dodecyl sulfate (SDS) to lyse the cells; 0.8 g of CsCl was then added to the lysate, which was layered over 2 ml of a 5.7 M CsCl cushion in an SW65 ultracentrifuge tube and centrifuged at 35,000 rpm for 12 h. The pellet containing the RNA was then suspended in 200 μl of 10 mM Tris–1 mM EDTA–0.2% SDS, pH 7.5, phenol-chloroform extracted, precipitated with ethanol, and suspended in water. The amount of PT mRNA was determined by dot-blot hybridization; the RNA was suspended in 10 μl of a solution containing 130 μl of 40% formaldehyde in 330 μl of water. Then, 10 μl of 4 M NaCl was added, and the RNA was spotted onto the nitrocellulose filter. Three fourfold dilutions (2 μg, 0.5 μg, and 0.125 μg) were used for each RNA sample. Filters were hybridized with a single-stranded RNA probe complementary to the S1 mRNA obtained from a clone of the S1 gene in the SP6 vector. The SP6 vector was obtained from Promega Biotech (Madison, Wis.) and used as specified by the manufacturer. Hybridization was performed under standard conditions (12), followed by extensive washings at high stringency in 0.2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate) at 68°C.

Assays for PT. The Chinese hamster ovary cell assay (4) was used to test the biological activity of PT. Quantitative determination of PT in culture supernatants of cell extracts was performed by enzyme-linked immunosorbent assay (ELISA) (23). Purified goat anti-PT immunoglobulins or fetuin was used to coat microtiter plates. Following incubation with antigen-containing preparations, a conjugate made of affinity-purified anti-PT rabbit gammaglobulins covalently coupled to alkaline phosphatase was added. PT was estimated by the resulting alkaline phosphatase activity.

Production of recombinant S1 subunits and ADP-ribosylation assay. The *Sau*3a-*Xba*I fragment from nucleotides 612 to 1317 (18), encoding the native S1 subunit, was cloned in the vector PEX34b (24) digested with *Bam*HI and *Xba*I. The resulting plasmid encoded a fusion protein of 36,000 daltons, which was temperature induced and purified as described previously (17, 24). ADP-ribosylation of transducin by the recombinant S1 subunits was performed as described by Manning et al. (13).

RESULTS

***B. parapertussis* and *B. bronchiseptica* contain DNA sequences homologous to the PT genes.** Following identification of the genes coding for PT (11, 18), we used them as a probe on Southern blots (12) of chromosomal DNA from *B. bronchiseptica*, *B. parapertussis*, *B. avium*, and *Alcaligenes* spp. *B. parapertussis* and *B. bronchiseptica* gave strong hybridization, suggesting that the two species contained at least part of the genes. No homology was detected with *B. avium* or *Alcaligenes* DNA (Table 1). To further characterize the regions homologous to the PT genes, we cloned the genomic DNA of *B. bronchiseptica* and *B. parapertussis* into phage EMBL4 and isolated positive overlapping clones, which were characterized by restriction mapping and hybridization analysis. A comparison of the genomic fragments of the three *Bordetella* species showed homology within the PT gene and the region downstream from it, except for the deletion of an *Eco*RI site downstream from the PT genes which fused fragments C and D of *B. pertussis* into a single fragment C-D (Fig. 1, fragments C, C-D, E, and F). In contrast, the sequences upstream from the PT genes (fragment G) of *B. parapertussis* and *B. bronchiseptica*, although similar to each other, were different from the equivalent regions of *B. pertussis* DNA (fragments A and B).

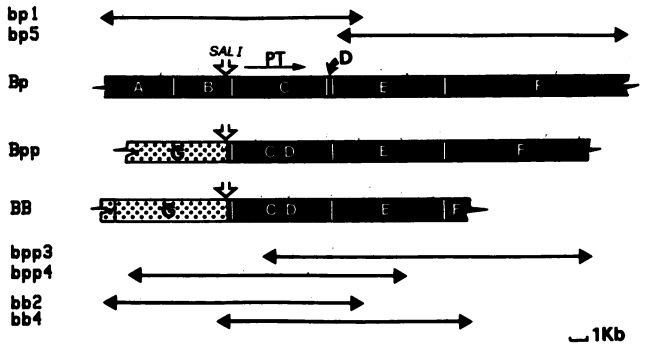
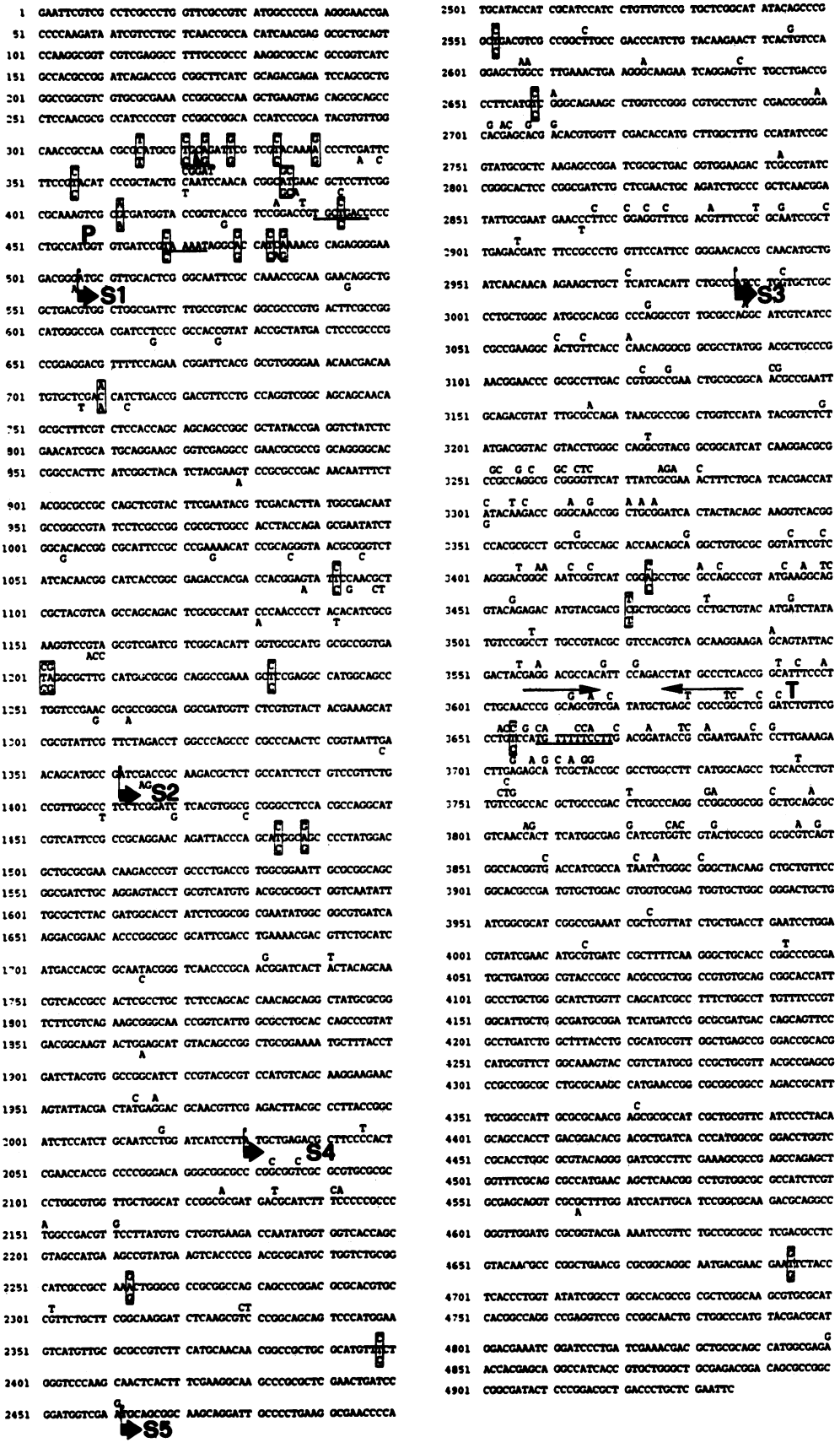


FIG. 1. *Eco*RI restriction map of the chromosomal region containing the PT genes in *B. pertussis* BP165 (BP), *B. parapertussis* ATCC 9305 (Bpp), and *B. bronchiseptica* ATCC 4617 (BB). The fragments of DNA from *B. parapertussis* and *B. bronchiseptica* which are homologous to the corresponding fragments of *B. pertussis* are shown as black boxes. The PT genes are contained in fragment C. In *B. parapertussis* and *B. bronchiseptica*, fragments C and D are fused into a single fragment C-D. Fragment A and most of fragment B are replaced in both species by a new fragment G (dotted box). It has been shown by Southern blot hybridization that fragments A and B are present somewhere else in the chromosome of *B. parapertussis* and *B. bronchiseptica* and that fragment G is also present in the chromosome of *B. pertussis*. Above and below the restriction endonuclease maps, the arrows indicate the overlapping recombinant phages which were used to construct the map; numbers identify the clones.

Nucleotide sequence. A detailed analysis of fragment C-D showed that it contained sequences homologous to each of the five genes for PT, raising the possibility that *B. parapertussis* and *B. bronchiseptica* may encode a functional PT. To answer this question, fragment C-D from both species was subcloned in the plasmid vector PEMBL8$^+$ (1) and sequenced (22). Figure 2 shows a comparison of the sequences of the PT genes of *B. pertussis* with those of *B. parapertussis* and *B. bronchiseptica*. The sequence conservation is considerable; in this region, the homology of *B. pertussis* to *B. bronchiseptica* and *B. parapertussis* was 96 and 98.5%, respectively. The mutations were mostly base substitutions, except for a 5-base-pair (bp) insertion found at position 323 of the *B. parapertussis* gene. It may be noted that a large proportion of the mutations of the *B. parapertussis* and *B. bronchiseptica* sequences were common to both species (boxed nucleotides in Fig. 2), and 50% of the common mutations were clustered within 72 bp comprising the region at the 5′ end of the promoter and the promoter itself. Two of these mutations (one T-to-C transition in the −35 region and one in the −10 region) were severe downmutations for *E. coli* promoters (28) and severely affect the efficiency in *E. coli* of the promoters of *B. parapertussis* and *B. bronchiseptica* (19).

Another mutation of interest was that at position 4694, where the T-to-G transversion deleted the *Eco*RI site which is present in *B. pertussis*. This change, which fused the *Eco*RI fragments C and D of *B. pertussis* into a single fragment C-D (Fig. 1), was conserved in all the isolates that we tested (Fig. 3 and Table 1) and can be used to differentiate *B. pertussis* from *B. parapertussis* and *B. bronchiseptica*.

Amino acid sequences. The amino acid sequences of the five subunits, deduced from the nucleotide sequences, are


P
S1
S2
S3
S4
S5

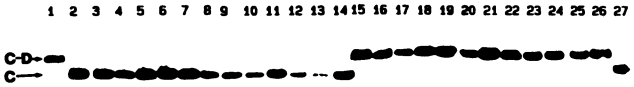


FIG. 3. Southern blot of *Eco*RI-digested chromosomal DNA from *Bordetella* species hybridizd to the *Eco*RI fragment C of Fig. 1. All the *B. pertussis* tested had a fragment comigrating with fragment C, while the strains of *B. parapertussis* and *B. bronchiseptica* had a fragment comigrating with fragment C-D. Lanes: 1, *B. parapertussis* ATCC 3715; 2 to 14, *B. pertussis* isolates as listed in Table 1; 15 to 20, *B. parapertussis* isolates as listed in Table 1; 20 to 26, *B. bronchiseptica* isolates as listed in Table 1; 27, *B. pertussis* 165.

shown in Fig. 4. *B. parapertussis* encoded a subunit S4 which was identical to that of *B. pertussis* and subunits S3, S5, and S1 with 2, 1, and 11 mutations, respectively. Most of these mutations involved conservative changes of amino acids which should not interfere with the function of the subunits. In marked contrast, subunit S2 contained a stop codon in position 170. *B. bronchiseptica* encoded subunits S1, S2, and S4 containing four, three, and seven mutations, respectively. Again, these mutations involved conservative changes of amino acids which should not interfere with the function of the subunits. Subunit S5 contained 11 mutations. The first one involved the ATG start codon, which became GTG (GTG can also be used as a start codon). Some of the other mutations involved changes in charged amino acids and could interfere with the structure or function of this subunit. Subunit S3 was heavily mutated, containing 35 mutations of all kinds which could interfere with its function.

PT operons of *B. parapertussis* and *B. bronchiseptica* encode functional proteins but are transcriptionally silent. The results obtained so far have shown that *B. parapertussis* and *B. bronchiseptica* encode the five subunits of PT and that most of them, if produced, might be functional. To determine whether the mutated proteins are active, we assayed the enzymatic activities of the recombinant S1 subunits of *B. parapertussis* and *B. bronchiseptica* and compared them with that of the wild type. Function assays for the other subunits are not available. The sequences coding for the S1 subunits of *B. parapertussis* and *B. bronchiseptica* were introduced into the plasmid vector PEX31 and expressed as fusion proteins in *E. coli* (17). The resulting proteins were assayed for ADP-ribosylation activity, and no difference was found between the activity of the proteins from the three *Bordetella* species (Fig. 5). These data suggested that the PT genes, if expressed, could play some role in the pathogenicity of *B. parapertussis* and *B. bronchiseptica*, and therefore we investigated the expression of the PT genes in these two species. Phase I *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were grown in parallel, and the amount of PT in the culture supernatant, periplasmic fraction, and total cell lysate and the amount of PT mRNA were determined by ELISA and dot-blot hybridization, respectively (17). In no case could PT mRNA or PT be detected in *B. parapertussis* or *B. bronchiseptica* (data not shown).

DISCUSSION

The results reported in this paper show that *B. parapertussis* and *B. bronchiseptica* contain but do not transcribe the PT operon. The lack of expression is most likely due to a cluster of mutations common to *B. parapertussis* and *B. bronchiseptica* which severely affect the efficiency of the promoter and could interfere with the action of the *trans*-acting regulatory element encoded by the *vir* locus, which has been shown to be necessary for transcription of the PT operon (19, 25). Expression of the S1 subunits from both species in *E. coli* showed that the silent S1 genes encoded functional proteins. The findings reported in this paper propose the question of why the PT genes have been maintained in *B. parapertussis* and *B. bronchiseptica* if they are not expressed and therefore do not confer any evolutionary advantage. A possible explanation could be that *B. parapertussis* and *B. bronchiseptica* have diverged recently from *B. pertussis* and have not had enough time for complete evolution; in this case, we should expect these two species to lose these silent genes in the future. Alternatively, we could speculate that the mutations in the promoter region may not really inactivate its function but simply alter its regulation and that the PT genes of *B. bronchiseptica* and *B. parapertussis* are positively activated by a gene different from *vir* and are expressed in growth conditions different from those of *B. pertussis*. This last hypothesis would offer an explanation for the facts that the entire operon is conserved in both *B. bronchiseptica* and *B. parapertussis* and that most of the amino acid substitutions are conservative. The fact that subunit S2 of *B. parapertussis* contains a stop codon would not be in disagreement with this hypothesis, because subunits S2 and S3 are homologous and could substitute for each other, as they do in *B. pertussis* BP356, a mutant which lacks subunit S3 but produces active PT (19).

Analysis of the nucleotide sequence of the PT operon in both species showed that many of the mutations were common to both *B. parapertussis* and *B. bronchiseptica*. This finding strongly suggests that these two species derive from a common ancestor. Alternatively, the PT genes could have been introduced by transposition, transduction, or other mechanisms into two separate species or transferred from one species to the other.

The observation that *B. parapertussis* contains 70 mutations scattered all over the PT operon allowed us to conclude that the proposed conversion from *B. pertussis* to *B. parapertussis* and vice versa is impossible because many independent mutations or major recombinational events would be required. This finding is in agreement with a recent study (16), in which the authors reached the same conclusion by studying the isoenzymes of several isolates of *Bordetella*.

FIG. 2. Nucleotide sequence of the *Eco*RI restriction fragments C and D (Fig. 1). Fragment C-D from *B. parapertussis* ATCC 9305 and *B. bronchiseptica* ATCC 4617 was subcloned in the plasmid vector PEMBL8$^+$ (1) and sequenced by the method described by Sanger et al. (22). The sequence of fragment C from *B. pertussis* BP165 (nucleotides 1 to 4696) has been published (18). The sequence from nucleotides 4696 to 4936 is reported here for the first time. The bases which are different in *B. bronchiseptica* ATCC 4617 and *B. parapertussis* ATCC 9305 are reported above and below the main sequence, respectively. For *B. parapertussis*, the sequence has been determined only from nucleotides 200 to 4700. The promoter region of the PT operon is indicated (P), and the −35 and −10 regions are underlined. The beginning of the genes coding for each of the five subunits are indicated by arrows S1 through S5. The termination of the PT operon (T) is also indicated by arrows above an inverted repeat followed by a stretch of T's (underlined). *B. bronchiseptica* and *B. parapertussis* differ from *B. pertussis* by 192 and 72 bp, respectively, and therefore in this region homology to *B. pertussis* is 96% for *B. bronchiseptica* and 98.5% for *B. parapertussis*. Thirty of the mutations are common to *B. parapertussis* and *B. bronchiseptica* (boxed), and 50% of the common mutations are concentrated in 72 bp comprising the promoter and the region immediately preceding it.

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S1
  1 MRCTRAIRQT ARTGWLTWLA ILAVTAPVTS PAWADDPPAT VYRYDSRPPE
                 R
                     E
 51 DVFQNGFTAW GNNDNVLDHL TGRSCQVGSS NSAFVSTSSS RRYTEVYLEH
                     E
101 RMQEAVEAER AGRGTGHFIG YIYEVRADNN FYGAASSYFE YVDTYGDNAG
                               I
                                                       P
151 RILAGALATY QSEYLAHRRI PPENIRRVTR VYHNGITGET TTTEYSNARY
                                 T                   P L
                                       T           P
201 VSQQTRANPN PYTSRRSVAS IVGTLVRMAP VIGACMARQA ESSEAMAAWS
          T          T                T           P
251 ERAGEAMVLV YYESIAYSF*
      T

S2
                                                       G
  1 MPIDRKTLCH LLSVLPLALL GSHVARASTP GIVIPPQEQI TQHGSPYGRC
     S                F  C                           G
 51 ANKTRALTVA ELRGSGDLQE YLRHVTRGWS IFALYDGTYL GGEYGGVIKD
                               R F
101 GTPGGAFDLK TTFCIMTTRN TGQPATDHYY SNVTATRLLS STNSRLCAVF
151 VRSGQPVIGA CTSPYDGKYW SMYSRLRKML YLIYVAGISV RVHVSKEEQY
                          *
201 YDYEDATFET YALTGISICN PGSSLC*

S3
                    L          R    S         L K
  1 MLINNKKLLH HILPILVLAL LGMRTAQAVA PGIVIPPKAL FTQQGGAYGR
              A     T                                 S
 51 CPNGTRALTV AELRGNAELQ TYLRQITPGW SIYGLYDGTY LGQAYGGIIK
    R AGAL   QKP         Y    DT                     G
101 DAPPGAGFIY RETFCITTIY KTGQPAADHY YSKVTATRLL ASTNSRLCAV
                    M
     A   KPL     TR  QSS  G    V        V
151 FVRDGQSVIG ACASPYEGRY RDMYDALRRL LYMIYMSGLA VRVHVSKEEQ
                           V
              E          I
201 YYDYEDATFQ TYALTGISLC NPAASIC*

S4
       L                P                T M  H   Q
  1 MLRRFPTRTT APGQGGARRS RVRALAWLLA SGAMTHLSPA LADVPYVLVK
 51 TNMVVTSVAM KPYEVTPTRM LVCGIAAKLG AAASSPDAHV PFCFGKDLKR
     S
101 PGSSPMEVML RAVFMQQRPL RMFLGPKQLT FEGKPALELI RMVECSGKQD
151 CP*

S5
  V                                                    T
  1 MQRQAGLPLK ANPMHTIASI LLSVLGIYSP ADVAGLPTHL YKNFTVQELA
        D    L        P              E RTRG
 51 LKLKGKNQEF CLTAFMSGRS LVRACLSDAG HEHDTWFDTM LGFAISAYAL
                     P
101 KSRIALTVED SPYPGTPGDL LELQICPLNG YCE*
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FIG. 4. Deduced amino acid sequence of the five subunits of PT in the three species of *Bordetella*. The sequence reported is for *B. pertussis* (18). The differences in *B. bronchiseptica* and *B. parapertussis* are reported above and below the main sequence, respectively. *, Stop codon. The arrows indicate the end of the leader peptides and the beginning of the native subunits.

The hybridization pattern of the PT genes (Fig. 3 and Table 1) shows that the mutations reported in Fig. 2 are conserved in a wide variety of strains isolated from different countries and over a period of 70 years, indicating that the origin of these three pathogens is clonal. The other two species related to *Bordetella* spp. (*B. avium* and *Alcaligenes* spp.) did not contain any DNA homologous to the PT genes, and therefore, by this analysis, they do not appear to be related to the other three species of *Bordetella*.

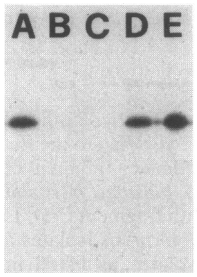

FIG. 5. ADP-ribosylation of transducin by the recombinant S1 subunits from *B. pertussis* (A), *B. parapertussis* (D), and *B. bronchiseptica* (E) produced in *E. coli* as fusion proteins. B and C are negative controls containing the recombinant S3 and S4 fusion proteins from *B. pertussis*, respectively (19).

The conservation of the restriction pattern associated with the PT genes that we have observed could be of use in the epidemiological typing of *Bordetella* strains from whooping cough epidemics.

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