Genetic Relatedness of *Bordetella* Species as Determined by Macrorestriction Digests Resolved by Pulsed-Field Gel Electrophoresis

M. N. KHATTAK AND R. C. MATTHEWS*

Pertussis Reference Laboratory and Department of Medical Microbiology, Manchester University Medical School, Stopford Building, Oxford Road, Manchester M13 9PT, United Kingdom

The genetic relatedness of the three medically important *Bordetella* species was examined by macrorestriction digestion of DNA with the rarely cutting enzyme *Xba*I and resolution by pulsed-field gel electrophoresis. Our data showed that *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* produced species-specific macrorestriction profiles and that there was some variation between different isolates of the same species. Conserved bands at 130 and 155 kb occurred with *B. pertussis* (130 isolates tested), but the nine variable bands between 200 and 412 kb distinguished 21 types. The 10 isolates of *B. parapertussis* tested produced up to 11 bands at 118 to 375 kb, 4 of which were variable, giving three types. The eight *B. bronchiseptica* isolates examined produced up to 16 bands at 118 to 394 kb, 11 of which were variable, giving three types. The results of this work are compared with the results of previous DNA-DNA hybridization and multilocus enzyme electrophoresis studies which suggested that these three species are closely related and should be considered members of the same species.

The following three medically important *Bordetella* species have been described: *Bordetella pertussis*, which causes whooping cough in humans; *Bordetella parapertussis*, the agent which can be responsible for a mild whooping cough-like disorder in humans; and *Bordetella bronchiseptica*, an animal respiratory pathogen which is very rarely isolated from humans. These are serologically related bacteria with similar morphologies, sizes, and staining reactions (3). They can be differentiated on the basis of their cultural characteristics, nutritional requirements, biochemical activities, and ranges of surface antigens (12).

For in vitro culture from nasopharyngeal secretions, *B. pertussis* is grown on a medium such as cephelexin charcoal blood agar, on which it produces small, shiny colonies. *B. pertussis* is a slowly growing, nonmotile organism that produces oxidase and catalase. The urease test is negative. *B. parapertussis* grows well on blood agar and nutrient agar and is nonmotile. Urease is produced after 24 h. Catalase is produced, while oxidase is not produced. *B. bronchiseptica* grows as glistening beta-hemolytic colonies on blood agar. This organism is motile, urease is produced after 4 h, and oxidase and catalase tests are positive. All three species ferment glucose and lactose with acid production (2).

DNA-DNA hybridization (7) and multilocus enzyme electrophoresis studies (10) have shown that these three species are closely related. It has been suggested that they should be considered members of a single species. Restriction fragment length polymorphism, generated by frequently cutting enzymes, has been described as a highly discriminatory system for determining phylogenetic relationships among isolates of the same species and related species (14). This technique failed to discriminate among isolates of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* when DNAs were cut with *Eco*RI; the three organisms were indistinguishable (data not shown).

Pulsed-field gel electrophoresis (PFGE) provides an alternative tool for analyzing relationships among strains and

species by facilitating the study of large fragments of DNA (1, 8). This technique was used to examine *B. parapertussis* and *B. bronchiseptica* and their relationship to *B. pertussis*. The chromosomal DNAs of *B. parapertussis* and *B. bronchiseptica* were digested with the rarely cutting restriction enzyme *Xba*I, and the DNA fragments were resolved by PFGE. The restriction fragment patterns of these organisms were compared with each other and with the patterns of *B. pertussis* isolates obtained in this and a previous study (6) in which an analysis of 105 isolates distinguished 17 DNA types identified by PFGE on the basis of 8 to 11 intense bands at 200 to 412 kb. A characteristic fingerprint for *B. pertussis* was defined on the basis of these predominant bands. We were able to differentiate the three *Bordetella* species, and each organism produced a species-specific fingerprint.

MATERIALS AND METHODS

Isolates. A total of 25 isolates of *B. pertussis* (Table 1), 10 isolates of *B. parapertussis*, and 8 isolates of *B. bronchiseptica* (Table 2) were examined. Isolates were obtained from the Manchester University Collection of Bacteria (MUCOB). Species were identified by Gram staining, cultural characteristics (*B. bronchiseptica* produces visible colonies after overnight incubation on nutrient agar, *B. parapertussis* produces a brown diffusible pigment after 2 days, and *B. pertussis* does not grow), and slide agglutination with rabbit polyvalent species-specific antiserum (11, 13). *B. bronchiseptica* was further characterized by its motility, its production of an obvious alkaline reaction in Hugh-Leifson medium, and its rapid hydrolysis of urea. *B. pertussis* vaccine strain 3700 was the control used for preparation of chromosomal DNA and molecular weight DNA markers previously sized by comparison with a bacteriophage lambda DNA ladder (Promega) (6).

Growth of bacteria. Freeze-dried cultures of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* were suspended in 100- μ l portions of sterile nutrient broth; these preparations were plated on charcoal blood agar, nutrient agar, and blood agar plates, respectively. The plates were incubated for 48 h

* Corresponding author.

TABLE 1. *B. pertussis* strains used in this study

Isolate	Serotype(s)	Year isolated
11/48	1, 2	1948
12/48	1, 2, 3	1948
13/48	1, 3	1948
2893	1, 2	1955
2991	1, 2	1955
3771	1, 2, 3	1955
4/55	1, 2, 3	1955
66/4	1, 2, 3	1967
57/6	1, 2, 3	1967
66/7	1, 3	1967
57/3	1, 3	1967
66/7	1	1967
11/9	1, 2	1968
3/8	1, 2, 3	1968
11/10	1, 3	1968
S/2	1	1968
489/12	1, 2	1970
70/6	1, 2	1970
489/123	1, 2, 3	1970
70/5	1, 2, 3	1970
489/13	1, 3	1970
70/2	1, 3	1970
D25/14	1, 2	1980
D25/12	1, 2, 3	1980
D25/13	1	1980

at 37°C. *B. pertussis* isolates were identified by their colonial morphology on charcoal blood agar, Gram staining, and serotyping with monospecific agglutinating sera prepared at the Pertussis Reference Laboratory as previously described (11). *B. paraptussis* isolates were identified on the basis of production of brown pigmentation on nutrient agar plates, while *B. bronchiseptica* produced glistening beta-hemolytic colonies on blood agar plates.

A colony of each isolate was resuspended and grown for 48 h at 37°C in 25 ml of modified Stainer-Scholte broth (5) with vigorous shaking. This synthetic medium supplemented with heptakis (2,6-O-dimethyl) β -cyclodextrin (catalog no. H6379; Sigma) is a significant growth stimulant. The cells were harvested by centrifugation at 4,000 rpm for 10 min. The resulting pellets were resuspended in 10-ml portions of 50 mM EDTA (pH 8.0), the preparations were centrifuged at 4,000 rpm for 10 min, and the turbidity, measured at 600 nm, was adjusted to an optical density of 1.5 in 50 mM EDTA (pH 8.0).

Chromosomal DNA preparations. In order to prevent shearing of high-molecular-weight DNA, 1-ml portions of the cell suspensions described above were mixed with equal volumes of 2% low-temperature-gelling molten agarose. Each cell suspension-agarose mixture was dispensed into a 10-plug mold (Bio-Rad). The plugs were allowed to solidify at 4°C for 10 min. Each plug was divided into four blocks with a clean glass coverslip.

For each strain, eight blocks were incubated overnight with shaking at 37°C in a sterile screw-cap bottle containing 5 ml of EC lysis buffer (6 mM Tris-HCl [pH 7.5], 1 M sodium chloride, 100 mM EDTA [pH 8.0], 0.5% [wt/vol] Brij 58 [Sigma], 0.2% [wt/vol] sodium deoxycholate [BDH], 0.5% [wt/vol] *N*-lauryl sarcosine [Sigma], 1 mg of lysozyme (Sigma) per ml, 20 mg of RNase [Sigma] per ml) to disrupt the cell wall and membrane. Subsequently, lysis buffer was substituted for 3 ml of ESP solution (0.5 M EDTA [pH 9.0], 1% [wt/vol] *N*-lauryl sarcosine, 1 mg of proteinase K per ml) for 48 h at 50°C (15). The blocks were washed for at least 15 min three times in 3 ml of 50 mM EDTA (pH 8.0) and stored at 4°C until they were used.

Restriction digestion of chromosomal DNA. Prior to restriction digestion of the chromosomal DNA, dialysis was necessary to eliminate the cell debris and proteinase K activity, as well as EDTA and detergents. The presence of trace amounts of these substances interferes with restriction endonuclease digestion (16). The DNA blocks were first treated with 5 ml of TE buffer (10 mM Tris-HCl [pH 7.5], 1 mM EDTA [pH 8.0]) containing 1 mM phenylmethylsulfonyl fluoride for 2 h at room temperature to completely inactivate

TABLE 2. *B. paraptussis* and *B. bronchiseptica* isolates used in this study

Species	Isolate	Geographic location	Year isolated	Serotype
<i>B. paraptussis</i>	9718	Manchester, United Kingdom	1980	
	6890	Manchester, United Kingdom	1990	
	MUCOB 76	Manchester, United Kingdom	1948	
	PP10	Manchester, United Kingdom	1955	
	M/S180	Manchester, United Kingdom	1967	
	M93	Manchester, United Kingdom	1969	
	MUCOB 70	United States	— ^a	
	BR18	Australia	—	
	7283DK	Copenhagen, Denmark	—	
<i>B. bronchiseptica</i>	PP145	Czechoslovakia	—	
	MUCOB 9	Copenhagen, Denmark		c
	MUCOB 241	—		d
	MUCOB 4	Manchester, United Kingdom	1948	c
	BB3	Manchester, United Kingdom	1948	c
	MUCOB 74	—		d
	8	—		d
	73	—		d
	8760/52	—		—

^a —, unknown.

TABLE 3. Characterization of the 21 DNA types found in 130 isolates of *B. pertussis*

DNA type	Sizes of DNA fragments ^a											No. of isolates
	412 kb	340 kb	315 kb	280 kb	271 kb	256 kb	240 kb	224 kb	200 kb	155 kb	130 kb	
1	+	—	+	+	+	+	—	+	+	+	+	40
2	+	—	+	+	—	+	—	— ^b	+	+	+	12
3	—	—	+	+	+	+	+	+	DB	+	+	20
4	— ^c	—	+	+	+	—	+	+	DB	+	+	4
5	—	+	+	+	+	—	+	+	+	+	+	9
6	+	+	+	+	+	+	—	+	+	+	+	3
7	+	+	— ^d	+	— ^e	—	—	+	+	+	+	1
8	+	+	+	+	—	+	+	—	+	+	+	4
9	+	—	+	+	+	+	—	—	DB	+	+	8
10	+	+	+	+	+	+	+	+	+	+	+	9
11	— ^c	—	+	+	—	+	+	+	+	+	+	3
12	+	—	+	—	+	+	—	—	DB	+	+	4
13	+	+	+	+	—	+	—	+	+	+	+	2
14	+	—	— ^f	+	—	+	—	— ^b	+	+	+	1
15	— ^c	—	—	+	+	+	+	+	+	+	+	1
16	+	—	+	+	—	—	—	+	— ^g	+	+	1
17	+	+	— ^f	+	—	—	—	+	+	+	+	1
18	+	—	+	+	+	+	—	+	—	+	+	3
19	+	—	+	+	—	+	—	+	+	+	+	2
20	+	—	+	+	—	+	— ^h	+	+	+	+	1
21	+	—	+	+	—	—	—	+	+	+	+	1

^a +, band present; —, band absent; DB, double band.

^b Band occurs at 215 kb.

^c Band occurs at 375 kb.

^d Band occurs at 297 kb.

^e Band occurs at 275 kb.

^f An extra band is present at 297 kb.

^g An extra band is present at 178 kb.

^h Band occurs at 244 kb.

the proteinases present in the lysis solution (8). The agarose blocks were then washed three times (15 min each time) in TE buffer without phenylmethylsulfonyl fluoride. For each strain three blocks were transferred to a microcentrifuge tube and equilibrated in 300 µl of *Xba*I reaction buffer for 20 min on ice. Subsequently, 25 U of restriction enzyme *Xba*I (Northumbria Biologicals) was added, and digestion was carried out overnight at 37°C. The reaction was stopped by the addition of 0.5 ml of 50 mM EDTA (pH 8.0).

PFGE. PFGE was performed with a CHEF-DRII system (Bio-Rad). The gels (13 by 13 cm) were made up of 1% agarose in TBE buffer (10.3 g of Tris-HCl per liter, 5.5 g of boric acid per liter, 0.93 g of EDTA 0.93 per liter). The agarose blocks were loaded into wells along with *B. pertussis* vaccine strain 3700 DNA, which was used as molecular weight marker DNA. Electrophoresis was performed with a pulse time of 25 s for 40 h (16) and with a field strength of 4.5 V/cm. The gels were stained with 0.5 µg of ethidium bromide per ml in 300 ml of distilled water for 20 min; this was followed by destaining for 3 h in distilled water. The gels were photographed under a UV transilluminator.

RESULTS

Macrorestriction fingerprinting by PFGE combined clear and reproducible resolution of restriction fragments with demonstration of a high degree of polymorphism. When the DNAs of 25 isolates of *B. pertussis* were cut with *Xba*I, 8 to 11 large fragments in the size range from 130 to 412 kb and multiple fragments smaller than 110 kb were generated. Fragment bands at 130 and 155 kb were conserved. Differentiation was based on variable bands at 200, 224, 240, 256,

271, 280, 315, 340, and 412 kb. DNA type 1 was the most common DNA type, as found in the previous study (6); 17 of the 25 isolates had this DNA type. DNA type 2 was found in only one isolate. Four new DNA types were observed. These were designated DNA types 18, 19, 20, and 21 (Table 3). Three isolates had DNA type 18, while two isolates had DNA type 19. DNA types 20 and 21 were each found in one isolate. The results for these 25 isolates are compared with the results obtained previously (6) in Table 3, and the profiles of DNA types 1 through 5 and 9 are illustrated in Fig. 1.

Restriction digestion of the DNAs of the *B. paraptussis*

FIG. 1. *B. pertussis* DNA types 1 through 5 and 9. Lanes 1 and 7, DNA type 1; lane 2, DNA type 2; lanes 3 and 8, DNA type 3; lanes 4 and 9, DNA type 4; lane 5, DNA type 5; lane 6, DNA type 9. Fragment sizes are indicated on the left.

FIG. 2. *B. parapertussis* DNA types 1 through 3. Lane 1, molecular weight marker DNA; lanes 4, 5, and 7 through 9, DNA type 1; lanes 3 and 6, DNA type 2; lane 2, DNA type 3. The sizes of fragments are indicated on the right.

FIG. 3. *B. bronchiseptica* DNA types 1 through 3. Lane 1, molecular weight marker DNA; lanes 3 through 8, DNA type 1; lane 2, DNA type 2; lane 9, DNA type 3. The sizes of fragments are indicated on the right.

isolates with *Xba*I generated 9 to 11 large bands in the size range from 118 to 375 kb. A cluster of smaller bands corresponding to sizes less than 97 kb long was also observed; these bands separated suboptimally under the running conditions selected to resolve the larger bands. Differentiation was based on variation in the four bands at 180, 200, 280, and 297 kb. For the 10 isolates examined, three different DNA types were defined on the basis of these variable bands (Fig. 2). DNA type 1 predominated; this DNA type was found in 7 of the 10 isolates. There were two DNA type 2 isolates and one DNA type 3 isolate (Table 4). There was no apparent correlation between phenotypic traits and genetic type. Bands at 118, 155, 166, 235, 261, 268, and 375 kb were conserved.

When the genomes of *B. bronchiseptica* isolates were digested with *Xba*I under identical conditions, up to 16 large bands in the 118- to 394-kb range were produced along with multiple small bands corresponding to sizes less than 97 kb long. The DNA typing system was based on variation in the large bands at 118, 166, 200, 230, 240, 250, 256, 261, 268, 297, and 340 kb (Fig. 3). Five of the eight isolates of *B. bronchiseptica* examined had DNA type 1, two isolates had DNA type 2, and DNA type 3 was found in only one isolate (Table 5). There was no correlation between phenotypic and genotypic differences. Bands at 130, 143, 180, 235, and 394 kb were conserved.

All of the isolates were typeable by this technique. The reproducibility of the technique was established by repeat testing, which produced identical fingerprint patterns under similar running conditions. Five isolates of each species were tested at least three times, and the repeat fingerprint patterns were unchanged for each of these isolates.

DISCUSSION

The heterogeneity of the three species of the genus *Bordetella* identified by phenotypic characteristics was demonstrated by the generation of species-specific macrorestriction profiles with the rarely cutting enzyme *Xba*I. The 25 isolates of *B. pertussis* examined in this study produced six different DNA profiles. These isolates were collected between 1940 and 1980 (Table 1). DNA type 1 predominated in this study, accounting for 17 isolates. Among the seven isolates collected before the introduction of pertussis vaccines (Table 1, up to 1955), five were DNA type 1 isolates. Among the remaining 18 isolates, which were collected after the introduction of mass vaccination, 12 were DNA type 1 isolates. Only one isolate was a DNA type 2 isolate. Four additional DNA types were observed which were different from the 17 DNA types described previously on the basis of an analysis of 105 isolates of *B. pertussis* (6). These new types were designated DNA types 18, 19, 20, and 21 (Table 3). The profiles of DNA types 1 to 5 and 9 are shown in Fig. 1. No correlation was found between the serotypes determined by agglutination and the DNA fingerprints determined by PFGE.

All 10 isolates of *B. parapertussis* produced similar DNA fingerprint patterns; 9 to 11 large bands were produced in the size range from 118 to 375 kb, and 7 of these bands (118, 155, 166, 235, 261, 268, and 375 kb) were conserved. Differentiation within the species was based on four variable bands. The restriction digestion patterns of 7 of the 10 isolates were indistinguishable from each other and were designated *B. parapertussis* DNA type 1 (Fig. 2 and Table 4). Three of these isolates were collected in 1948, 1955, and 1967 from Manchester, United Kingdom. The other four DNA type 1

TABLE 4. Details of *B. parapertussis* DNA types recognized

DNA type	Sizes of DNA fragments ^a											No. of isolates
	375 kb	297 kb	280 kb	268 kb	261 kb	235 kb	200 kb	180 kb	166 kb	155 kb	118 kb	
1	+	+	—	+	+	+	+	+	+	+	+	7
2	+	—	+	+	+	+	+	+	+	+	+	2
3	+	—	—	+	+	+	DB	—	+	+	+	1

^a +, band present; —, band absent; DB, double band.

TABLE 5. Details of *B. bronchiseptica* DNA types recognized

DNA type	Sizes of DNA fragments ^a																No. of isolates
	394 kb	340 kb	297 kb	268 kb	261 kb	256 kb	250 kb	240 kb	235 kb	230 kb	200 kb	180 kb	166 kb	143 kb	130 kb	118 kb	
1	+	—	—	+	+	+	+	+	+	+	—	+	+	+	+	+	5
2	+	+	+	—	—	+	+	—	+	+	DB	+	—	+	+	—	2
3	+	—	+	+	+	—	—	—	+	—	+	+	+	+	+	+	1

^a +, band present; —, band absent; DB, double band.

isolates were from different continents (Table 2). Similarly, the eight isolates of *B. bronchiseptica* produced similar restriction fragment patterns (Fig. 3 and Table 5). Unlike *B. parapertussis*, this species had five conserved bands (at 130, 143, 180, 235 and 394 kb), and differentiation was based on 11 variable bands (118, 166, 200, 230, 240, 250, 256, 261, 268, 297, and 340 kb). The restriction fragment patterns of these two species were different from each other even though there was sometimes considerable variation within a species. For example, the *B. bronchiseptica* DNA type 2 pattern (Fig. 3, lane 2) shared just 9 of 16 bands with the *B. bronchiseptica* DNA type 1 or 3 pattern but had only four bands in common with the *B. parapertussis* pattern. Bands at 394, 340, 256, 250, 230, 143, and 130 kb were found in *B. bronchiseptica* DNA type 2 isolates but not in isolates of *B. parapertussis*.

The restriction fragment patterns of these two species were compared with the restriction fragment patterns of 130 isolates of *B. pertussis* obtained in this study and a previous study (6). *B. pertussis* had two conserved bands (at 130 and 155 kb), and differentiation was based on variation in 8 to 11 intense bands between 200 and 412 kb. There was striking interspecies diversity; none of the 130 isolates of *B. pertussis* showed similarity to either of the other two *Bordetella* species (Fig. 1 through 3). In contrast, PFGE revealed considerable genetic relatedness among strains of the same species.

Multilocus enzyme electrophoresis revealed only a limited degree of genetic diversity among 60 strains of the three *Bordetella* species. It was concluded that these organisms were related too closely to warrant recognition as three separate species (10). However, multilocus enzyme electrophoresis is a measure of phenotypic expression and measures differences in only a few enzymes (4). Arbeit et al. (1) demonstrated that PFGE could differentiate epidemiologically independent but evolutionarily related isolates of *Escherichia coli* that were indistinguishable by multilocus enzyme electrophoresis.

Our data confirm that macrorestriction fingerprinting by PFGE of three *Bordetella* species can identify considerable polymorphism among the species which was missed by multilocus enzyme electrophoresis. Similarly, the results of DNA-DNA hybridization studies suggested that these three species are closely related (7). However, DNA samples indistinguishable on the basis of homology data are not necessarily identical, and DNA samples differing by less than 5% in homology values cannot be accurately resolved (9).

In conclusion, in this study we found that *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* had species-specific macrorestriction fingerprints when the organisms were examined by PFGE under the same conditions. Isolates of the same species exhibited various degrees of similarity to each other but were easily distinguishable from the other two

species. This heterogeneity in the restriction profiles of the three species, together with the phenotypic differences of the species, argues against the proposal of Kloos et al. (7) and Musser et al. (10) that these organisms should be regarded as members of the same species. Macrorestriction genomic fingerprinting by PFGE reflects the overall organization of the bacterial genome and may for some species be a better indicator of clonal origin and genetic relatedness than data from other types of nucleic acid homology studies (4). It remains to be determined whether further macrorestriction fingerprints generated by other restriction enzymes will generate some fingerprints common to two or more species of the genus *Bordetella*. Ultimately, genomic maps, such as the one produced for *B. pertussis* by Stibitz and Garlettts (17), will help establish the genetic relationships of these three species.

REFERENCES

1. Arbeit, R. D., M. Arthur, R. Dunn, C. Kim, R. K. Selander, and R. Goldstein. 1990. Resolution of recent evolutionary divergence among *Escherichia coli* from related lineages: the application of pulsed-field electrophoresis to molecular epidemiology. *J. Infect. Dis.* **161**:230–235.
2. Cheesbrough, M. 1984. Genus *Bordetella*, p. 280–281. In M. Cheesbrough (ed.), *Medical laboratory manual for tropical countries—1983*. Cambridge University Press, Cambridge.
3. Finger, H. 1986. *Bordetella*, p. 540–546. In S. Baron (ed.), *Medical microbiology—1986*. Addison-Wesley Publishing Co., Reading, Mass.
4. Grothues, D., and B. Tummler. 1991. New approaches in genome analysis by pulsed-field gel electrophoresis: application to the analysis of *Pseudomonas* species. *Mol. Microbiol.* **5**:2763–2776.
5. Imiazumi, A., Y. Suzuki, S. Ono, H. Sato, and Y. Sato. 1983. Heptakis (2,6-O-dimethyl) β -cyclodextrin: a novel growth stimulant for *Bordetella pertussis* phase I. *J. Clin. Microbiol.* **17**:781–786.
6. Khattak, M. N., R. C. Matthews, and J. P. Burnie. 1992. Is *Bordetella pertussis* clonal? *Br. Med. J.* **304**:813–815.
7. 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.
8. McClelland, M., M. R. Jones, Y. Patel, and N. Nelson. 1987. Restriction endonuclease for pulsed field mapping of bacterial genomes. *Nucleic Acids Res.* **15**:5985–6005.
9. McFadden, J. J., and A. Knight. 1991. DNA probes for detection and identification of bacteria, p. 99–111. In J. M. Grange, A. Fox, and N. L. Morgan (ed.), *Genetic manipulation*. Blackwell Scientific Publications, Oxford.
10. Musser, J. M., E. L. Helwett, M. S. Peppler, and R. K. Selander. 1986. Genetic diversity and relationships in populations of *Bordetella* spp. *J. Bacteriol.* **166**:230–237.
11. Preston, N. W. 1970. Technical problems in the laboratory diagnosis and prevention of whooping cough. *Lab. Pract.* **19**:482–486.
12. Preston, N. W. 1988. Pertussis today, p. 1–18. In A. C. Wardlaw and R. Parton (ed.), *Pathogenesis and immunity in pertussis*.

- John Wiley and Sons, London.
13. **Preston, N. W.** 1992. *Bordetella*: whooping cough, p. 381–388. In D. Greenwood, R. C. B. Slack, and J. F. Peutherer (ed.), Medical microbiology. A guide to microbial infections: pathogenesis, immunity, laboratory diagnosis and control. Churchill Livingstone, Edinburgh.
 14. **Saunders, N. A.** 1991. Analysis of restriction fragment length polymorphisms in the study of bacteria, p. 227–244. In J. M. Grange, A. Fox, and N. L. Morgan (ed.), Genetic manipulation. Blackwell Scientific Publications, Oxford.
 15. **Smith, C. L., and C. R. Cantor.** 1987. Purification, specific fragmentation, and separation of large DNA molecules. *Methods Enzymol.* **155**:449–467.
 16. **Smith, C. L., S. R. Kalco, and C. R. Cantor.** 1988. Pulsed-field gel electrophoresis and the technology of large DNA molecules, p. 41–77. In R. E. Davis (ed.), *Genome analysis: a practical approach*, 1st ed. IRL Press, Oxford.
 17. **Stibitz, S., and T. L. Garlettts.** 1992. Derivation of a physical map of the chromosome of *Bordetella pertussis* Tohama I. *J. Bacteriol.* **174**:7770–7777.