INFECTION AND IMMUNITY, Mar. 1987, p. 497–501
0019-9567/87/030497-05$02.00/0
Copyright © 1987, American Society for Microbiology
Vol. 55, No. 3

Nucleotide Sequence Homology to Pertussis Toxin Gene in *Bordetella bronchiseptica* and *Bordetella parapertussis*

KEVIN S. MARCHITTO, SUSAN G. SMITH, CAMILLE LOCHT, AND JERRY M. KEITH*

Section of Pathobiology, Laboratory of Molecular Pathobiology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

Received 14 August 1986/Accepted 17 November 1986

Multiple strains of *Bordetella parapertussis* and *B. bronchiseptica* were examined for the presence of nucleotide sequences which hybridized with a cloned 4.5-kilobase (kb) fragment of *B. pertussis* DNA containing the genes responsible for pertussis toxin expression. All six *B. parapertussis* strains tested had nucleic acid sequences that hybridized with the cloned 4.5-kb fragment in Southern blot analyses. When the *B. parapertussis* DNA was digested with restriction endonuclease *Pst*I, the pattern of hybridization was identical to that obtained with *B. pertussis*. Only five of the seven *B. bronchiseptica* strains tested had sequences that hybridized with the 4.5-kb fragment. Three of these *B. bronchiseptica* strains had a hybridization pattern identical to *B. pertussis* upon *Pst*I digestion and Southern blot analyses. Two *B. bronchiseptica* strains were shown to lack a *Pst*I cleavage site downstream from the region analogous to that coding for the pertussis toxin structural genes. Monoclonal antibody analyses were unable to detect pertussis toxin subunits S1 and S2 in Western blots with cellular material or culture supernatant from several *B. bronchiseptica* and *B. parapertussis* strains that possessed the DNA homologies. In addition, preliminary Northern hybridizations with RNA isolated from *B. bronchiseptica* and *B. parapertussis* strains suggested that the homologous regions were not transcribed. The data show that the gene coding for the toxic component of *B. pertussis* is common in other *Bordetella* species, though the gene probably is not expressed.

Species in the genus *Bordetella* are closely related phenotypically, possess common antigens (23), and share a high degree of DNA homology (9, 10). Virulence determinants of *Bordetella pertussis* common to *B. bronchiseptica* and *B. parapertussis* include an extracellular adenylate cyclase (4, 21), dermonecrotic toxin (12, 23), a heat-labile toxin (22, 23), and hemolysins (5, 23). Despite such similarities, significant differences do exist. Of particular importance is the fact that the host range of these organisms varies widely; *B. bronchiseptica* can infect humans, dogs, swine, and other animals (6), whereas *B. pertussis* and *B. parapertussis* are recognized exclusively as human pathogens (7, 16). *B. pertussis* is considered the causative agent of whooping cough, yet *B. bronchiseptica* and *B. parapertussis* sometimes appear as clinical isolates in mild forms of the disease (3, 6, 7, 16).

Activity of pertussis toxin (pertussigen, lymphocytosis-promoting factor), a major virulence determinant of *B. pertussis*, is conspicuously absent in *B. bronchiseptica* and *B. parapertussis* (6, 24). The toxin has a variety of biological effects, including histamine sensitization, lymphocytosis-promoting activity, and an ADP-ribosylation activity, to name a few (for a review, see reference 26). The role of the toxin in the pathogenesis process is not completely understood and has recently attracted a great deal of interest.

Recently, investigators in this and another laboratory were successful in cloning the entire pertussis toxin gene (14, 20). The complete nucleotide sequence was determined, and the amino acid sequences of each of the individual subunits were deduced (15). All five subunits are coded by closely linked cistrons in a region of the genome 3.1 kilobases (kb) long.

* Corresponding author.

The cloning of the *B. pertussis* toxin gene presented a unique opportunity to look for specific homologies in the genomes of *B. bronchiseptica* and *B. parapertussis*. When pertussis toxin gene fragments were hybridized with *B. bronchiseptica* and *B. parapertussis* DNA, a high degree of homology was detected. This suggests that although these species are considered to be deficient in toxin activity, they apparently harbored the genes encoding the toxin. A survey of *B. bronchiseptica* and *B. parapertussis* strains was therefore undertaken to ascertain the occurrence of the gene and the extent of homology in a number of strains.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used in this study are listed in Table 1. All strains were provided by Leonard Mayer (Centers for Disease Control, Atlanta, Ga.), except for *B. bronchiseptica* 7668 and 899L and *B. pertussis* 3779, which were provided by John Munoz (Rocky Mountain Laboratories). Bacteria were inoculated from frozen stocks onto Bordet-Gengou agar supplemented with 15% defibrinolated horse blood and cultivated at 37°C. For isolation of DNA, strains were inoculated from Bordet-Gengou plates to Stainer-Scholte liquid cultures and incubated at 37°C with agitation at 250 rpm.

Enzymes and DNA fragment isolation. Enzymes used in this study were obtained from Bethesda Research Laboratories, and digests were performed according to the instructions of the suppliers. A 4.5-kb cloned fragment of *B. pertussis* DNA containing the toxin gene was excised from plasmid pPTX42 (14, 15) by digestion with *Eco*RI and *Bam*HI and isolated by 0.8% low-melting-point agarose gel electrophoresis followed by purification on Elutip-d columns (Schleicher & Schuell, Inc.). Four fragments generated by *Pst*I digestion of the 4.5-kb insert were isolated by the same

TABLE 1. Sources of strains used in this study

Species	Strain	Origin	Source or reference[a]
B. bronchiseptica	K1011	Unknown	ATCC (9)
	K1020	Guinea pig	ATCC (9)
	4617	Unknown	ATCC (9)
	5376	Unknown	2
	19395	Dog	ATCC (9)
	7668	Unknown	J. Munoz
	899L	Unknown	J. Munoz
B. parapertussis	K1010	Human	ATCC
	5952	Human	NCTC (9)
	15237	Human	9
	15311	Human	ATCC
	17879	Unknown	L. Mayer
B. pertussis	9797	Human	ATCC
	3779	Vaccine strain	Eli Lilly & Co.

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md., NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London NW9 5HT, England.

procedure. Each DNA fragment was labeled by nick translation (Bethesda Research Laboratories) with [α-^{32}P]dCTP (800 Ci/mmol; New England Nuclear Corp.).

Colony hybridizations. Genomic DNA was extracted according to the procedure of Hull et al. (8). Bordet-Gengou plates were overlaid with a colony/plaque screen hybridization transfer membrane (New England Nuclear), individual colonies were transferred to the membrane with a toothpick, and the plates incubated overnight at 37°C. Each disc was hybridized according to the instructions of the manufacturers except that herring sperm DNA (Sigma Chemical Co.) was substituted for salmon sperm DNA. Filters were probed with the nick-translated labeled 4.5-kb *Pst*I fragment (about 1 μCi/ml). The dried filters were exposed to Kodak X-ray film with a Lightning-Plus (Du Pont Cronex) intensifying screen.

Southern blot analyses. Approximately 540 ng of genomic DNA from each strain was digested with *Pst*I, loaded onto 1% (wt/vol) agarose gels, and electrophoresed at 26 V for 15 h. DNA from the gels was transferred to nitrocellulose filters and hybridized (14) to the nick-translated probes as follows. Filters were prehybridized at 68°C for 4 h in 6× sodium chloride-sodium phosphate-EDTA buffer (SSPE [17]) 0.5% (wt/vol) sodium dodecyl sulfate (SDS), 5× modified Denhardt (all concentrations given are vol/vol: 0.1% Ficoll 400, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, and 0.3× SSPE), and 100 μg of herring sperm DNA per ml. The hybridization buffer was the same as the prehybridization buffer, except EDTA was added to a final concentration of 10 mM. Nick-translated probes were hybridized at a concentration of about 1 μCi/ml for 48 h at 68°C. Filters were then washed in 2× SSPE and 0.5% (wt/vol) SDS at room temperature for 5 min and then in 2× SSPE and 0.1% (wt/vol) SDS at room temperature for 15 min, and finally in 0.1× SSPE and 0.5% (wt/vol) SDS at 68°C for 2 h. The washed filters were air dried and exposed to X-ray film with an intensifying screen.

Monoclonal antibodies and Western blots. Monoclonal antibodies were isolated as described elsewhere (K. S. Marchitto, J. J. Munoz, and J. M. Keith, manuscript submitted). Western blots were performed by a modification of the methods of Towbin et al. (25) and Burnette (1) as described by Marchitto et al. (18).

RESULTS

A 4.5-kb fragment of *B. pertussis* DNA, encoding the pertussis toxin gene sequence (Fig. 1) was labeled by nick translation and used to probe colonies of *B. bronchiseptica*, *B. parapertussis*, and *B. pertussis*. The probe hybridized to all *B. pertussis* and *B. parapertussis* strains, and five of the seven *B. bronchiseptica* strains (data not shown). The results suggest that strains of *B. bronchiseptica* and *B. parapertussis*, previously thought to be deficient in pertussis toxin, possess at least a portion of the pertussis toxin gene sequence.

To further assess this apparent homology, DNA from several strains of each of the three species was digested completely with *Pst*I, subjected to electrophoresis, blotted onto nitrocellulose, and probed with the same labeled 4.5-kb

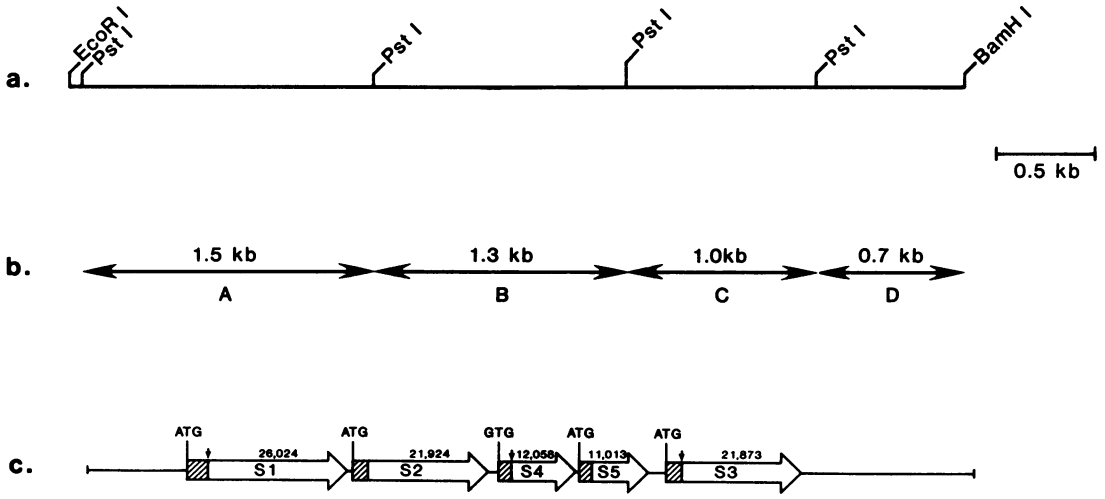

FIG. 1. Organization of the cloned 4.5-kb *Eco*RI-*Bam*HI *B. pertussis* DNA fragment containing the pertussis toxin structural genes. (a) Restriction map depicting sites of *Pst*I cleavage; (b) *Pst*I fragments derived from digestion of 4.5-kb DNA; (c) organizational map of the pertussis toxin gene. The open arrows show the translational direction and length of the protein-coding regions for the individual subunits. The hatched boxes indicate signal peptides. Molecular weights for the respective subunits are given above the arrows.

fragment that contains the pertussis toxin gene. *Pst*I digestion of *B. pertussis* DNA was shown to yield four fragments of sizes 1.5, 1.5, 1.3, and 1.0 kb (Fig. 1) which hybridize with the 4.5-kb insert (14). Four of the *Pst*I sites are shown in Fig. 1; a fifth site lies 0.8 kb downstream from the *Bam*HI site and generates the second 1.5-kb piece.

When the *B. parapertussis* DNA was digested with *Pst*I, the hybridization patterns of all five strains were identical to those of *B. pertussis* strains 3779 and 9797 (Fig. 2A).

Hybridization of the 4.5-kb fragment with *Pst*I-digested DNA of *B. bronchiseptica* yielded different patterns (Fig. 2B). Strains 19395, K1020, and 5376 produced a pattern similar to that of the *B. pertussis* and *B. parapertussis* strains. Strains K1011 and 4617 apparently lacked a restriction site, as suggested by the disappearance of the 1.0-kb band and the presence of a new 2.5-kb band. This is consistent with the absence of a *Pst*I site in the region analogous to that lying between the 1.0- and 0.7-kb fragments of the original cloned 4.5-kb insert (Fig. 1). Two of the *B. bronchiseptica* strains, 7668 (Fig. 2B) and 899L (not shown), had no detectable sequences homologous with the 4.5-kb piece of DNA.

The initially observed hybridization patterns were further assessed by first digesting the 4.5-kb fragment completely with *Pst*I to yield four pieces of sizes 1.5, 1.3, 1.0, and 0.7 kb (Fig. 1), and then by using the labeled fragments to probe Southern blots with *B. bronchiseptica* DNA from each of the strains above (Fig. 3). It should be noted that incomplete purification of the fragments may have resulted in the observation of background reactions in the blots. As expected, hybridization was not observed in any of the experiments with strains 899L and 7668 (Figs. 2 and 3).

The 1.5-kb *Pst*I fragment A and 1.3-kb fragment B hybridized with the corresponding *Pst*I fragments in five of the seven strains of *B. bronchiseptica* tested (Fig. 3A and 3B). The hybridization patterns of *B. pertussis* DNA are shown in the first lane of each panel for comparison.

The results with the *Pst*I 1.0-kb fragment C and 0.7-kb fragment D are shown in Fig. 3C and D. These fragments

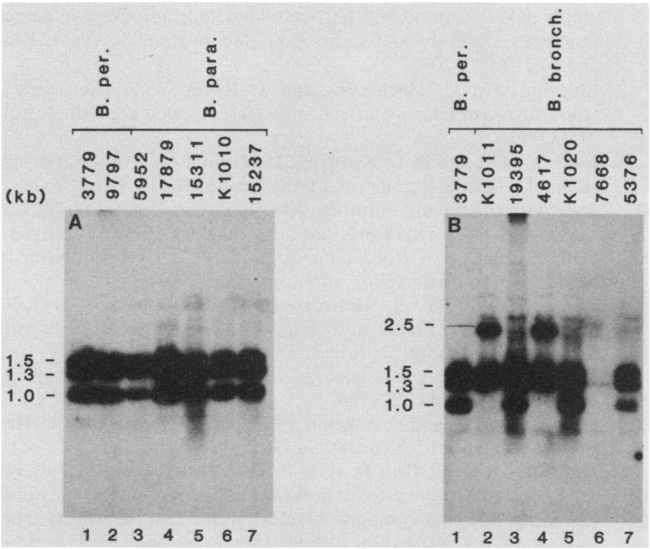

FIG. 2. Southern blot analyses of (A) *B. pertussis* and *B. parapertussis* and (B) *B. pertussis* and *B. bronchiseptica* genomic DNA with a cloned probe. DNA was digested with *Pst*I and analyzed by Southern blot with the nick-translated 4.5-kb cloned fragment containing the pertussis toxin structural genes.

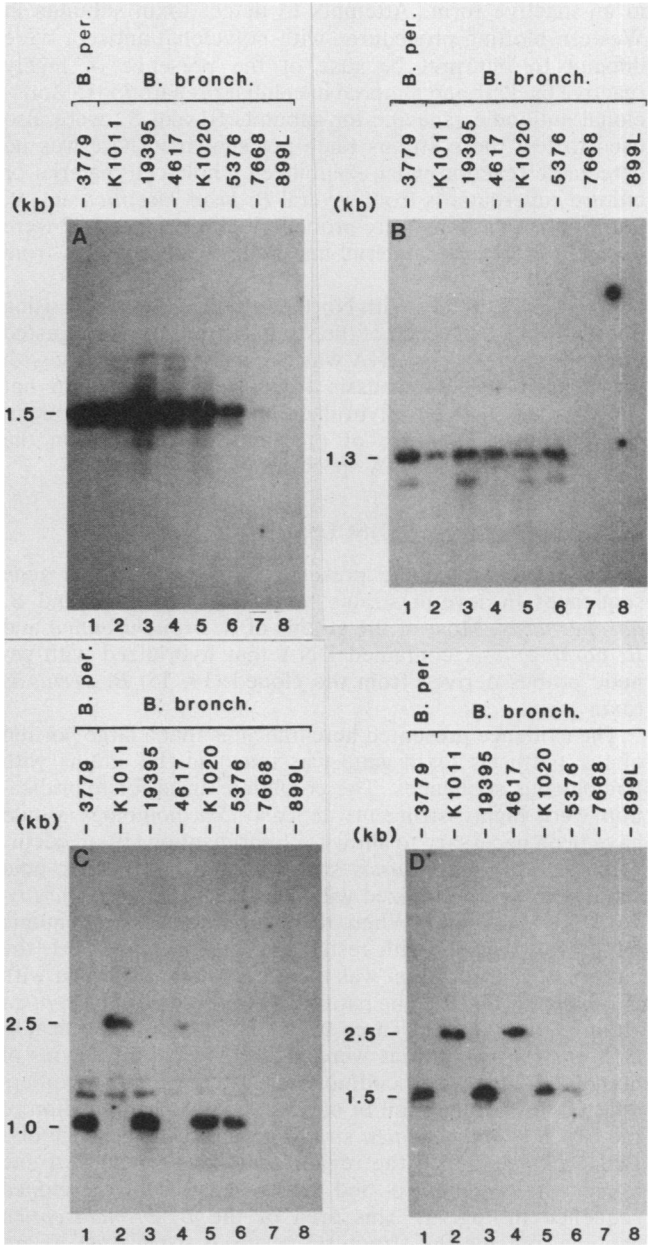

FIG. 3. Southern blot analyses of *B. pertussis* and *B. bronchiseptica* genomic DNA with fragments derived from a cloned probe. The 4.5-kb cloned *B. pertussis* DNA fragment was digested with *Pst*I to yield four pieces of (A) 1.5 kb, (B) 1.3 kb, (C) 1.0 kb, and (D) 0.7 kb length. Each fragment was nick translated and used as a probe to produce the blots in each panel.

each hybridized with the respective 1.0- and 1.5-kb pieces in *B. bronchiseptica* 19395, K1020, and 5376 and *B. pertussis* 3779. The two *Pst*I fragments hybridized with the 2.5-kb band in strains K1011 and 4617, although the bands were somewhat faint in some cases. This result confirmed that the *Pst*I site located between fragments C and D of the 4.5-kb pertussis toxin gene was absent in these strains.

The highly stringent reaction conditions used in these studies required that at least 75% homology must be present between the pertussis toxin genes and the *B. bronchiseptica* and *B. parapertussis* DNA sequences for hybridization to occur (19). It is possible that toxin subunits were made but

in an inactive form. Attempts to detect toxin subunits in Western blotting procedures with polyclonal antisera were difficult to interpret because of the presence of highly reactive background material in cellular preparations. Monoclonal antibodies specific for subunits S1 and S2 were also used to test these strains (data not shown). There was no detectable reaction when solubilized whole-cell material or culture supernatants from several *B. bronchiseptica* and *B. parapertussis* strains were probed. Subunits S1 and S2 were detected in cellular material and culture supernatants from *B. pertussis* 3779.

Preliminary studies with Northern hybridizations by using purified RNA from each of the strains in this study suggested that only *B. pertussis* RNA was capable of hybridizing with the labeled 4.5-kb pertussis toxin gene probe (data not shown). The Northern hybridization studies were inconclusive, however, because of problems encountered in the isolation of stable mRNA species.

DISCUSSION

We demonstrated the presence of pertussis toxin gene sequences in several strains of *B. bronchiseptica* and *B. parapertussis*. Most of the strains of *B. bronchiseptica* and *B. parapertussis* contained DNA that hybridized with genetic probes derived from the cloned (14, 15) *B. pertussis* toxin gene.

The evidence presented here indicates that a large portion of the pertussis toxin gene was intact in the strains with homologous sequences. The conditions used for hybridization were highly stringent; at least 75% homology would have been necessary to allow for hybridization (19) to occur.

All six *B. parapertussis* strains tested had nucleic acid sequences that hybridized with the cloned 4.5-kb *B. pertussis* DNA fragment. When the *B. parapertussis* genomic DNA was digested with restriction endonuclease *Pst*I, the pattern of hybridization was identical to that observed with *B. pertussis* (Fig. 2). The patterns with the *B. bronchiseptica* strains were variable. Three strains had patterns identical to *B. pertussis*, two strains were found that had no regions of homology under the conditions used here, and two others differed in the migration of one band. Close examination of the two *B. bronchiseptica* strains revealed the absence of a *Pst*I cleavage site in the region analogous to the *Pst*I site found between the 1.0- and 0.7-kb segment of the cloned sequence in Fig. 1. This area of the *B. bronchiseptica* genome, outside the structural pertussis toxin gene, is apparently variable among different strains. It is interesting that in most cases the region containing the toxin structural genes was conserved.

The existence of the conserved regions has interesting implications. The less virulent strains of *Bordetella* are generally considered to be deficient in toxin activity (6, 24). Several explanations are possible for the lack of detectable toxin in these strains. The toxin gene sequences in *B. parapertussis* or *B. bronchiseptica* may be altered or cryptic. Alternatively, the toxin may be synthesized but unable to escape the cell. A third possibility is that an incomplete or altered toxin may be synthesized and secreted but may be biologically inactive in standard tests of pertussis toxin activity.

We were unable to detect pertussis toxin subunits S1 and S2 in the cells and culture supernatants of *B. bronchiseptica* and *B. parapertussis* with monoclonal antibodies specific for these peptides. These subunits were, however, detected in *B. pertussis* 3779. This suggests that if the toxin is made in the less virulent *Bordetella* species, it must be altered in some way. Even a single amino acid change would be sufficient to alter a specific epitope recognized by a monoclonal antibody probe. These studies are being extended further to include polyclonal antisera and a larger panel of monoclonal antibodies. However, preliminary results with Northern hybridizations suggested that mRNA which hybridized with the toxin gene probe was present only in *B. pertussis* and not in the other species tested here. Such a result indicates that the toxin genes of *B. parapertussis* and *B. bronchiseptica* are not expressed.

It is possible these species arose from a single, common ancestor. Alternatively, the less virulent species of this family are the result of selective pressures on *B. pertussis*. Kumazawa and Yoshikawa (11) have suggested that *B. parapertussis* appears in a population through antibiotic selection after treatment of *B. pertussis* infections. It is possible that a pleiotropic mutation affecting characteristics indicative of each of these species may also have affected toxin expression. Lack of toxin expression would result in the absence of selective pressure to maintain the toxin gene and hence could have given rise to the variability in the nucleotide sequence. Changes in the reverse direction—that is, from *B. bronchiseptica* or *B. parapertussis* to *B. pertussis*—may also be a possibility. If this is the case, mild *B. parapertussis* infections in humans may represent a human reservoir for whooping cough. *B. bronchiseptica* infections in animals may represent a nonhuman reservoir, thus raising questions about the potential for complete eradication of the disease.

ACKNOWLEDGMENTS

We thank Betty Kester for her help in preparing the manuscript. We also thank Leonard Mayer and John J. Munoz for supplying bacterial strains and Charlotte Parker for supplying anti-S2 subunit monoclonal antibodies.

LITERATURE CITED

1. **Burnette, W. N.** 1980. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein A. Anal. Biochem. **112**:195–203.
2. **Eldering, G., C. Hornbeck, and J. Baker.** 1957. Serological study of *Bordetella pertussis* and related species. J. Bacteriol. **74**:133–136.
3. **Eldering, G., and P. L. Kendrick.**1952. Incidence of parapertussis in the Grand Rapids area as indicated by 16 years' experience with diagnostic cultures. Am. J. Public Health **42**:27–31.
4. **Endoh, M., T. Takezawa, and Y. Nakase.** 1980. Adenylate cyclase activity of *Bordetella* organisms. I. Its production in liquid medium. Microbiol. Immunol. **24**:95–104.
5. **Ezzel, J. W., W. J. Dobrogosz, W. E. Kloos, and C. R. Manclark.** 1981. Phase-shift markers in *Bordetella*: alterations in envelope proteins. J. Infect. Dis. **143**:562–569.
6. **Goodnow, R. A.** 1980. Biology of *Bordetella bronchiseptica*. Microbiol. Rev. **44**:722–738.
7. **Granström, M., and P. Askelof.** 1982. Parapertussis: an abortive pertussis infection? Lancet **2**:1249–1250.
8. **Hull, R. A., R. E. Gill, P. Hsu, B. H. Minshew, and S. Falkow.** 1981. Construction and expression of recombinant plasmids encoding type 1 or D-mannose-resistant pili from a urinary tract infection *Escherichia coli* isolate. Infect. Immun. **33**:933–938.
9. **Kloos, W. E., W. J. Dobrogosz, J. E. Ezzell, B. R. Kimbro, and C. R. Manclark.** 1978. DNA-DNA hybridization, plasmids, and genetic enchange in the genus *Bordetella*, p. 70–80. *In* C. R. Manclark and J. C. Hill (ed.), International Symposium on Pertussis. Department of Health, Education, and Welfare pub-

lication no. (NIH) 79-1830. U.S. Government Printing Office, Washington, D.C.

10. **Kloos, W. E., N. Mohapatra, W. J. Dobrogosz, J. W. Ezzell, and C. R. Manclark.** 1981. Deoxyribonucleotide sequence relationships among *Bordetella* species. Int. J. Syst. Bacteriol. **31:** 173–176.
11. **Kumazawa, N. H., and M. Yoshikawa.** 1978. Conversion of *Bordetella pertussis* to *Bordetella parapertussis*. J. Hyg. **81:** 15–23.
12. **Kume, K., T. Nakai, Y. Samejima, and C. Sugimoto.** 1986. Properties of dermonecrotic toxin prepared from sonic extracts of *Bordetella bronchiseptica*. Infect. Immun. **52:**370–377.
13. **Lax, A. J., and C. A. Walker.** 1986. Plasmids related to RSF 1010 from *Bordetella bronchiseptica*. Plasmid **15:**210–216.
14. **Locht, C., P. A. Barstad, J. E. Coligan, L. Mayer, J. J. Munoz, S. G. Smith, and J. M. Keith.** 1986. Molecular cloning of pertussis toxin genes. Nucleic Acids Res. **14:**3251–3261.
15. **Locht, C., and J. Keith.** 1986. Pertussis toxin gene: nucleotide sequence and genetic organization. Science **232:**1258–1264.
16. **Manclark, C. R., and J. L. Cowell.** 1984. Pertussis, p. 69–106. *In* R. Germanier (ed.), Bacterial vaccines. Academic Press, Inc., Orlando, Fla.
17. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
18. **Marchitto, K. S., S. A. Jones, R. F. Schell, P. L. Holmans, and M. V. Norgard.** 1984. Monoclonal antibody analysis of specific antigenic similarities among pathogenic *Treponema pallidum* subspecies. Infect. Immun. **45:**660–666.
19. **Mason, P. J., and J. G. Williams.** 1985. Hybridization in the analysis of recombinant DNA, p. 113–137. *In* B. D. Hames, and S. J. Higgins (ed.), Nucleic acid hybridisation: a practical approach. IRL Press, Oxford.
20. **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.
21. **Novotny, P., A. P. Chubb, K. Cownley, and J. A. Montaraz.** 1985. Adenylate cyclase activity of a 68,000-molecular-weight protein isolated from the outer membrane of *Bordetella bronchiseptica*. Infect. Immun. **50:**199–206.
22. **Oddy, J. G., and D. G. Evans.** 1940. The effects produced by toxic and nontoxic extracts of *H. pertussis* and *Br. bronchiseptica* on the blood sugar of rabbits. J. Pathol. Bacteriol. **50:**11–16.
23. **Pittman, M.** 1984. Genus *Bordetella*, p. 388–393. *In* N. R. Krieg and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
24. **Ross, R., J. Munoz, and C. Cameron.** 1969. Histamine-sensitizing factor, mouse-protective antigens, and other antigens of some members of the genus *Bordetella*. J. Bacteriol. **99:**57–64.
25. **Towbin, J., R. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
26. **Wardlaw, A. C., and R. Parton.** 1983. *Bordetella pertussis* toxins. Pharmacol. Ther. **19:**1–53.