JOURNAL OF CLINICAL MICROBIOLOGY, June 1997, p. 1550–1555 Vol. 35, No. 6
0095-1137/97/$04.00+0

Molecular Characterization of Two *Bordetella bronchiseptica* Strains Isolated from Children with Coughs

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Received 24 October 1996/Returned for modification 16 December 1996/Accepted 28 February 1997

During a surveillance program associated with the Italian clinical trial for the evaluation of new acellular pertussis vaccines, two bacterial isolates were obtained in cultures of samples from immunocompetent infants who had episodes of cough. Both clinical isolates were identified as *Bordetella bronchiseptica* by biochemical criteria, although both strains agglutinated with antisera specific for *Bordetella parapertussis*, suggesting that the strains exhibited some characteristics of both *B. bronchiseptica* and *B. parapertussis*. Both children from whom these strains were isolated exhibited an increase in serum antibody titer to pertussis toxin (PT), a protein that is produced by *Bordetella pertussis* but that is not thought to be produced by *B. bronchiseptica*. We therefore examined whether the clinical isolates were capable of producing PT. Neither strain produced PT under laboratory conditions, although both strains appeared to contain a portion of the *ptx* region that encodes the structural subunits of PT. In order to determine whether the *ptx* genes may encode functional proteins, we inserted an active promoter directly upstream of the *ptx* region of one of these strains. Biologically active PT was produced, suggesting that this strain contains the genetic information necessary to encode an active PT molecule. Sequence analysis of the *ptx* promoter region of both strains indicated that, while they shared homology with the *B. bronchiseptica* ATCC 4617 sequence, they contained certain sequence motifs that are characteristic of *B. parapertussis* and certain motifs that are characteristic of *B. pertussis*. Taken together, these findings suggest that variant strains of *B. bronchiseptica* exist and might be capable of causing significant illness in humans.

Bordetella pertussis, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are closely related species that are each capable of producing upper respiratory tract disease in humans. *B. pertussis* is the etiologic agent of whooping cough, which can be especially severe in infants. *B. parapertussis* is usually responsible for milder forms of disease in humans, although parapertussis can also present in a severe form (12). *B. bronchiseptica* primarily produces disease in mammals other than humans. This organism causes disease in both domestic and wild animals including dogs, swine, and rabbits (35). On very rare occasions, however, *B. bronchiseptica* has been known to infect humans. Investigators have reported that *B. bronchiseptica* is associated with upper respiratory tract infections in animal caretakers (9), pertussis-like illnesses in children (24), subacute bacterial endocarditis (17), and infections in immunocompromised patients (5, 9, 29, 30, 35).

These three *Bordetella* spp. share many of the same virulence factors, including toxins such as adenylate cyclase toxin, dermonecrotic toxin, and tracheal cytotoxin (7), as well as adhesins such as filamentous hemagglutinin (FHA) and pertactin (2, 23). One notable exception is the virulence factor pertussis toxin (PT), which is thought to be produced only by *B. pertussis*. While *B. parapertussis* and at least certain strains of *B. bronchiseptica* contain the *ptx* genes that encode the structural subunits for PT (1, 26), the genes are not expressed under conditions that have been examined so far, presumably due to the presence of a cluster of mutations in the promoter region of the *ptx* operons that render those promoters silent (1).

In this report, we describe the isolation and identification of two variant strains of *B. bronchiseptica*. These strains were isolated from two children with episodes of cough who were participating in the clinical trial for the evaluation of acellular pertussis vaccines conducted in Italy. During the course of their illnesses, although culture and PCR of nasopharyngeal aspirates did not reveal the presence of *B. pertussis*, both children showed an increase in serum antibody titers for FHA and, surprisingly, PT. Thus, these strains are interesting in two aspects in that they were isolated from immunocompetent children, instead of one of the more common animal hosts for this pathogen, and they were isolated from children who exhibited increases in antibodies to PT during the course of their illnesses.

Despite the wide host range of *B. bronchiseptica* and despite the fact that many aspects of its biology have been studied, few reports have been written concerning the nature and extent of genetic variation in natural populations. The isolation of two *B. bronchiseptica* strains from children who exhibited increases in antibody titers to PT during the course of their illnesses prompted us to examine the phenotypic and genotypic differences between these two clinical isolates and other *B. bronchiseptica* strains.

MATERIALS AND METHODS

Patients and sampling. Nasopharyngeal aspirates (NPAs) and capillary blood samples (obtained 6 to 8 weeks apart) were collected by established procedures (10) from two infants participating in the Italian clinical trial for the efficacy of acellular pertussis vaccines.

Clinical and epidemiological data for each child were recorded. Both infants lived in Piemonte, one of the four regions involved in the clinical trial, but in two

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different locations. The first infant, code 03145, coughed for 15 days without paroxysms at the age of 18 months, 10 months after having completed the vaccination cycle with three doses of diphtheria and tetanus toxoids and acellular pertussis vaccine manufactured by Chiron-Biocine (Siena, Italy). At the onset of the cough the patient was treated with azithromycin for 2 days and with cefaclor for 7 days. The second infant, code 15374, had 19 days of paroxysmal cough at the age of 14 months, 9 months after having completed the vaccination cycle with three doses of diphtheria and tetanus toxoids and acellular pertussis vaccine manufactured by SmithKline Beecham Biologicals (Rixensart, Belgium). Starting at the onset of the cough, the child was treated for 10 days with rokitamycin. This infant was admitted to the hospital because of a high temperature 1 day before the onset of cough and remained in the hospital for 8 days; the diagnosis was laryngopharyngitis.

No immunocompromised status and no household contacts suffering from pertussis was recorded for either of the infants. The NPAs were collected 13 days after the onset of cough for study infant 03145 and 12 days after the onset of cough for the second infant, infant 15374. No animal contacts were reported for either child.

Primary isolation and identification of *Bordetella* spp. For primary isolation from NPAs, bacteria were grown on charcoal agar plates supplemented with cephalexin (20 μg/ml; Unipath, Milan, Italy), incubated at 35°C in a moist atmosphere, and observed for up to 7 days. All suspected colonies were identified by biochemical tests including tests for oxidase and urease production and motility, and they were evaluated by the API 20 NE system (bioMerieux, Marcy l'Etoile, France) and by testing for agglutination with antisera for *B. pertussis* and *B. parapertussis* (Murex Diagnostics, Dartfort, England). For confirmation by PCR, *Bordetella* strains which had grown for 48 h on charcoal agar plates were suspended in 1 ml of distilled water, heated for 10 min at 100°C, and diluted to a concentration of 10^4 cells/reaction mixture.

Description of bacterial strains. The following strains were used as reference strains: the American Type Culture Collection (ATCC) strains *B. pertussis* ATCC 9797 (18-323), *B. parapertussis* ATCC 9305, and *B. bronchiseptica* ATCC 4617. *B. bronchiseptica* RB50 was obtained from Jeff Miller (University of California, Los Angeles) and has been described previously (4). *B. parapertussis* 10978 was obtained from James Cherry (University of California, Los Angeles) (11).

PCR for strain confirmation. Two oligonucleotide primer pairs from the sequence of the *ptx* region were selected for amplification of *B. pertussis* and *B. parapertussis-B. bronchiseptica* DNA. They were synthesized in an automated DNA synthesizer (model 380A; Applied Biosystems) by Biocine Research Center, Siena, Italy, and were used without further purification. Primers Ptox1 and Ptox2 flank positions 345 and 695 of the *ptx* region numbering system as described in Nicosia et al. (31), and primers Ptox3 and Ptox4 flank positions 749 and 1053. The two sets of primers define two amplification products of 406 and 346 bp, respectively (32). Both PCR products are detected when *B. pertussis* DNA is used as the template; however, only the 346-bp amplified product is detected when either *B. parapertussis* or *B. bronchiseptica* DNA is used as the template. PCR analysis and detection of PCR products were carried out as described previously (32).

PCR assay with NPAs. As with other NPAs collected during the trial, PCR was performed to detect *B. pertussis* DNA by using the procedure previously described by Mastrantonio et al. (27). Two primers derived from the PT promoter region were used to amplify a 191-bp PCR product specific for *B. pertussis*. To investigate the presence of *B. parapertussis* DNA, primers BPPA (21 bp) and BPPZ (21 bp), derived from the insertion sequence IS*1001*, were used (33).

PCR products were subjected to electrophoresis performed with 3% agarose gels and 500 μg of ethidium bromide per ml and were visualized under UV light.

Serologic assays. A standardized enzyme-linked immunoabsorbent assay (ELISA) was used to measure immunoglobulin G (IgG) and IgA titers against PT and FHA (28). The reference line method (25) was used to calculate ELISA units (EUs) with standardized software (Unitcalc, Biosys inova, Stockholm, Sweden, 1992). The results were expressed in EUs per milliliter. U.S. Reference Pertussis Antiserum (human), lot 3, was used to calibrate an internal reference serum sample for the assays for these antigens.

PT neutralizing antibodies were measured by Chinese hamster ovary (CHO) cell assay as described previously (8). U.S. Reference Pertussis Antiserum (human), lot 3, was used to calibrate an internal reference serum sample. The amount of toxin (2 ng/ml) used in the test corresponded to four cytopathogenic units. The results of the assay were expressed as the reciprocal of the highest serum dilution causing complete inhibition of the clustering activity induced by the native toxin.

An increase of ≥100% from the acute-phase to the convalescent-phase serum of IgG PT, IgG FHA, IgA PT, or IgA FHA or a fourfold increase in PT neutralizing antibodies was considered to be serologic evidence supportive of a diagnosis of pertussis, according to the case definition adopted in the trial (10).

Chromosomal DNA preparations for PFGE. DNA was prepared as described by Khattak and Matthews (19), with some minor modifications. Briefly, bacteria were resuspended in TE buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA [pH 8.0]) to an optical density of 0.7. One milliliter of cell suspension was mixed with an equal volume of 1% low-melting-point agarose (Sigma). The mixture was dispensed in a 12-plug mold (Bio-Rad). The plugs were incubated overnight with shaking at 37°C in 25 ml of lysis buffer (6 mM Tris-HCl [pH 7.5], 1 M NaCl, 100 mM EDTA [pH 8.0], 0.5% *N*-lauroylsarcosine [Sigma], lysozyme [1 mg/ml; Boehringer]). Lysis buffer was substituted with 10 ml of ESP buffer (0.5 M EDTA [pH 8.0], 1% *N*-lauroylsarcosine, proteinase K [2 mg/ml; Sigma]), and the plugs were incubated for 48 h at 50°C. The blocks were then washed three times for at least 15 min each time with 20 ml of TE buffer. Restriction digestion was performed with 40 U of *Xba*I (New England Biolabs) in a final volume of 200 μl per plug, and the plug mold was incubated overnight at 37°C. Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF Mapper II (Bio-Rad). A 1% agarose gel (15 by 15 cm) was prepared in 0.5× Tris-borate-EDTA buffer (TBE). The agarose blocks were loaded into wells along with lambda ladder PFGE Markers (New England Biolabs). Electrophoresis was performed with 5- to 45-s ramping times for 27 h at a field strength of 6.0 V/cm, and an included angle of 120°. The gel was stained with ethidium bromide (0.5 μg/ml) and was photographed under UV light.

Antimicrobial susceptibility assay. Antimicrobial susceptibility testing was performed by the disk agar diffusion method with Mueller-Hinton agar (Unipath) and eight different antimicrobial agents: ampicillin, amikacin, ceftazidime, cephaloridine, imipenem, gentamicin, piperacillin, and sulfamethoxazole-trimethoprim. *Staphylococcus aureus* ATCC 25923 was used as a quality control strain; *B. bronchiseptica* ATCC 4617 was used to compare the performance of *B. bronchiseptica* isolates. The interpretative criteria were those recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for rapidly growing aerobic organisms.

Construction of *B. bronchiseptica* 03145::pSZH8. The *ptx* promoter and a portion of the gene for the S1 subunit of the PT of *B. bronchiseptica* 03145 were replaced by the corresponding region of *B. pertussis* by introducing a plasmid containing the promoter-S1 region of *B. pertussis*(pSZH8) into the chromosome of *B. bronchiseptica* 03145 essentially as described previously (11).

Electrophoresis and immunoblotting analysis of *B. bronchiseptica* cell extracts and culture supernatants. Cell extracts were prepared by suspending bacteria, which had grown for 5 days on Bordet-Gengou agar (either in the presence or the absence of 20 mM $MgSO_4$ and nicotinic acid), in phosphate-buffered saline to an A_{600} of 2. $MgSO_4$ and nicotinic acid modulate the cells from the Bvg^+ phase to the Bvg^- phase (34) such that they do not express genes regulated by the *bvg* (*Bordetella* virulence genes) locus. Samples of the cell extracts (35 μl) were precipitated with an equal volume of 20% trichloroacetic acid. After centrifugation, the precipitates were suspended in sodium dodecyl sulfate (SDS) sample buffer (0.2 M Tris containing 50 mM dithiothreitol, 20% SDS, and 0.012 bromophenol blue).

Culture supernatants from cells grown to an A_{600} of 2 were collected after centrifugation of cells at 18,500 × *g* for 15 min. Samples of the supernatants (2 ml) were precipitated with an equal volume of 20% trichloroacetic acid. After centrifugation, the precipitates were suspended in SDS sample buffer.

Samples were subjected to SDS-polyacrylamide gel electrophoresis performed essentially as described by Laemmli (21) with 4 to 20% gradient polyacrylamide gels (Integrated Separation System, Natick, Mass.) for FHA and adenylate cyclase and 15% polyacrylamide gels for flagellin and PT. After electrophoresis, the proteins were electrophoretically transferred to nitrocellulose. Immunoblot analysis was conducted as described previously (16) by using monoclonal antibody 9D4 (13) to visualize the adenylate cyclase toxin (provided by Erik Hewlett, University of Virginia, Charlottesville), monoclonal antibody X3C to visualize FHA (22), monoclonal antibody 15D8 (4) to visualize flagellin (provided by Jeff Miller, University of California, Los Angeles), P11B10 (6) to visualize the S2 subunit of PT, and 6DX3 (18) to visualize the S4 subunit of PT.

CHO cell assay for the detection of biologically active PT. Bacteria were grown in Cohen-Wheeler medium at 37°C to an A_{550} of 1.25. Cultures were centrifuged at 18,500 × *g*. The supernatants were collected and filtered through 0.22-μm-pore-size filters. The biologically active PT in the preparation was assayed by measuring the ability of these fractions to alter the morphology of CHO cells, as first described by Hewlett et al. (14). The assay was conducted essentially as described above.

DNA sequence analysis. The DNA sequence of the *ptx* promoter region of clinical *B. bronchiseptica* isolates was determined by first amplifying by PCR a piece of DNA consisting of nucleotides 72 to 1821 of the *ptx-ptl* region of *B. bronchiseptica* 03145 and 15374. These fragments of DNA were then cloned into a pBluescript vector (Stratagene, La Jolla, Calif.). DNA sequences were determined by Lark Sequencing Technologies (Houston, Tex.).

RESULTS

Identification of clinical isolates. Between April and May 1994, two bacterial strains were isolated from two infants with suspected pertussis participating in the clinical trial for the evaluation of new acellular pertussis vaccines conducted in Italy (10). The bacterial growth from the NPAs on Bordet-Gengou agar showed the presence of small hemolytic colonies composed of gram-negative rods. Both clinical isolates were identified as variants of *B. bronchiseptica* on the basis of biochemical tests as well as use of the PCR, as outlined below. Upon further passage, there appeared a second, distinctly

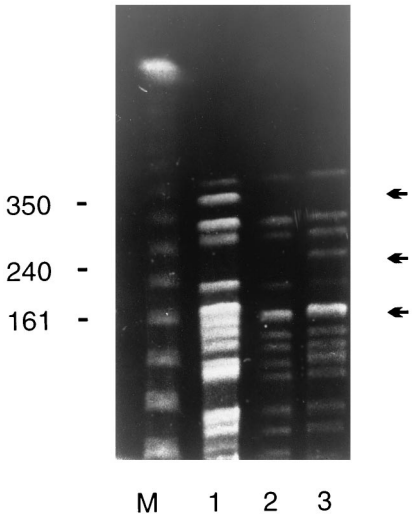

FIG. 1. PFGE patterns of *Bordetella* chromosomal DNA digested with the restriction enzyme *Xba*I. The results of macrorestriction analysis are shown for DNAs of *B. bronchiseptica* ATCC 4617 (lane 1), the *B. bronchiseptica* strain from infant 03145 (lane 2), and the *B. bronchiseptica* strain from infant 15374 (lane 3). Lane M, Lambda Ladder molecular size marker for PFGE (New England Biolabs). Fragment sizes are indicated on the left (in kilobases).

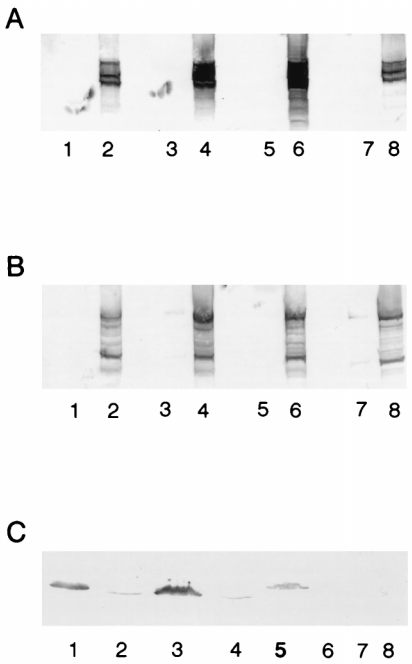

FIG. 2. Immunoblot analysis for the production of adenylate cyclase (A), FHA (B), and flagellin (C) by clinical *B. bronchiseptica* isolates. Cell extracts were prepared from the *B. bronchiseptica* strain from infant 03145 (lanes 1 and 2), the *B. bronchiseptica* strain from infant 15374 (lanes 3 and 4), *B. bronchiseptica* RB50 (lanes 5 and 6), and *B. parapertussis* 10978 (lanes 7 and 8) which had been grown in either the presence (lanes 1, 3, 5, and 7) or the absence (lanes 2, 4, 6, and 8) of $MgSO_4$ and nicotinic acid. (A) Lanes 1 and 3 contain immunoblot artifacts that do not represent protein bands.

larger colony phenotype which was nonhemolytic on Bordet-Gengou agar. These larger colonies were found to be the modulated form of the organism in which virulence genes controlled by the *bvg* locus (15, 20, 34) were no longer expressed. Therefore, care was taken to isolate the small colonies and to use only those colonies for further analyses.

The two clinical isolates were urease positive, oxidase positive, and motile. *B. bronchiseptica* strains are motile, whereas *B. pertussis* and *B. parapertussis* are nonmotile (35). Analysis of the two clinical isolates with the commercial API 20 NE system yielded, for both strains, the numerical code 1200067, which was compatible with the identification of *B. bronchiseptica* (percent identification, 95.6).

The two strains did not agglutinate with *B. pertussis* antiserum but showed a strong reactivity with *B. parapertussis* antiserum. In contrast, *B. bronchiseptica* ATCC 4617 did not agglutinate with either antiserum.

Identification of strains was confirmed by PCR, revealing on the gel only the presence of the 346-bp amplified product, indicative of *B. parapertussis* or *B. bronchiseptica* DNA. Attempts to identify *B. pertussis* or *B. parapertussis* directly from NPAs by PCR did not yield positive results.

Characterization of the *B. bronchiseptica* isolates. The macrorestriction fingerprinting by PFGE performed with the two *B. bronchiseptica* isolates showed different patterns. DNAs of the *B. bronchiseptica* isolates from infants 15374 and 03145 (the DNAs had been digested with *Xba*I) exhibited 11 to 13 bands in the 90- to 400-kb range, along with multiple small bands not sufficiently resolved around 97 kb. Figure 1 shows the restriction profiles of the two isolates compared with that of *B. bronchiseptica* ATCC 4617. The DNA patterns differed among all three strains. Differentiation between *B. bronchiseptica* ATCC 4617 and clinical *B. bronchiseptica* isolates was based on the absence of a band corresponding to a molecular size of about 350 kb and on the presence of only one band at 161 kb instead of the double band visible for the reference strain. Moreover, the *B. bronchiseptica* strain from infant 15374 showed a new band corresponding to 240 kb. The DNAs were then digested with *Spe*I, using the same conditions, and each of the isolates exhibited different patterns (data not shown).

No differences in behavior were detected in the antibiotic susceptibility test between the two *B. bronchiseptica* isolates and *B. bronchiseptica* ATCC 4617. As determined by the disk agar diffusion method, all the strains were sensitive to amikacin, imipenem, gentamicin, piperacillin, and sulfamethoxazole-trimethoprim and were resistant to ampicillin, ceftazidime, and cephaloridine.

The clinical isolates were examined for the ability to produce virulence factors that are found in the virulent phase of the organism (Bvg^+ phase) or to produce flagellin, which is produced in the Bvg^- phase by motile strains of *B. bronchiseptica*. As in the case of the control strain *B. bronchiseptica* RB50, both clinical isolates produced adenylate cyclase toxin as well as FHA in the Bvg^+ phase and flagellin in the Bvg^- phase (Fig. 2). In Fig. 2, multiple bands are observed for both adenylate cyclase toxin and FHA. The presence of multiple bands is likely due to the fact that both of these proteins are easily proteolyzed, generating lower-molecular-weight forms.

Analysis of the *ptx* locus of the clinical isolates. Table 1 shows the serology results obtained by ELISA and the CHO cell assay for infants 15374 and 03145. For both infants, a 100% or more increase in IgG titer to PT and FHA was evident between the acute- and convalescent-phase serum samples, while a positive result for IgA was obtained only against FHA. Positive results were observed also for PT neutralizing antibody on CHO cells, with at least a fourfold increase in the neutralizing antibody titers between the acute- and convalescent-phase sera.

The serology results were surprising since *B. bronchiseptica*

TABLE 1. Serology results between acute- and convalescent-phase serum samples by ELISA and CHO cell assay

Patient code	Sample	Antibody titers (EUs)				PT neutralizing antibody titer[a]
		PT		FHA		
		IgG	IgA	IgG	IgA	
03145	Acute phase	41	1	643	33	160
	Convalescent phase	240	2	1,297	258	2,560
15374	Acute phase	4	4	654	31	80
	Convalescent phase	30	4	3,982	398	640

[a] Values are reciprocals of end dilutions.

strains are not thought to produce PT. Therefore, we investigated whether these strains were capable of producing PT. We were unable to detect the production of PT by these strains either using immunoblot analysis or analyzing the ability of culture supernatants to cluster CHO cells, a sensitive assay for PT.

Since we were unable to detect the production of PT, we next examined whether these strains contain the genes encoding this toxin and, if so, whether the genes would encode an active toxin. While many *B. bronchiseptica* strains contain the genes for PT, some strains do not (26). PCR analysis had suggested that both strains contained at least a portion of the *ptx* region since we were able to amplify a region corresponding to nucleotides 749 to 1053 of the *ptx* locus in the strains. We examined the *B. bronchiseptica* strain from infant 03145 in some detail to determine whether this strain contained the entire *ptx* region and, if so, whether the genes would encode a functional toxin. In order to do this, we replaced the *ptx* promoter region and a portion of the S1 subunit of this strain with the corresponding region from *B. pertussis* using homologous recombination, as described previously (11). Since the *ptx* promoter from *B. pertussis* is active under normal laboratory conditions, replacement of the promoter of the *B. bronchiseptica* strain from infant 03145 with the *B. pertussis* promoter should result in active transcription of the *ptx* locus of this strain. As shown in Fig. 3, the engineered strain which contained the active promoter did produce an S2 subunit and an S4 subunit which resembled the corresponding subunits from *B. pertussis*. Moreover, analysis of the culture supernatant from this engineered strain showed that it contained active PT, as determined by the CHO cell assay. Dilutions of the supernatant as high as 1:128 were capable of clustering CHO cells, demonstrating the presence of active PT. Thus, this strain contains all *ptx* genes needed for the production of an active toxin, but apparently lacks a promoter that is active under normal laboratory conditions.

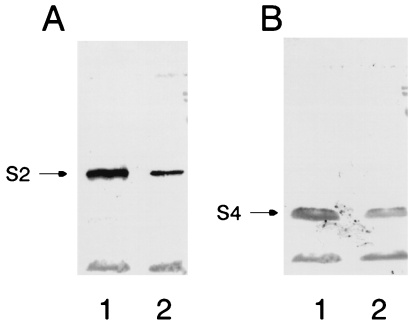

FIG. 3. Immunoblot analysis of PT subunits. Cell extracts of *B. bronchiseptica* 03145::pSZH8 (lane 1) and *B. pertussis* 536 (lane 2) were examined for the presence of the S2 subunit of PT (A) and the S4 subunit of PT (B) by using subunit-specific monoclonal antibodies and immunoblot analysis as described in Materials and Methods.

We examined the sequences of the *ptx* promoter regions of the strains from infants 03145 and 15374 in order to determine whether they differed from the published sequence of the *B. bronchiseptica ptx* promoter region. Both clinical isolates were found to have identical sequences (Fig. 4). The sequences, however, differed in certain respects from the published sequence of this region for *B. bronchiseptica* ATCC 4617 (1). At four positions, nucleotides 339, 434, 438, and 479, the sequence resembled that of *B. pertussis* rather than that of *B. bronchiseptica*. Interestingly, at four positions (nucleotides 349, 393, 427, and 506), the sequence resembled that of *B. parapertussis* rather than that of either *B. bronchiseptica* or *B. pertussis*. Finally, three new changes not seen in the other *Bordetella* spp. occurred at positions 340, 471, and 484.

DISCUSSION

In the present report, we provide information about two variant strains of *B. bronchiseptica* isolated from the NPAs of two infants during the follow-up of suspected cases of pertussis in the Italian clinical trial for the evaluation of new acellular pertussis vaccines (10). The isolation of *B. bronchiseptica* from the two infants was interesting since *B. bronchiseptica* is rarely isolated from immunocompetent humans. Both isolates showed many morphological and biochemical properties characteristic of *B. bronchiseptica* strains; however, they were not typical *B. bronchiseptica* strains since they both cross-agglutinated with *B. parapertussis* antiserum. Moreover, sequence analysis of the *ptx* promoter region of these strains indicated that, while their sequences shared homology with the *B. bronchiseptica* ATCC 4617 sequence, their sequences were not identical to that sequence and contained certain sequence motifs that are characteristic of *B. parapertussis* and certain motifs characteristic of *B. pertussis* rather than *B. bronchiseptica* ATCC 4617. These results would suggest that these strains are variants of *B. bronchiseptica* that share certain characteristics with other *Bordetella* spp. These findings are of interest since they suggest that a broader spectrum of bordetellae exists than was previously thought and that these "intermediate strains" may be able to cause significant illness in humans. Future studies of these strains will be needed to determine whether the host specificity of the two clinical isolates is typical of *B. bronchiseptica* or whether these strains may represent *B. bronchiseptica* strains that have adapted to a human host. Moreover, careful surveillance of strains isolated from patients affected by whooping cough-like disease should be continued to monitor the existence and emergence of variants of bordetellae that might be capable of causing disease in humans.

Although neither culture nor PCR with aspirates revealed the presence of *B. pertussis*, both infants exhibited a significant increase in PT neutralizing antibodies and IgG to PT between the acute- and convalescent-phase sera. While neither *B. bronchiseptica* variant was capable of producing PT under the normal laboratory conditions examined, both contained at least a portion of the *ptx* locus. Detailed analysis of the *ptx* region of the *B. bronchiseptica* strain from infant 03145 revealed that this strain contained all *ptx* genes essential for the production of an active toxin, although under normal laboratory conditions the promoter does not seem to be active.

Interestingly, both children from whom these strains were isolated had been vaccinated with acellular pertussis vaccines that each contained inactivated PT as one of their major com-

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PERTUSSIS    300   GCAACCGCCA   ACGCGCATGC   GTGCAGATTC   GTCGTACAAA   ACCCTCGATT
BB03145                         T            C  G   G    C            G         C
BPP9305                         T            C ^G   G    C    G             A  C
BB4617                          T            C  G   G    C    G

PERTUSSIS    350   CTTCCGTACA   TCCCGCTACT   GCAATCCAAC   ACGGCATGAA   CGCTCCTTCG
BB03145              C                                     GC           C
BPP9305              C                        T            GCA          C
BB4617               C                                     GC

PERTUSSIS    400   GCGCAAAGTC   GCGCGATGGT   ACCGGTCACC   GTCCGGACCG   TGCTGACCCC
BB03145              A            A                G                    C
BPP9305              A            A                G                    C
BB4617               A            A                         A   T        C

PERTUSSIS    450   CCTGCCATGG   TGTGATCCGT   AAAATAGGCA   CCATCAAAAC   GCAGAGGGGA
BB03145                           C          C            CGG
BPP9305                           C                  G     CAG
BB4617                            C                  G     C G

PERTUSSIS    500   AGACGGGATG
BB03145              A
BPP9305              A
BB4617
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FIG. 4. DNA sequence of the *ptx* promoter region of *Bordetella* strains. The DNA sequence is shown for the *B. bronchiseptica* strain from infant 03145 (BB03145) and *B. bronchiseptica* ATCC 4617 (BB4617), as well as for *B. parapertussis* ATCC 9305 (BPP9305). The sequencing of the *ptx* region from *B. bronchiseptica* ATCC 4617 and *B. parapertussis* ATCC 9305 were taken from Aricò and Rappuoli (1). The numbering system used is that originally described by Nicosia et al. (31). ^, *B. parapertussis* contains the insert CGGAT after nucleotide 322.

ponents. Thus, both children likely were immunologically primed with this antigen. Infection of the children with bacteria that transiently produce even small quantities of PT might be sufficient to boost their antibody responses during the course of their illness, although such quantities of PT might not be able to elicit a robust primary antibody response.

Recently, several strains of *B. parapertussis* were isolated from children who had a small but significant antibody rise to PT during the course of their illness (11). Like *B. bronchiseptica*, *B. parapertussis* is not thought to produce PT. These clinical isolates of *B. parapertussis* were shown to contain *ptx* genes that encode a functional toxin, although production of PT was not detected when these strains were examined in the laboratory. The present study reports very similar findings for two variant strains of *B. bronchiseptica*. Taken as a whole, these findings bring up the intriguing question of whether the *ptx* genes of *B. bronchiseptica* and *B. parapertussis* might be expressed under certain, as yet unknown, conditions.

Recently, others have reported the finding that certain bacterial genes are expressed only during infection of the host (3). Further studies are needed to determine whether the *ptx* genes of *B. bronchiseptica* and *B. parapertussis* are expressed transiently during the infection process.

ACKNOWLEDGMENTS

This work was partly supported by a contract (NO1.AI.25138) with the National Institute of Allergy and Infectious Diseases.

P.S. is indebted to Italian National Research Council for a short-stage fellowship connected to the Programme for Training and Mobility of Researchers.

REFERENCES

1. **Aricò, B., and R. Rappuoli.** 1987. *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. J. Bacteriol. **169:**2847–2853.
2. **Brennan, M. J., Z. M. Li, J. L. Cowell, M. E. Bisher, A. C. Steven, P. Novotny, and C. R. Manclark.** 1988. Identification of a 69-kilodalton nonfimbrial protein as an agglutinogen of *Bordetella pertussis*. Infect. Immun. **56:**3189–3195.
3. **Camilli, A., and J. J. Mekalanos.** 1995. Use of recombinase gene fusions to identify *Vibrio cholerae* genes induced during infection. Mol. Microbiol. **18:**671–683.
4. **Cotter, P. A., and J. F. Miller.** 1994. BvgAS-mediated signal transduction: analysis of phase-locked regulatory mutants of *Bordetella bronchiseptica* in a rabbit model. Infect. Immun. **62:**3381–3390.
5. **Delafuente, J., C. Albo, A. Rodriguez, B. Sapena, and C. Martinex.** 1994. *Bordetella bronchiseptica* pneumonia in a patient with AIDS. Thorax **49:**719–720.
6. **Frank, D. W., and C. D. Parker.** 1984. Interaction of monoclonal antibodies with pertussis toxin and its subunits. Infect. Immun. **46:**195–201.
7. **Gentry-Weeks, C. R., B. T. Cookson, W. E. Goldman, R. B. Rimler, S. B. Porter, and R. Curtiss.** 1988. Dermonecrotic toxin and tracheal cytotoxin, putative virulence factors of *Bordetella avium*. Infect. Immun. **56:**1698–1707.
8. **Gillenius, P., E. Jaatmaa, P. Askelof, M. Granstrom, and M. Tiru.** 1985. Standardization of an assay for pertussis toxin and antitoxin in microplate culture of Chinese hamster ovary cells. J. Biol. Stand. **13:**61–66.
9. **Goodnow, R. A.** 1980. Biology of *Bordetella bronchiseptica*. Microbiol. Rev. **44:**722–738.
10. **Greco, D., S. Salmaso, P. Mastrantonio, M. Giuliano, A. Tozzi, A. Anemona, M. Ciofi degli Atti, A. Giammanco, P. Panei, W. C. Blackwelder, D. L. Klein, and S. G. F. Wassilak.** 1996. A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. N. Engl. J. Med. **334:**341–348.
11. **Hausman, S. Z., J. D. Cherry, U. Heininger, C. H. Wirsing von Konig, and D. L. Burns.** 1996. Analysis of proteins encoded by the *ptx* and *ptl* genes of *Bordetella bronchiseptica* and *Bordetella parapertussis*. Infect. Immun. **64:**4020–4026.
12. **Heininger, U., K. Stehr, S. Schmitt-Grohe, C. Lorenz, R. Rest, P. D. Christensen, M. Uberall, and J. D. Cherry.** 1994. Clinical characteristics of illness caused by *Bordetella parapertussis* compared with illness caused by *Bordetella pertussis*. Pediatr. Infect. Dis. J. **13:**306–309.
13. **Hewlett, E. L., V. M. Gordon, J. D. McCaffery, W. M. Sutherland, and M. D. Gray.** 1989. Adenylate cyclase toxin from *Bordetella pertussis*. Identification and purification of the holotoxin molecule. J. Biol. Chem. **264:**19379–19384.
14. **Hewlett, E. L., K. T. Sauer, G. A. Myers, J. L. Cowell, and R. L. Guerrant.** 1983. Induction of a novel morphological response in Chinese hamster ovary cells by pertussis toxin. Infect. Immun. **40:**1198–1203.
15. **Idigbe, E. O., R. Parton, and A. C. Wardlaw.** 1981. Rapidity of antigenic modulation of *Bordetella pertussis* in modified Hornibrook medium. J. Med. Microbiol. **14:**409–418.
16. **Johnson, F. D., and D. L. Burns.** 1994. Detection and subcellular localization of three Ptl proteins involved in the secretion of pertussis toxin from *Bordetella pertussis*. J. Bacteriol. **176:**5350–5356.
17. **Jones, M.** 1950. Subacute bacterial endocarditis of non-streptococcal etiology: a review of the literature of thirteen year period 1936–1948 inclusive. Am. Heart J. **40:**106–116.
18. **Kenimer, J. G., K. J. Kim, P. G. Probst, C. R. Manclark, D. G. Burstyn, and J. L. Cowell.** 1989. Monoclonal antibodies to pertussis toxin: utilization as probes of toxin function. Hybridoma **8:**37–51.
19. **Khattak, M. N., and R. C. Matthews.** 1993. Genetic relatedness of *Bordetella* species as determined by macrorestriction digests resolved by pulsed-field gel electrophoresis. Int. J. Syst. Bacteriol. **43:**659–664.

20. **Lacey, B. W.** 1960. Antigenic modulation of *Bordetella pertussis*. J. Hyg. **58:**57–93.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Leininger, E., J. Kenimer, and M. J. Brennan.** 1993. Inhibition of *Bordetella pertussis* filamentous hemagglutinin mediated cell adherence with monoclonal antibodies. FEMS Microbiol. Lett. **106:**31–38.
23. **Locht, C., P. Bertin, F. D. Menozzi, and G. Renauld.** 1993. The filamentous haemagglutinin, a multifaceted adhesin produced by virulent *Bordetella* spp. Mol. Microbiol. **9:**653–660.
24. **Man, C. S.** 1950. Pertussis due to *Brucella bronchiseptica*. Pediatrics **6:**227–228.
25. **Manclark, C. R., B. M. Meade, and D. G. Burstyn.** 1986. Serological response to *Bordetella pertussis*, p. 388–396. *In* N. R. Rose, H. Friedman, and J. L. Fahey (ed.), Manual of clinical immunology, 3rd ed. American Society for Microbiology, Washington, D.C.
26. **Marchitto, K. S., J. J. Munoz, and J. M. Keith.** 1987. Nucleotide sequence homology to pertussis toxin gene in *Bordetella bronchiseptica* and *Bordetella parapertussis*. Infect. Immun. **55:**1309–1313.
27. **Mastrantonio, P., P. Stefanelli, and M. Giuliano.** 1996. Polymerase chain reaction for the detection of *Bordetella pertussis* in clinical nasopharyngeal aspirates. J. Med. Microbiol. **44:**261–266.
28. **Meade, B. M., A. Deforest, K. M. Edwards, T. A. Romani, F. Lynn, C. H. O'Brien, C. B. Swartz, G. F. Reed, and M. A. Deloria.** 1995. Description and evaluation of serologic assays used in a multicenter trial of acellular pertussis vaccines. Pediatrics **96:**570–575.
29. **Meis, J. F., A. J. A. van Griethuijsen, and H. L. Muytijens.** 1990. *Bordetella bronchiseptica* in an immunosuppressed patient. Eur. J. Clin. Microbiol. Infect. Dis. **9:**366–367.
30. **Mensnard, R., N. Guiso, C. Michelet, J. M. Sire, P. Paredas, P. Y. Donnio, and J. L. Avreil.** 1993. Isolation of *Bordetella bronchiseptica* from a patient with AIDS. Eur. J. Clin. Microbiol. Infect. Dis. **12:**304–306.
31. **Nicosia, A., M. Perugini, C. Franzini, M. C. Casagli, M. G. Borri, G. Antoni, M. Almoni, P. Neri, G. Ratti, and R. Rappuoli.** 1986. Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. Proc. Natl. Acad. Sci. USA **83:**4631–4635.
32. **Stefanelli, P., M. Giuliano, M. Bottone, P. Spigaglia, and P. Mastrantonio.** 1996. Polymerase chain reaction for the identification of *Bordetella pertussis* and *Bordetella parapertussis*. Diagn. Microbiol. Infect. Dis. **24:**197–200.
33. **Van der Zee, A., C. Agterberg, M. van Agterveld, M. Peeters, and F. R. Mooi.** 1993. Characterization of IS*1001*, an insertion sequence element of *Bordetella parapertussis*. J. Bacteriol. **175:**141–147.
34. **Weiss, A. A., A. R. Melton, K. E. Walker, C. Andraos-Selim, and J. J. Meidl.** 1989. Use of the promoter fusion transposon Tn*5 lac* to identify mutations in *Bordetella pertussis vir*-regulated genes. Infect. Immun. **57:**2674–2682.
35. **Woolfrey, B. F., and J. A. Moody.** 1991. Human infections associated with *Bordetella bronchiseptica*. Clin. Microbiol. Rev. **4:**243–255.