Genetic Diversity and Relationships in Populations of *Bordetella* spp.

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Genetic diversity in 60 strains of three nominal *Bordetella* species recovered from humans and other mammalian hosts was assessed by analyzing electrophoretically demonstrable allelic variation at structural genes encoding 15 enzymes. Eleven of the loci were polymorphic, and 14 distinctive electrophoretic types, representing multilocus genotypes, were identified. The population structure of *Bordetella* spp. is clonal, and genetic diversity is relatively limited compared with most other pathogenic bacteria and is insufficient to justify recognition of three species. All isolates of *Bordetella parapertussis* were of one electrophoretic type, which was closely similar to 9 of the 10 electrophoretic types represented by isolates of *Bordetella bronchiseptica*. *Bordetella pertussis* strains 18-323, which is used in mouse potency tests of vaccines, is more similar genetically to isolates of *B. bronchiseptica* and *B. parapertussis* than to other isolates currently assigned to the species *B. pertussis*. Apart from strain 18-323, the isolates of *B. pertussis* represented only two closely related clones, and all isolates of *B. pertussis* from North America (except strain 18-323) were genotypically identical. Strain Dejong, which has been classified as *B. bronchiseptica*, was strongly differentiated from all of the other *Bordetella* isolates examined.

Bacteria belonging to the genus *Bordetella* are of primary importance in pediatric and veterinary medicine because of their ability to colonize and multiply on the ciliated epithelium of the respiratory tract, thereby causing bronchial and pulmonary pathology (11, 31). Infection by *Bordetella pertussis* is especially severe in human infants, while *Bordetella parapertussis* produces a milder, less frequently diagnosed syndrome (31). *Bordetella bronchiseptica* infects many lower mammalian species, in which it causes a variety of pertussis-like respiratory diseases, most notably kennel cough in dogs and atrophic rhinitis in piglets (11).

Despite the existence of an effective whole-cell vaccine, pertussis continues to be a health problem worldwide. It is a major cause of childhood morbidity and mortality in developing countries (39), primarily because immunization is limited by vaccine availability and delivery. Also, in developed countries, concerns over adverse reactions to the vaccine have led to reduced vaccine acceptance and a concomitant increase in the incidence of the disease (10, 18).

Although many aspects of the biology of *Bordetella* spp. have been studied (11, 23, 31), few genetically oriented studies have been undertaken, and virtually nothing has been reported concerning the nature and extent of genetic variation in natural populations. Several species are recognized on the basis of phenotypic characteristics, despite evidence that strains of *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* constitute a single DNA homology group (20). In addition, subspecific classification historically has been based on serologic (8, 29) and other phenotypic characters (3, 4, 17, 23, 32). Consequently, certain basic questions concerning the epidemiology and taxonomy of *Bordetella*, such as the genetic relationships of isolates obtained from different host species, have not been answered.

Recently, multilocus enzyme electrophoresis has been used to assess the genetic structures of natural populations of several bacterial species (25, 26, 28, 33, 35). In this study, we measured the genetic diversity and relationships within and among three nominal *Bordetella* species by assaying electrophoretic variation in 15 metabolic enzymes. The recovery of isolates having the same multilocus genotypes at many geographic localities and at different times indicates that the genetic structure of populations is clonal. Our analysis also demonstrated that there is relatively limited genetic variation in populations of *Bordetella* spp. and that the current assignment of strains to species does not accurately reflect the overall genetic relatedness of these organisms.

**MATERIALS AND METHODS**

**Bacterial strains.** A total of 60 isolates of *Bordetella* spp. was examined, including 23 strains of *B. pertussis*, 21 strains of *B. parapertussis*, and 16 strains of *B. bronchiseptica* (Table 1). Of the 23 *B. pertussis* strains, 21 were originally recovered from humans with clinical disease; strain 3779 was derived in a laboratory from clinical isolate BB-114, and strain 1NA1 was in turn derived from strain 3779. All of the isolates of *B. parapertussis* were originally recovered from humans with clinical disease. The species of origin of *B. bronchiseptica* isolates were as follows: seven strains from dogs, one strain from a cat, two strains from pigs, one strain from a rat, one strain from a rabbit, one strain from a human, and three strains from unknown hosts.

Approximately one-half of the strains were received and analyzed under code. Many of the strains have been described previously, and some have been passaged in vitro more than 100 times (3, 4, 15, 27).

**Growth of bacteria and electrophoresis of enzymes.** The isolates were grown on Bordet-Gengou medium (Remel, Lenexa, Kans.) in a humidified atmosphere containing 5% CO2 and were examined visually for purity. Each isolate was then grown in 400 ml of modified Stanier-Scholle medium.
TABLE 1. Characteristics of 60 strains of three nominal *Bordetella* species

| Species          | ET* | Isolate | Locality | Year | Source | Additional information |
|------------------|-----|---------|----------|------|--------|-------------------------|
| *B. pertussis*   | 4   | 18-323  | Michigan | 1940s| Mouse challenge strain |
|                  | 12  | UVa-1   | Virginia | 1970s| Clinical isolate       |
|                  |     | UVa-2   | Virginia | 1983 | Clinical isolate       |
|                  |     | UVa-3   | Virginia | 1983 | Clinical isolate       |
|                  |     | BB-114  | Indiana  | 1955 | Clinical isolate; vaccine strain |
|                  | 3779| Laboratory | 1950s | Derived from BB-114  |
|                  | 1NA1| Laboratory | 1980  | Phase III variant of 3779 |
|                  | UT 25-80 | Texas   | 1970s  | Avirulent; 80 passages |
|                  | 134 | New York | 1950s  | Pillemer strain        |
|                  | BP-90-80 | New York | 1980  | Clinical isolate       |
|                  | BP-554-80 | New York | 1980  | Clinical isolate       |
|                  | BP-625-81 | New York | 1981  | Clinical isolate       |
|                  | BP-582-82 | New York | 1982  | Clinical isolate       |
|                  | BP-584-83 | New York | 1983  | Clinical isolate       |
|                  | BP-578-84 | New York | 1984  | Clinical isolate       |
|                  | 12742| Mexico   | 1980   | Clinical isolate       |
|                  | M-3  | Mexico   | 1984   | Clinical isolate       |
|                  | M-DJ | Mexico   | 1984   | Clinical isolate       |
|                  | M-AC | Mexico   | 1990   | Clinical isolate       |
|                  | L51  | United Kingdom | 1950s | Avirulent strain; clinical isolate |
| *B. parapertussis* | 5   | 21353   | Michigan | 1948 | Clinical isolate |
|                  | 17903| Michigan | 1949   | Clinical isolate       |
|                  | 23054| Michigan | 1950   | Clinical isolate       |
|                  | 23141| Michigan | 1950   | Clinical isolate       |
|                  | 23144| Michigan | 1950   | Clinical isolate       |
|                  | 23148| Michigan | 1950   | Clinical isolate       |
|                  | 23346| Michigan | 1950   | Clinical isolate       |
|                  | 32343| Michigan | 1967   | Clinical isolate       |
|                  | 21619| Michigan | 1967   | Clinical isolate       |
|                  | 33030| Michigan | 1968   | Clinical isolate       |
|                  | BP-626-83 | New York | 1983  | Clinical isolate       |
|                  | BP-675-84 | New York | 1984  | Clinical isolate       |
|                  | CDC 5-3124-1 | Michigan | 1984  | Clinical isolate       |
|                  | Ont 2-2 | Ontario, Canada | 1970s | Clinical isolate |
|                  | Ont 3-2 | Ontario, Canada | 1970s | Clinical isolate |
|                  | PL-1   | Alberta, Canada | 1984 | Clinical isolate |
|                  | M-2    | Mexico    | 1980   | Clinical isolate       |
|                  | CN 8234 | Czechoslovakia | pre-1970 | Clinical isolate |
|                  | 501    | Czechoslovakia | 1979  | Clinical isolate       |
|                  | 497    | Czechoslovakia | 1979  | Clinical isolate       |
|                  | 77     | Japan     | 1974   | Clinical isolate       |
| *B. bronchiseptica* | 1   | 110H     | New York | 1970s| Host species: dog      |
|                  | 2     | CN 7531  | United Kingdom | 1977| Host species: pig      |
|                  | 3     | Rab-10   | Montana  | 1970s| Host species: rabbit   |
|                  | 501   | New York | 1970s  | Host species: dog      |
|                  | BTS   | Iowa     | 1970s  | Host species: pig      |
|                  | Columbus | Ohio   | 1970s  | Host species: cat      |
|                  | 17640 | New York | 1970s  | Host species: dog      |
|                  | 469   | Ft. Collins | Colorado | 1970s| Host species: human    |
|                  | 8     | Rat-1    | New York | 1970s| Host species: dog      |
|                  | 214   | Michigan | 1970s  | Host species: rat      |
|                  | 8    | UT dog   | Tennessee | 1970 | Host species: dog      |
|                  | 10   | New York | 1970s  | Host species: dog      |
|                  | 11   | 19141 SAC | New York | 1970s| Host species: dog      |
|                  | 14   | Dejong  | Denmark | 1970s| Host species: pig      |

* See Table 2.
TABLE 2. Allele profiles at 15 enzyme loci in 14 ETs of Bordetella spp.

| ET   | Reference isolate | No. of isolates | Alleles at the following enzyme loci* |
|------|-------------------|-----------------|--------------------------------------|
| 1    | 110H              | 2               | MDH 1 2 1 2 2 2 1 1 3 1 3 1 3 1 2 1 2 |
| 2    | 213               | 1               | FUM 1 2 1 2 1 2 2 1 1 4 1 3 3 1 3 1 2 |
| 3    | Rah-10            | 3               | IPO 1 2 1 2 2 2 2 1 1 5 1 3 1 1 1 2 |
| 4    | 18-323            | 1               | HBD 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 5    | M-2               | 21              | ALP 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 6    | Columbus          | 2               | EST 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 7    | 469               | 2               | PGI 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 8    | Rat-1             | 2               | IDH 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 9    | UT dog            | 1               | PGM 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 10   | 87                | 1               | ADK 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 11   | 19141 SAC         | 1               | LAP 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 12   | BB-114            | 19              | GLD 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 13   | Tohama I          | 3               | PE1 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 14   | Dejong           | 1               | CAT 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |
| 15   |                   | 1               | GOT 1 2 1 2 2 2 2 1 1 3 1 3 1 3 1 2 |

* MDH, Malate dehydrogenase; FUM, fumarase; IPO, indophenol oxidase; HBD, hydroxybutyrate dehydrogenase; ALP, alkaline phosphatase; EST, esterase; PGI, phosphoglucone isomerase; IDH, isocitrate dehydrogenase; PGM, phosphoglucomutase; ADK, adenylate kinase; LAP, leucine aminopeptidase; GLD, glutamate dehydrogenase; PE1, leucylalanine peptidase; CAT, catalase; GOT, glutamic oxaloacetic transaminase.

(16) at 37°C on an orbital shaker (275 rpm) and harvested by centrifugation at 6,000 × g for 10 min at 4°C. Following suspension in 3 ml of 50 mM Tris hydrochloride buffer containing 5 mM EDTA (pH 7.5), the bacteria were sonicated with a model 200 Sonifer Cell Disrupter (Branson Sonic Power Co., Danbury, Conn.) equipped with a microtip for 30 s at 50% pulse, with ice water cooling, and centrifuged at 20,000 × g for 20 min at 4°C. The clear supernatant (lysat) was stored at −70°C.

Phenotypic modulation of the cell envelope proteins of B. pertussis was performed with Hornbrook medium, as described elsewhere (37).

Lysates were electrophoresed on starch gels and selectively stained for 15 metabolic enzymes by methods described by Selander et al. (33). The enzymes studied were NAD-dependent malate dehydrogenase, fumarase, indophenol oxidase, hydroxybutyrate dehydrogenase, alkaline phosphatase, esterase, phosphoglucone isomerase, isocitrate dehydrogenase, phosphoglucomutase, adenylyl kinase, leucine aminopeptidase, glutamate dehydrogenase, leucylalanine peptidase, catalase, and glutamic oxaloacetic transaminase. NAD-dependent malate dehydrogenase, esterase, isocitrate dehydrogenase, and glutamic oxaloacetic transaminase were electrophoresed in Tris-citrate buffer (pH 8.0). A phosphate buffer system (pH 7.0 gel buffer and pH 6.7 tray buffer) was used for electrophoresis of phosphoglucose isomerase, adenylyl kinase, leucine aminopeptidase, and catalase; a Poulik buffer system (pH 8.7 gel buffer and pH 8.2 borate tray buffer) was used for fumarase, indophenol oxidase, and hydroxybutyrate dehydrogenase; Tris-maleate buffer (pH 7.4) was used for alkaline phosphatase, phosphoglucomutase, and leucylalanine peptidase; and Tris-EDTA-borate buffer (pH 8.0) was used for glutamate dehydrogenase.

Distinctive electromorphs (mobility variants) of each enzyme were numbered in order of decreasing rate of anodal migration and were equated with alleles at the corresponding structural gene locus (Table 2). Because all of the isolates showed activity for all 15 enzymes, we presume that the corresponding structural gene loci are located on the chromosome rather than on plasmids.

Each isolate was characterized by its combination of alleles at the 15 enzyme loci, and distinctive profiles of electromorphs, corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs) (Table 2) (33).

Statistical analysis. Genetic diversity at a locus (h) among ETs or isolates was calculated as follows: 

$$h = \frac{1 - \sum x_i^2}{n(n-1)}$$

where $x_i$ is the frequency of the $i$th allele and $n$ is the number of ETs or isolates (33). Mean genetic diversity per locus ($\bar{h}$) is the arithmetic average of $h$ values for all loci. G statistics (35) were used to apportion genetic diversity within and among species.

Genetic distance between pairs of ETs was calculated as the proportion of loci at which different alleles were represented (mismatches), and clustering of ETs was performed from a matrix of genetic distances by the average-linkage method (33).

RESULTS

Overall genetic diversity. In the collection of 60 isolates as a whole, 11 enzymes were polymorphic, and 4 enzymes were monomorphic. The mean number of alleles per locus was 2.4 (range, 1 to 5) (Table 3). A total of 14 distinctive ETs was identified (Table 2), most of which differed from one another at only one locus or a few loci. $\bar{h}$ for the ETs was 0.284 (interlocus variance $s^2 = 0.061$). There was less diversity among the 58 isolates recovered from patients ($\bar{h} = 0.186; s^2 = 0.060$), reflecting the fact that eight of the ETs were represented by two or more isolates.

Genetic diversity within species. In B. bronchiseptica, 11 enzymes were polymorphic, and 10 ETs were identified. $\bar{h}$ was 0.277 ($s^2 = 0.054$) among the 10 ETs, and $\bar{h}$ was 0.232 ($s^2 = 0.042$) among the 16 isolates. Strain Dejong (ET 14) was very different from all of the other Bordetella isolates examined, possessing seven unique alleles. All of the isolates of B. parapertussis were of ET 5, which had alleles at the phosphoglucomutase, leucylalanine peptidase, and glutamic oxaloacetic transaminase loci that were not present in B. pertussis isolates.

Three ETs were identified among the 23 strains of B. pertussis examined; 12 loci were monomorphic, 3 loci were polymorphic, and $\bar{h}$ among the ETs was 0.133 ($s^2 = 0.076$). There was much less genetic diversity among the 21 isolates recovered from patients ($\bar{h} = 0.034; s^2 = 0.008$). Sixteen of 17 isolates recovered from patients in North America and one isolate from the United Kingdom were genotypically identical, representing ET 12. Also, all three Japanese iso-
TABLE 3. Genetic diversity at 15 enzyme loci in 14 ETs of three nominal *Bordetella* species

| Enzyme locus | No. of alleles | Variance component |
|--------------|----------------|--------------------|
|              | Within species | Total              | $G_{ST}^a$ |
| Malate dehydrogenase | 1 | 0.000 | 0.000 | 0.000 |
| Fumarase | 2 | 0.333 | 0.363 | 0.081 |
| Indophenol oxidase | 1 | 0.000 | 0.000 | 0.000 |
| Hydroxybutyrate dehydrogenase | 2 | 0.143 | 0.143 | 0.000 |
| Alkaline phosphatase | 3 | 0.429 | 0.473 | 0.093 |
| Esterase | 3 | 0.270 | 0.275 | 0.018 |
| Phosphoglucose isomerase | 2 | 0.143 | 0.143 | 0.000 |
| Isocitrate dehydrogenase | 1 | 0.000 | 0.000 | 0.000 |
| Phosphoglucomutase | 5 | 0.381 | 0.725 | 0.475 |
| Adenylate kinase | 3 | 0.286 | 0.385 | 0.257 |
| Leucine aminopeptidase | 3 | 0.524 | 0.615 | 0.149 |
| Glutamate dehydrogenase | 2 | 0.143 | 0.143 | 0.000 |
| Leucylalanine peptidase | 3 | 0.460 | 0.604 | 0.238 |
| Catalase | 1 | 0.000 | 0.000 | 0.000 |
| Glutamic oxaloacetic transaminase | 1 | 0.286 | 0.396 | 0.278 |
| Mean | 2.4 | 0.226 | 0.284 | 0.203 |

\* Mean genetic diversity among ETs within species.
\* Mean genetic diversity in the total sample of 14 ETs.
\* $G_{ST}$, Ratio of the probability of a mismatch of alleles at a locus for two ETs chosen randomly from different species to the probability of a mismatch in the pooled sample of ETs. Therefore, $G_{ST}$ is a measure of the relative magnitude of genetic differentiation among species.

lates were of ET 13, which differed at only one locus from ET 12.

The mean numbers of alleles per locus among isolates assigned to the three species were 2.1, 1.2, and 1.0 for *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis*, respectively.

**Relationship among multiocus genotypes.** The relationships among the 14 ETs are shown in Fig. 1. The smallest observed genetic distance (0.07) between ETs in the dendrogram corresponds to a single-locus difference, and the largest distance (0.57) corresponds to differences at 10 of the 15 loci assayed.

At a genetic distance of approximately 0.25 there are three groups of isolates (Fig. 1, groups A, B, and C), each represented by a cluster of ETs or, in the case of strain Dejong, a single ET. Most ETs belong to the larger of the two multistrain clusters, bounded by ET 1 and ET 11, which in turn is divided into several subgroups. This cluster (group A) contains predominantly *B. bronchiseptica* isolates, but also includes all of the *B. parapertussis* isolates and *B. pertussis* strain 18-323. The second group (group B) consists of two similar ETs represented by isolates of *B. pertussis*. A third line (group C), which diverges at a genetic distance of 0.57, contains one strain, strain Dejong.

Clustering of ETs by the same average-linkage method used to construct the dendrogram shown in Fig. 1, but from a matrix of coefficients of weighted genetic distance (proportion of mismatches, with the contribution of each locus to the coefficient weighted inversely by the genetic diversity at the locus [35]) produced several minor changes in the pattern of branching at genetic distances ranging from 0.10 to 0.25 but did not alter the compositions of the three major branches.

In summary, our analysis revealed the following three major divisions in the genus *Bordetella*: (i) group A, ET 1 through ET 11, containing 15 isolates of *B. bronchiseptica*, all 21 isolates of *B. parapertussis* (ET 5), and one isolate (ET 4) of *B. pertussis* (strain 18-323); (ii) group B, ETs 12 and 13, containing 22 of the 23 strains of *B. pertussis*; and (iii) group C, ET 14, composed of one *B. bronchiseptica* isolate, strain Dejong.

**Lack of effect of phenotypic modulation on electromorph profile.** Growth of *B. pertussis* BB-114 in modified Stanier-Scholte medium, Hornibrook X-mode medium, and C-mode medium (37) did not affect the electromorph mobilities of any of the 15 enzymes assayed, as shown for phosphoglucomutase in Fig. 2.

![Genetic relationships among 14 ETs of *Bordetella* spp.](http://jb.asm.org/)

FIG. 1. Genetic relationships among 14 ETs of *Bordetella* spp. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of unweighted genetic distance, based on 15 enzyme loci. The ETs are numbered from top to bottom in the order of listing in Table 2. ETs 1 through 3, 6 through 11, and 14 were represented by strains currently assigned to the species *B. bronchiseptica*, ET assignments for some of the better-known laboratory strains are as follows: *B. bronchiseptica* 110H, ET 1; *B. bronchiseptica* Columbus, ET 6; *B. bronchiseptica* Rat-1, ET 8; *B. bronchiseptica* Dejong, ET 14; *B. parapertussis* 17903, ET 5; *B. pertussis* 18-323, ET 4; *B. pertussis* BB-114, ET 12; and *B. pertussis* Tohama 1, ET 13.
DISCUSSION

For *Escherichia coli* and *Shigella* spp. (28), *Legionella* spp. (35), and many higher organisms (36), estimates of genetic relatedness based on multilocus enzyme electrophoresis have been shown to be positively correlated with measures of similarity in total nucleotide sequence derived from DNA hybridization experiments. Therefore, we have reason to believe that the 15 enzyme loci examined in this study are a representative sample of *Bordetella* spp. genomes and, hence, provide a basis for estimating both overall genetic relationships among strains and levels of genetic diversity within populations. Because evolutionary convergence to the same multilocus enzyme genotype is highly improbable (35), isolates of the same ET are considered members of the same cell line or clone.

**Genetic relationships among strains and species.** The results of our multilocus electrophoretic study of *Bordetella* spp. are fully compatible with previous data on variation at the molecular level (20) and confirm previous lines of evidence, derived from analyses of serotypes (8, 29), metabolic properties (17), and other characteristics (9, 23), showing close relatedness among strains currently assigned to the species *B. pertussis, B. parapertussis*, and *B. bronchiseptica*. The molecular evidence originates from DNA reassociation experiments (20), plasmid analysis (23), serologic schemes (8), transformation studies (23), and electrophoresis of cell envelope proteins (9). Although most of these studies compared relatively small numbers of strains and therefore did not adequately determine the extent of genetic variation within species in relation to that among species, the cumulative evidence clearly demonstrates that isolates of *B. pertussis, B. bronchiseptica*, and *B. parapertussis* are not sufficiently dissimilar to warrant classification as different species. For comparison, an analysis of variation at 16

| Taxon | No. of ETs | Average no. of alleles per locus | $\bar{n}$ |
|-------|------------|----------------------------------|---------|
| *Bordetella* spp. | 14 | 2.4 | 0.284 |
| *B. bronchiseptica* | 10 | 2.1 | 0.277 |
| *B. parapertussis* | 1 | 1.0 | 0.000 |
| *B. pertussis* | 3 | 1.2 | 0.133 |
| *H. influenzae* | Serotype b | 32 | 3.1 | 0.342 |
| Nontypable | 94 | 5.0 | 0.500 |
| *Legionella pneumophila* | 50 | 3.2 | 0.312 |
| *Neisseria meningitidis* | 55 | 6.1 | 0.615 |
| *Neisseria gonorrhoeae* | 100 | 4.1 | 0.393 |
| *E. coli* | Strains from neonatal septicemia and meningitis | 39 | 3.9 | 0.369 |
| Strains from urinary tract infections | 140 | 4.2 | 0.448 |

* Data from this study.
† Data from reference 26.
‡ Data from reference 25.
§ Data from reference 35.
¶ Data from Caugant, unpublished data.
¶ Data from reference 24.
∥ Data from reference 36.
* Data from reference 34.
† Data from reference 35.
§ R. K. Selander, unpublished data.
enzyme loci in 177 isolates of serotype b Haemophilus influenzae identified 32 ETs, with a $h$ of 0.342, which is significantly larger than the comparable value of 0.284 calculated for the 14 ETs in this study (Table 4). Hence, our findings support the view that continued recognition of three species for these bacteria can be justified only on clinical and historical grounds (20). In contrast, we shall present data elsewhere supporting the observation that some avian isolates, recently distinguished as Bordetella avium (19), are in fact genetically divergent enough to warrant specific status (Musser, unpublished data).

If the current practice of formally distinguishing species is to be continued, it should be clearly recognized that the nominal species B. pertussis and B. bronchiseptica as now constituted are polytypic assemblages of strains (Fig. 1). Strains currently assigned to B. pertussis fall into two distinctive genetic clusters, one of which includes most of the isolates of B. bronchiseptica. Moreover, the highly divergent strain Dejong of B. bronchiseptica is no more closely allied to other strains currently assigned to B. bronchiseptica than to strains of B. pertussis and B. parapertussis. Also, B. parapertussis is in overall genetic character merely a strain of B. bronchiseptica.

Evidence of a close genetic relationship between B. parapertussis and B. bronchiseptica and of limited divergence of B. pertussis from these two species was obtained previously by a DNA reassociation analysis (20), but the results were regarded as tentative because of the large experimental error associated with this technique (35). The observations that bacteriophages isolated from B. bronchiseptica lysed strains of B. parapertussis but not strains of B. pertussis (32) and that B. parapertussis and B. bronchiseptica are more closely similar antigenically and metabolically to each other than to B. pertussis (8) support our finding of genetic divergence of the two clones of B. pertussis in group B (ETs 12 and 13) from the strains of B. parapertussis and B. bronchiseptica (Fig. 1).

There has been considerable speculation (13) and some experimental evidence (21, 24) that B. pertussis and B. parapertussis are interconvertible by gain or loss of a lysogenic bacteriophage or by chemical mutagenesis. If interconversion occurs to any appreciable extent in nature, we might expect to find sharing of ETs between these two species. However, all of the isolates received as B. pertussis were genotypically distinct from all of the isolates of B. parapertussis examined (Fig. 1). Thus, we found no evidence of switching of B. pertussis to B. parapertussis or vice versa in natural populations. Inasmuch as ET 5 (B. parapertussis) differs from both ET 12 and ET 13 (B. pertussis) at five enzyme loci (phosphoglucomutase, adenylate kinase, leucine aminopeptidase, leucylalanine peptidase, and glutamic oxaloacetic transaminase) (Table 2), the possibility of interconversion of these multilocus genotypes by a single mutagenic event seems remote. Analysis of additional isolates will be required to determine whether some strains currently classified as B. pertussis and B. parapertussis have a closer genetic relationship than shown here.

Our finding that all of the isolates of B. parapertussis analyzed had an identical multilocus enzyme genotype agrees with the results of DNA reassociation tests (20) and serologic studies (8). A small amount of variation in metabolic characters has been detected among B. parapertussis isolates (17), but the extent to which this diversity represents in vitro phenotypic alteration is unclear.

Our identification of 10 distinctive ETs among the 16 B. bronchiseptica strains examined is not surprising in view of previous reports of diversity among isolates of B. bronchiseptica in plasmid profiles (23), serologic reactivities (29), and several other phenotypic characters (3, 4). An examination of 250 additional isolates of B. bronchiseptica from diverse host species and geographic areas in North America and Japan identified few additional ETs, suggesting that much of the total genetic diversity in natural populations of B. bronchiseptica was represented by the isolates analyzed in this study (Musser, unpublished data).

Because strain Dejong (ET 14), which has unique alleles at seven loci, was the most genetically divergent strain examined, it is noteworthy that this organism was recovered from a pig without atrophic rhinitis and lacked both adenylate cyclase activity and a 68-kilodalton protein that is believed to be important in virulence (27). Other investigators have noted antigenic uniformity among isolates recovered from pigs (29), but genetically diverse strains can infect the same host species, as indicated by our identification of seven ETs among isolates recovered from dogs (Table 1). Isolates having the same ET have been recovered from dogs, rabbits, and pigs, but an analysis of a larger sample of isolates will be required to determine the extent, if any, of host specificity of ETs.

**Stability of electrophoroms.** During cultivation in vitro, bordetellae frequently express changes in many of their surface components and in some other biological properties commonly found in fresh clinical isolates (9, 23, 30, 37). Serotype variation in strains of B. pertussis also occurs spontaneously in vitro and in vivo (6). This propensity for phenotypic modification has limited the conclusions that can be drawn from epidemiological studies in which serologic typing techniques are used and thus has hampered research in this area. As we have shown, phenotypic modulation of B. pertussis BB-114 to avirulent C-mode cells by growth in different media had no effect on the electrophorom profile (Fig. 2). Moreover, strain BB-114 and its isogenic derivatives, strains 3779 (2) and 1N1A1 (30), which have very different histories of in vitro passage, all had identical electrophorom profiles (Table 1). Isolates of strain Tohama obtained from two different laboratories had identical profiles, and three derivatives of strain Tohama produced by Tn5 insertional mutagenesis (38) and grown in modified Stanier-Scholte media were of the same ET (data not shown). Many of the strains examined in this study were analyzed under code, and in all cases the multilocus technique accurately discriminated among isolates of the three species. On one occasion, an analysis of the electrophoretic enzyme profiles of strains received under code revealed an error in strain labeling. Our results showed that the electrophoretic mobilities of metabolic enzymes are stable characters that can be used for epidemiological as well as genetic studies of this bacterium.

**Clonal structure of populations.** The recovery of ET 11 isolates (B. pertussis) and ET 5 isolates (B. parapertussis) and most of the ETs of B. bronchiseptica at a number of different localities and at different times (Table 1) (Musser, unpublished data) indicates that the genetic structure of Bordetella spp. is basically clonal. Although laboratory experiments have shown that bordetelloeae are naturally competent and capable of recombinational exchange of chromosomal material (23), our data suggest that chromosomal recombination occurs very infrequently in natural populations. The recovery of ET 12 isolates (B. pertussis) in Virginia, Texas, Michigan, Mexico, and the United Kingdom and of ET 5 isolates (B. parapertussis) in North America, Europe, and Japan demonstrated that these two
common clones have achieved very widespread geographic distributions, presumably by human dispersal, since no nonhuman hosts are known and no environmental reservoir for either organism has been documented (12).

It is noteworthy that all three ET 13 isolates (B. pertussis) were initially recovered in Japan. By clearly showing that members of this ET are genetically distinct from the clone of B. pertussis which occurs in the United States and Mexico (ET 12), our analysis suggests that there is a geographic component of population structure, a phenomenon previously noted in serotype b strains of H. influenzae (26). However, larger samples of isolates will be required to determine whether the observed geographic variation is real or only an artifact of inadequate sampling.

The genetic diversity among strains of Bordetella spp. is lower than that among strains of most other species of pathogenic bacteria that have been examined in our laboratory (Table 4). This restricted diversity could be explained by small effective population size, niche specialization, or recent evolutionary origin, and evidence can be advanced in support of all three possibilities. Restricted population size is suggested by the fact that humans are the only documented hosts of B. pertussis and B. parapertussis; little evidence exists for a chronic carrier state of either organism (22), and there is no evidence for an environmental reservoir (12). However, the population size of the group of clones representing B. bronchiseptica may be relatively large, inasmuch as this species has been recovered from a variety of mammalian hosts (11) and the carriage rates for dogs, rabbits, and swine can be high (31). On the assumption that much of the electrophoretically demonstrable enzyme variation in bacteria is selectively neutral or nearly so (14), the restricted single-locus genetic diversity in B. pertussis and B. parapertussis suggests that the clones assigned to these species have evolved rather recently. This hypothesis is supported by evidence that, in marked contrast to a variety of other pediatric infectious diseases, the earliest recorded description of whooping cough was in 1578 (7), and even this account failed to mention the characteristic inspiratory whoop. An alternative hypothesis is that widespread vaccination has significantly reduced the effective population size of this group of clones.

It has long been recognized that B. pertussis 18-323 possesses virulence for mice unequalled by other B. pertussis isolates (1, 5), but the molecular basis for this property has not been determined. Perhaps our demonstration that strain 18-323 is genetically more closely related to isolates of B. bronchiseptica than to the isolates of B. pertussis analyzed in this study (Fig. 1) will provide an impetus for further comparative genetic and physiologic studies of strain 18-323, an organism of signal importance because it is a standard challenge strain used for potency testing of pertussis vaccines in the United States and elsewhere.

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