JOURNAL OF BACTERIOLOGY, June 1987, p. 2793–2803
0021-9193/87/062793-11$02.00/0

Vol. 169, No. 6

Clonal Diversity and Host Distribution in *Bordetella bronchiseptica*

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Received 29 December 1986/Accepted 20 March 1987

A total of 303 isolates of *Bordetella bronchiseptica* recovered from 11 host species were characterized by the electrophoretic mobilities of 15 metabolic enzymes, and 21 distinctive multilocus genotypes (electrophoretic types) were distinguished on the basis of allele profiles at the enzyme loci. The population structure of *B. bronchiseptica* is clonal, and its genetic diversity is limited in comparison with most other pathogenic bacteria, perhaps reflecting a relatively recent origin of the species. Electrophoretic types mark clones which are, in many cases, nonrandomly associated with host species. Clones differing only slightly in overall chromosomal genetic character may have pronounced differences in virulence potential. There was considerable variation among individual clones and clone families in degree of host specificity and among various species of hosts in the diversity of clones causing disease. The diversity of clones infecting dogs was an order of magnitude greater than that of clones infecting pigs. Most bordetellosis in pigs in the United States and Japan was found to be caused by strains of a single multilocus genotype.

Bordetella bronchiseptica, a gram-negative bacterium recognized since 1910 as a pathogen of mammals (11), is a major etiological agent of atrophic rhinitis in pigs and acute tracheobronchitis (kennel cough) in dogs (14, 38). Other domesticated and wild animals may contract bordetellosis, and epidemics have occasionally been reported among vivarium-maintained guinea pigs, rats, rabbits, marmosets, and monkeys (2, 14). *B. bronchiseptica* is highly viscerotropic for ciliated epithelial cells of the respiratory tract, to which it adheres and then multiplies. The clinical syndrome is caused primarily by injury to surface mucosal cells, and the bronchial and pulmonary histopathology described at necropsy has been attributed to several toxins (14, 26, 38).

Interest in *B. bronchiseptica* has increased in recent years because bordetellosis is responsible for substantial economic loss in the swine, canine, and laboratory animal industries. Many aspects of the biology of this pathogen have been studied (14, 29, 38), including colony morphology (3, 19, 37), hemolysin production (3), hemagglutination (4, 36), plasmid content (16), and others (5, 18, 20, 28, 39, 47, 50, 51), but these analyses have not yielded estimates of genetic relationships among strains recovered from the same or different host species or provided much insight into the epidemiology of bordetellosis.

Electrophoretic and biochemical methods of detecting polymorphic variation in 15 metabolic enzymes were recently used to measure genetic diversity and relationships among 60 strains of *Bordetella*, including a small sample of *B. bronchiseptica* strains (33). This study suggested that multilocus enzyme electrophoresis could profitably be applied on a larger scale to address several basic questions about the population genetics of *B. bronchiseptica*. Is the species clonal, and if so, what is the extent of clonal diversity among isolates recovered from a variety of host species? Are strains of particular clones especially likely to be associated with certain host species? Are there ecological or natural history correlates of the genetic structure of natural populations? Answers to these questions may be directly relevant to the formulation and institution of programs for the control of bordetellosis.

We here present the results of an electrophoretic examination of enzyme polymorphism in *B. bronchiseptica*. Our results confirm preliminary data (33) indicating that there is relatively limited genetic variation in natural populations of *B. bronchiseptica* and that the genetic structure of the species is clonal. We have determined that most atrophic rhinitis in swine in the United States and Japan is caused by organisms of a single clone and that a distinct but related clone is responsible for nearly half the cases of bordetellosis in dogs.

MATERIALS AND METHODS

Bacterial isolates. We examined a total of 303 isolates of *B. bronchiseptica*, including 229 isolates from 19 states and the District of Columbia, 68 isolates from 15 prefectures on five islands in Japan, 2 isolates from the Netherlands, and 4 isolates from the United Kingdom. A partial list of the strains is presented in Table 1, and a complete list is available upon request from J.M.M. The host species distribution was as follows: 168 isolates from pigs, 57 from dogs, 7 from cats, 38 from rabbits, 4 from rats, 3 from guinea pigs, 1 from a porcupine, 4 from horses, 5 from monkeys, 3 from humans, 5 from turkeys, and 8 from unknown hosts (Table 1). Many of the strains have been described previously (3, 5, 33, 34), and some have been passaged in vitro more than 100 times.

Most of the isolates were recovered from animals with respiratory tract disease, but 33 of the 38 isolates from rabbits were cultured from the nasal passages of apparently healthy animals, and 10 of 20 isolates from swine in Wisconsin were obtained from a herd of animals that had no history of atrophic rhinitis. Strains recovered from diseased animals were presumed to be pathogenic and those from asymptomatic hosts were designated carrier isolates.

Culturing of bacteria and electrophoresis of enzymes. Each isolate was grown in 150 ml of tryptic soy broth (Difco Laboratories) overnight at 37°C on an orbital shaker (250

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TABLE 1. ET, host species, and collection locality and date for 130 strains of *B. bronchiseptica*[a]

ET	Isolate	Locality[b]	Year	Host species
1	B-4	New Jersey	1970s	Pig
	Bemis 116	Kentucky	1980s	Pig
	82-1711	Tennessee	1980s	Pig
	S. Madrid	Iowa	1970s	Pig
	7-8 NADL	Iowa	1970s	Pig
	495 NADL	Iowa	1970s	Pig
	7-11 NADL	Iowa	1970s	Pig
	2-9	Iowa	1970s	Pig
	5-8	Iowa	1970s	Pig
	5-4	Iowa	1970s	Pig
	B133	Iowa	1970s	Pig
	S-55	Iowa	1960s	Pig
	2576	Iowa	1960s	Pig
	S-2	Iowa	1960s	Pig
	S-3	Iowa	1960s	Pig
	S-4	Iowa	1960s	Pig
	85-17833	Iowa	1985	Pig
	85-18589	Iowa	1985	Pig
	85-18187	Iowa	1985	Pig
	Bemis 126	Indiana	1980s	Pig
	Bemis 132	Wisconsin	1980s	Pig
	CN7531	United Kingdom	1977	Pig
	MBORD 302	Hokkaido, Japan	1980	Pig
	MBORD 303	Hokkaido, Japan	1983	Pig
	MBORD 315	Ibaraki, Japan	1985	Pig
	MBORD 319	Gunma, Japan	1980	Pig
	MBORD 320	Gunma, Japan	1981	Pig
	MBORD 321	Gunma, Japan	1983	Pig
	MBORD 324	Saitama, Japan	1981	Pig
	MBORD 325	Chiba, Japan	1981	Pig
	MBORD 328	Chiba, Japan	1984	Pig
	MBORD 331	Niigata, Japan	1985	Pig
	MBORD 335	Ishikawa, Japan		Pig
	MBORD 337	Yamanashi, Japan	1985	Pig
	MBORD 338	Mie, Japan	1976	Pig
	MBORD 339	Mie, Japan	1985	Pig
	MBORD 342	Hyogo, Japan	1984	Pig
	MBORD 344	Tottori, Japan	1984	Pig
	MBORD 346	Shimane, Japan	1982	Pig
	MBORD 347	Tokushima, Japan	1983	Pig
	MBORD 348	Nagasaki, Japan	1984	Pig
	MBORD 349	Nagasaki, Japan	1985	Pig
	MBORD 366	Oita, Japan	1984	Pig
	MBORD 367	Okinawa	1972	Pig
	B-205-BT	Iowa	1980s	Pig
	1120A-83103	Indiana	1980s	Pig
	CSU-P-1	Colorado	1980s	Pig
	110H	New York	1970s	Dog
	PR8053	Missouri	1970	Dog
	PR8125	Missouri	1970	Dog
	D54	Kansas	1970	Dog
	B-001	Iowa	1960s	Dog
	B-013	Iowa	1960s	Dog
	Ct-1	Iowa	1960s	Cat
	Ct. Madrid	Iowa	1970s	Cat
	MBORD 172	Washington, D.C.	1985	Porcupine
	NCTC 10540	United States	1949	Human
2	213			
3	BTS	Iowa	1970s	Pig
	501	New York	1970s	Dog
	PR78A	Missouri	1970	Dog
	Rab-10	Montana		Rabbit
4	138 AnHus	New York	1970s	Dog
	H-475	New York	1970s	Dog

Continued

TABLE 1—*Continued*

ET	Isolate	Locality[b]	Year	Host species
	ATCC 19395[c]	Michigan	1910s–1920s	Dog
	ATCC 780	Michigan	1910s–1920s	Dog
	ATCC 10580	Michigan	1910s–1920s	Dog
	Ft. Collins	Colorado	1970s	Dog
	469			Human
5	MBORD 116	Iowa	1984	Pig
6	BB 241168	New York	1960s	Dog
	BB Ithaca	New York	1960s	Dog
	17640 SAC	New York	1970s	Dog
	18731 SAC	New York	1970s	Dog
	19022 SAC	New York	1970s	Dog
	19705 SAC	New York	1970	Dog
	23153 (Meisel)	New York	1970	Dog
	Brook	New York	1970	Dog
	35992 M	New York	1970	Dog
	36043 M	New York	1970	Dog
	RoMark	New York	1970	Dog
	Lutton	New York	1970	Dog
	1388-B3	New Jersey	1970s	Dog
	JS34682	Kansas	1970	Dog
	482-74	Kansas	1970	Dog
	695-74	Kansas	1970	Dog
	D-2	Iowa	1960s	Dog
	SS-232-75	Michigan	1970	Dog
	Dahlund DR-64	California	1970s	Dog
	Hopper DS-43	California	1970s	Dog
	Phase-1 Tuskegee	Alabama	1970s	Pig
	52190	New York	1970s	Cat
	Columbus	Ohio	1970s	Cat
7	MBORD 334	Ishikawa, Japan		Pig
8	Ross 64-C-0406 (D-1)	Iowa	1960s	Dog
9	MBORD 215	Virginia	1980s	Rabbit
10	CN8593	United Kingdom		Human
11	Bemis 47	Tennessee	1980s	Dog
12	MBORD 440	Washington	1986	Rabbit
13	87	New York	1970s	Dog
	J696/107	New York	1970s	Dog
	K704/174	New York	1970s	Dog
	CD-C47	New York	1970s	Dog
	899L	Michigan	1950s	
	NYS#4			
	NYS#10			
14	19141 SAC	New York	1970s	Dog
	Bemis 221	Iowa	1985	Turkey
15	MBORD 173	Arkansas	1985	Turkey
16	Rat-1	New York	1970s	Rat
	Rat-2	New York	1970s	Rat
	SHGP-1	New York	1970s	Guinea pig
	GPA	New Jersey	1970s	Guinea pig
	VPI GP-1	Virginia	1980s	Guinea pig
	MBORD 177	Oregon	1985	Rabbit
	MBORD 216	Virginia	1980s	Rabbit
	MBORD 219	Virginia	1980s	Cat
	MBORD 445	Arkansas	1986	Rabbit
	BB Manhattan	New York	1960s	Dog
	VPI EQ-1	Virginia	1980s	Horse

Continued

TABLE 1—*Continued*

ET	Isolate	Locality[b]	Year	Host species
	UT 115	Tennessee	1980s	Horse
	UT 143	Tennessee	1980s	Horse
	ILG	New Jersey	1970s	Monkey
	214	Michigan	1930s	
17	MBORD 282	Tennessee	1980s	Dog
18	Bemis 48	Tennessee	1980s	Dog
19	Rt-1	Iowa	1960s	Dog
20	UT dog	Tennessee	1970	Dog
21	AR non-path[d]	The Netherlands	1970s	Pig
	CN8594	The Netherlands	1970s	Pig

[a] For the number of isolates in each ET and their distribution among hosts, see Table 6.
[b] Isolates MBORD 302 and 303 are from Hokkaido, isolates MBORD 315 through 346 are from Honshu, isolate MBORD 347 is from Shikoku, and isolates MBORD 348, 349, and 366 are from Kyushu.
[c] ATCC 19395 is the American Type Culture Collection type strain for *B. bronchiseptica*.
[d] Referred to as "Dejong" by Musser et al. (33).

rpm) and harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. Following suspension in 2 ml of 50 mM Tris hydrochloride buffer containing 5 mM EDTA (pH 7.5), the bacteria were sonicated with a model 200 Sonifier-Cell Disrupter (Branson Sonic Power Co., Danbury, Conn.) equipped with a microtip for 45 s at 50% pulse, with ice-water cooling, and centrifuged at 20,000 × *g* for 20 min at 4°C. The clear supernatant (lysate) was stored at −70°C.

Lysates were electrophoresed on starch gels and selectively stained for 15 metabolic enzymes by methods described elsewhere (33, 43). The enzymes studied were NAD-dependent malate dehydrogenase, fumarase, indophenol oxidase, hydroxybutyrate dehydrogenase, alkaline phosphatase, esterase, phosphoglucose isomerase, isocitrate dehydrogenase, phosphoglucomutase, adenylate kinase, leucine aminopeptidase, glutamate dehydrogenase, leucylalanine peptidase, catalase, and glutamic oxaloacetic transaminase.

Distinctive electromorphs (mobility variants) of each enzyme, numbered in order of decreasing rate of anodal migration, were equated with alleles at the corresponding structural gene locus (Table 2). Because all 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 combinations of electromorphs, corresponding to unique multilocus genotypes, were designated electrophoretic types (ETs) (Table 2) (43).

TABLE 2. Allele profiles at 15 enzyme loci in 21 ETs of *B. bronchiseptica*

ET	Reference isolate	No. of isolates	Allele at indicated enzyme locus[a]														
			MDH	FUM	IPO	HBD	ALP	EST	PGI	IDH	PGM	ADK	LAP	GLD	PE1	CAT	GOT
1	B-4	193	1	2	1	2	2	2	1	1	3	1	3	1	3	1	2
2	213	1	1	2	1	2	1	2	1	1	3	1	3	1	3	1	2
3	BTS	4	1	2	1	2	2	2	1	1	4	1	3	1	3	1	2
4	138 AnHus	8	1	1	1	2	2	2	2	1	3	1	3	1	3	1	2
5	MBORD 116	1	1	2	1	2	2	2	2	1	3	1	3	1	3		
6	BB 241168	32	1	1	1	2	2	1	1	1	3	1	3	1	3	1	2
7	MBORD 334	1	1	2	1	2	2	1	1	1	3	1	3	1	3	1	2
8	Ross 64-C-0406 (D-1)	1	1	1	1	2	2	2	1	1	3	1	3	1	3	1	2
9	MBORD 215	1	1	1	1	2	2	4	1	1	3	1	3	1	3	1	2
10	CN8593	1	1	1	1	2	2	1	1	1	3	1	2	1	3	1	2
11	Bemis 47	1	1	1	1	2	2	1	1	1	3	1	3	2	3	1	2
12	MBORD 440	1	1	1	1	2	2	2	2	1	4	1	2	1	3	1	2
13	87	7	1	2	1	2	1	2	1	1	1	1	2	1	1	1	2
14	19141 SAC	7	1	2	1	2	1	2	1	1	3	1	2	1	1	1	2
15	MBORD 173	1	1	2	1	2	1	2	1	1	3	1	2	1	3	1	2
16	Rat-1	38	1	2	1	2	2	2	1	1	3	1	2	1	1	1	2
17	MBORD 282	1	1	2	1	2	4	2	1	1	3	1	2	1	1	1	2
18	Bemis 48	1	1	2	1	2	5	2	1	1	3	1	2	1	1	1	2
19	Rt-1	1	1	2	1	1	2	2	1	1	3	1	2	1	1	1	2
20	UT dog	1	1	2	1	2	2	3	1	1	3	1	2	1	1	1	2
21	AR non-path	2	1	1	1	1	3	2	1	1	2	2	3	2	2	1	1

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

TABLE 3. Allele frequencies and genetic diversity at 15 enzyme loci in 21 ETs of *B. bronchiseptica*

Enzyme locus[a]	Allele					Genetic diversity (*h*)
	1	2	3	4	5	
MDH	1.000					0.000
FUM	0.381	0.619				0.495
IPO	1.000					0.000
HBD	0.095	0.905				0.181
ALP	0.190	0.667	0.048	0.048	0.048	0.538
EST	0.190	0.714	0.048	0.048		0.471
PGI	0.857	0.143				0.257
IDH	1.000					0.000
PGM	0.048	0.048	0.810	0.095		0.348
ADK	0.952	0.048				0.095
LAP	0.476	0.524				0.524
GLD	0.095	0.905				0.181
PE1	0.333	0.048	0.619			0.529
CAT	1.000					0.000
GOT	0.048	0.952				0.095
Mean diversity per locus (*H*)						0.248

[a] See Table 2, footnote *a*. The mean diversity per locus (*H*) was 0.248.

Statistical analysis. Genetic diversity at an enzyme locus (*h*) among either ETs or isolates was calculated from allele frequencies as $h = (1 - \Sigma x_i^2)(n/n - 1)$, where x_i is the frequency of the *i*th allele and *n* is the number of ETs or isolates (43). Mean genetic diversity per locus (*H*) is the arithmetic average of *h* values for all loci.

Genetic distance between pairs of ETs was expressed as the proportion of enzyme 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 (43).

RESULTS

Overall genetic diversity. In the collection of 303 isolates of *B. bronchiseptica* examined, 11 of the 15 enzyme loci assayed were polymorphic for alleles encoding electrophoretically distinguishable variant polypeptides, and four loci (malate dehydrogenase, indophenol oxidase, isocitrate dehydrogenase, and catalase) were monomorphic (Table 2). A total of 21 distinctive ETs were identified (Table 2), most of which differed at only one or a few loci. Mean genetic diversity per locus (*H*) among the ETs was 0.248 (interlocus variance [s^2] = 0.047) (Table 3). There was much less genetic diversity among the isolates ($H = 0.095$; $s^2 = 0.014$), reflecting the circumstance that eight of the ETs were represented by two or more isolates, with three ETs (ET 1, ET 6, and ET 16) accounting for 263 (87%) of the isolates examined.

Genetic relationships among multilocus genotypes. The dendrogram in Fig. 1 summarizes estimates of the genetic relationships of the 21 ETs. The smallest observed genetic distance (0.07) between ETs corresponds to a single-locus difference, and the largest distance (0.57) corresponds to differences at 8 of the 15 loci. At a distance of 0.15, there were five lineages or clusters, designated A through E; clusters A, B, and D were each composed of five or more ETs, and the C and E lineages were each represented by a single ET. Clusters A, B, and D contained 68, 12, and 19%, respectively, of the isolates, and there was little variation in the level of genetic diversity per locus among ETs in these clusters (Table 4). ETs of clusters A and B differed, on

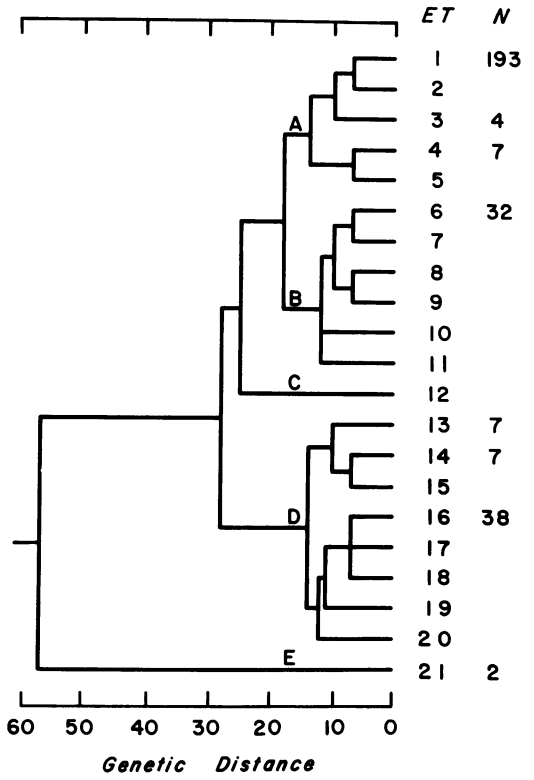

FIG. 1. Genetic relationships among 21 ETs of *B. bronchiseptica*. The dendrogram was generated by the average linkage method of clustering from a matrix of coefficients of pairwise genetic distance, based on 15 enzyme loci. ETs are numbered sequentially from top to bottom in the order of their listing in Table 1. *N* is the number of isolates in each ET represented by multiple strains; all other ETs were represented by single isolates.

average, at three loci; cluster D was somewhat more divergent from clusters A and B, with a mean genetic distance of 0.28, reflecting the occurrence of dissimilar alleles at an average of four loci. Lineage C (ET 12), which was separated from clusters A and B at a distance of 0.24 and diverged from cluster D at a distance of 0.28, was represented by a single isolate (MBORD 440) from the nasal passage of a healthy rabbit.

The most divergent lineage, ET 21, which included strain AR non-path (also known as CN8164, 53453, and Dejong) and MBORD 201 (also known as CN8594 and 26177) (34), separated from other ETs at a genetic distance of 0.57. These two strains can be distinguished from other *B. bronchiseptica* by the possession of distinctive alleles at five loci (alkaline phosphatase, phosphoglucomutase, adenylate

TABLE 4. Mean genetic diversity at 15 enzyme loci in 21 ETs of *B. bronchiseptica* classified by lineage or cluster

Lineage or cluster	Genetic diversity in ETs		
	Sample size (*n*)	Mean (*H*)	Variance (s^2)
A	5	0.120	0.045
B	6	0.107	0.037
C	1	0.000	0.000
D	8	0.119	0.047
E	1	0.000	0.000

TABLE 5. Mean genetic diversity per locus in *B. bronchiseptica* recovered from different host species

Host species	ETs			Isolates		
	n	Mean diversity (*H*)	Variance (s^2)	*n*	Mean diversity (*H*)	Variance (s^2)
Pig[a]	6	0.307	0.047	168	0.020	0.000
Pig[b]	5	0.173	0.051	166	0.007	0.000
Dog	12	0.215	0.057	57	0.148	0.033
Rabbit	5	0.200	0.069	38	0.088	0.030
Other	10	0.201	0.051	40	0.126	0.031
Total[a]	21	0.248	0.047	303	0.095	0.014
Total[b]	20	0.214	0.050	301	0.089	0.014

[a] Includes the two isolates of ET 21.
[b] Excludes the two isolates of ET 21.

kinase, leucylalanine peptidase, and glutamic oxaloacetic transaminase) (Table 2).

In summary, enzyme electrophoresis revealed five major divisions in *B. bronchiseptica*: (i) cluster A (ETs 1 through 5), containing 206 isolates; (ii) cluster B (ETs 6 through 11), 37 isolates; (iii) lineage C (ET 12), 1 isolate; (iv) cluster D (ETs 13 through 20), 57 isolates; and (v) lineage E (ET 21), 2 isolates.

Distribution of clones among host species. Single-locus variation among ETs was estimated for each of the three host species (dog, pig, and rabbit) for which 10 or more isolates were available (Table 5). For the 12 ETs identified among the 57 isolates from dogs and the six ETs distinguished among the 168 pig isolates, mean genetic diversity was 0.215 and 0.307, respectively. The 38 isolates from rabbits were assigned to five ETs, among which the mean genetic diversity was 0.200. Thus, the genetic diversity among ETs recovered from each of these host species was similar to that of the total sample (Table 5). Of the 168 pig isolates, 162 (96%) were of multilocus genotypes in cluster A, and 160 of them (95%) were ET 1 (Table 6). Fifty-two percent of the dog isolates were in cluster B, 33% in cluster A, and 15% in cluster D. However, of the 37 isolates in cluster B recovered from all species, 30 (81%) were cultured from dogs, and most (93%) dog isolates were ET 6. Clusters A and D contained 40 and 55%, respectively, of the rabbit isolates; 14 of 15 isolates from rabbits in cluster A were ET 1; and all isolates from rabbits in cluster D represented ET 16.

All horse isolates (three isolates from unassociated animals in Tennessee and an isolate from Virginia) were ET 16, of cluster D. Similarly, the three guinea pig isolates (one isolate each from animals in New York, New Jersey, and Virginia) were identical in multilocus genotype (ET 16). Isolates from rats were confined to two ETs in cluster D, and all turkey isolates represented either ET 14 or ET 15, which differed at only one locus (leucylalanine peptidase). Each of the three isolates from humans had a distinctive multilocus genotype (ETs 1, 4, and 10).

In sum, there is striking variation in the frequency of

TABLE 6. Numbers of *B. bronchiseptica* isolates from various animal hosts, classified by ET

Cluster or lineage	ET	Total no. of isolates	No. of isolates from indicated source host										
			Dog	Pig	Rat	Guinea pig	Monkey	Cat	Horse	Turkey	Human	Rabbit	Porcupine	Unknown
A	1	193	9	160			4	2			1	14	1	2
	2	1												1
	3	4	2	1								1		
	4	7	6								1			
	5	1		1										
B	6	32	28	2				2						
	7	1		1										
	8	1	1											
	9	1										1		
	10	1									1			
	11	1	1											
C	12	1										1		
D	13	7	4											3
	14	7	2	1						4				
	15	1								1				
	16	38	1		3	3	1	3	4			21		2
	17	1	1											
	18	1	1											
	19	1			1									
	20	1	1											
E	21	2		2										

recovery of particular multilocus genotypes from different host species. Some 96% of the isolates from pigs represented one multilocus genotype, ET 1. Most (83%) dog isolates were of ETs in clusters A and B, whereas most rabbit isolates (95%) were of ETs in clusters A and D.

Of the eight ETs represented by two or more isolates, seven were recovered from two or more host species (mean, 3.9 hosts; range, 2 to 7). ET 1 and ET 16 were each represented by isolates from seven host species.

Clonal structure in relation to disease. Isolates of the single ETs of lineages C and E were not associated with disease episodes, but all ETs in clusters A and D were represented by one or more isolates recovered from diseased animals. Strain MBORD 215, the single isolate of ET 9, in cluster B, was obtained from the nasal wash of a healthy rabbit, but all other isolates of ETs in cluster B were cultured from diseased hosts. Seventeen isolates of ET 1 were from carriers (11 isolates from pigs, including 10 from one herd in Wisconsin, and 7 isolates from rabbits), and 20 of the isolates of ET 16 were from healthy rabbits.

Geographic distribution of ETs. Individual ETs may have wide, even intercontinental, distribution. For example, isolates of ET 1 have been recovered from pigs on five islands in Japan, in eight states of the continental United States, and in the United Kingdom; isolates of ET 6 have been obtained from dogs in New York, New Jersey, Tennessee, Michigan, Kansas, and California.

DISCUSSION

Estimating genetic diversity and relatedness among isolates. For several genera of bacteria and many groups of higher organisms, estimates of genetic relatedness based on multilocus enzyme electrophoresis have been shown to be strongly correlated with measures of similarity in total nucleotide sequence derived from DNA hybridization experiments (43). Consequently, there is reason to believe that the 15 enzyme loci examined in the present study are a representative sample of the chromosomal genome of *B. bronchiseptica* and therefore provide a useful basis for estimating both levels of genetic diversity in populations and overall genetic relationships among strains.

Nature of the sample studied. Because porcine isolates from the United States and Japan were obtained from several geographic regions, including those with large pig-rearing facilities, the samples from these countries are believed to be representative of the populations of strains causing atrophic rhinitis. The canine isolates, which were collected in 10 states, in most cases in the 1960s or 1970s, although three strains were obtained in the 1910s or 1920s and some in the 1980s, presumably are representative of the population associated with dogs in the continental United States. The only readily identifiable sources of sampling bias involve the rabbit isolates, 32 of 38 of which were obtained from inbred New Zealand White animals, and the five monkey isolates, four of which were recovered from animals maintained by one commercial supplier and were probably epidemiologically associated.

Clonal population structure. Enzyme variants detected by electrophoresis have been used as chromosomal markers to measure genetic relatedness among strains and to identify clones in studies of the genetic structure of *Escherichia coli* (44) and other bacteria (6, 32, 42, 46). Because evolutionary convergence to the same multilocus genotype is highly improbable (45), isolates of identical multilocus genotype are considered members of the same clone or cell line.

B. bronchiseptica is naturally competent and capable of recombinational exchange of chromosomal material (23), but the recovery of the same multilocus genotype at different localities and at different times suggests that chromosomal recombination occurs very infrequently in natural populations and that, consequently, the genetic structure of *B. bronchiseptica* is clonal. For example, isolates of ET 1 have been recovered from swine in the continental United States, the United Kingdom, and Japan, and isolates of ET 4 were collected in the early decades of this century and in the 1970s.

Diversity of genotypes causing disease. A relatively small number of clones are associated with bordetellosis. The highly divergent strains AR non-path and MBORD 201 (ET 21), which were recovered from pigs without atrophic rhinitis, lack both adenylate cyclase activity and a 68-kilodalton protein that is believed to be important in virulence (34). Moreover, they were unable to induce atrophic rhinitis in specific-pathogen-free pigs (8). Our analysis indicated that these two strains bear little resemblance to those of *B. bronchiseptica* causing disease in the United States and Japan. Indeed, they are sufficiently divergent in chromosomal genotype from other *B. bronchiseptica* isolates to suggest that DNA hybridization experiments would show nucleotide sequence similarity below the 70% level now conventionally taken as the criterion of species limits for many groups of bacteria (25). The results of investigations with these strains, which have been extensively characterized (8, 34) and used in in vivo tests of pathogenicity, should be interpreted accordingly.

Genetic diversity compared with that of other bacteria. *B. bronchiseptica* is genetically less diverse than most other species of pathogenic bacteria that have been examined (33). Because *B. bronchiseptica* has worldwide distribution and can be recovered from a variety of mammalian hosts (14, 38), we might have expected it to be at least as genetically variable as serotype b *Haemophilus influenzae*, a pathogen whose only known natural host is the human. However, our estimate of mean genetic diversity among ETs of *B. bronchiseptica* (H = 0.248) is only 73% of a comparable estimate (0.342) obtained for ETs of serotype b *H. influenzae* isolates from the United States (32). When the highly divergent ET 21 is excluded, mean genetic diversity for *B. bronchiseptica* decreases to 0.214, or only 63% of that for serotype b *H. influenzae* (Table 5).

Previously we demonstrated that genetic variation among strains currently assigned to *Bordetella pertussis*, *B. parapertussis*, and *B. bronchiseptica* is insufficient to justify recognition of three species (33); data from DNA hybridization studies are in agreement (21, 24). When the three ETs of *B. pertussis* and the one ET of *B. parapertussis* described previously (33) were pooled with the 21 ETs of *B. bronchiseptica*, genetic diversity increased only slightly, to 0.257 (data not shown), a value which is still low relative to estimates for other species of pathogenic bacteria (33).

Restricted genetic diversity could, in theory, be caused by small effective population size (perhaps connected with a recent evolutionary origin of the species), ecological niche specialization, or a low mutation rate. The intuitively appealing notion that genetic diversity and niche width are in some fashion related has long been an area of debate in eucaryotic population genetics (22), but apart from information provided by studies of enzyme polymorphism, there are no data for bacterial species that bear on such a relationship. Inasmuch as *B. bronchiseptica* is highly viscerotropic for ciliated respiratory epithelial cells and has not been recovered from

environmental sources, we might be tempted to invoke niche specialization as an explanation for restricted genetic diversity, but studies in the laboratory of R. K. Selander on a variety of procaryotic species have failed to demonstrate a relationship between niche width and genetic diversity. For example, for *E. coli*, which occupies the bowel of virtually every mammalian species, and for *Neisseria meningitidis*, whose natural habitat is confined to human nasopharyngeal surfaces, estimates of mean genetic diversity among ETs are 0.510 (44) and 0.615 (6), respectively. The comparable estimate for *Legionella pneumophila*, which is ubiquitous in freshwater environments worldwide (12), is 0.313, or only 54% of that of *E. coli*.

The contribution of mutation frequency to the restricted genetic diversity of *B. bronchiseptica* is difficult to assess because there are no data on the spontaneous mutation rate of this pathogen, apart from estimates of the frequency of phase variation (27), a phenomenon which may not represent the average rate of mutation in the genome as a whole.

Another possible explanation for limited genetic diversity in *B. bronchiseptica* is that isolates recovered from diseased animals represent only a limited subset of clones of the species. (A similar hypothesis has been advanced to explain the restricted genetic diversity of *L. pneumophila* from clinical cases and man-made environments [46].) According to this interpretation, the relatively low genetic diversity recorded for *B. bronchiseptica* may be an artifact of analyses based primarily on samples of disease isolates or of the criteria used for species definition. The observation that isolates of the highly divergent ET 21 failed to cause bordetellosis in specific-pathogen-free pigs suggests that there are additional clones of *B. bronchiseptica* occurring in the carrier state and infrequently if ever associated with clinical disease.

If much or all of the enzyme polymorphism in bacteria is selectively neutral or nearly so (17), the amount of variation carried by a population or species at equilibrium is expected to be a function of the evolutionary effective population size, which is roughly equivalent to the harmonic mean of the number of clones or colonies that have existed each generation since the origin of the species (22). The association of *B. bronchiseptica* with respiratory tract disease in a large variety of host species (14), together with the demonstration that the carriage rates for dogs, rabbits, and pigs can be high (38), suggests that the present population size of the species is large and therefore is not a major factor responsible for its low genetic diversity. However, the evolutionary effective population size could be small if the clones assigned to the species evolved rather recently from one or a small number of ancestral clones (30). We favor the hypothesis of a recent evolutionary origin to account for the relatively limited genetic variation observed in *B. bronchiseptica*.

Clonal variation in relation to pathogenicity. That particular strains of *B. bronchiseptica* vary in pathogenicity for different host species was demonstrated 20 years ago (40) in a study of the ability of eight isolates, recovered from five different host species, to cause disease in purebred Yorkshire pigs and has been confirmed in experiments with gnotobiotic pigs (7).

We determined the enzyme genotypes of six strains (S-2, S-3, S-4, Ct-1, Rt-1, and D-1) that were previously used in a series of in vivo pathogenicity tests (40). Three strains (S-2, S-3, and S-4) isolated from swine with atrophic rhinitis and one strain (Ct-1) recovered from the tracheal exudate of a kitten were of multilocus genotype ET 1 (cluster A). Fifteen of 16 pigs inoculated intranasally with these four strains developed turbinate atrophy or pneumonia by 4 weeks postinoculation, and two of the pigs died of pneumonia. Of the four pigs inoculated with strain Rt-1, which represents ET 16, in cluster D (Fig. 1), three had normal turbinates and lungs at 4 weeks postinoculation, and the fourth pig, which died of enteritis 18 days after inoculation, had only slight turbinate atrophy. All four pigs inoculated with strain D-1 (ET 8, cluster B) had normal turbinates and lungs 4 weeks after inoculation. *B. bronchiseptica* was recovered in large numbers from the nasal and tracheal exudates of almost all pigs inoculated with strains Rt-1 and D-1, but fewer organisms were recovered from pigs inoculated with either of these strains than from pigs inoculated with S-2, S-3, S-4, or Ct-1. Thus, strains Rt-1 and D-1 apparently have properties permitting colonization of swine but fail to express cellular moieties required for production of the gross lesions characteristic of atrophic rhinitis. In sum, regardless of host species of origin, isolates of ET 1 were pathogenic for pigs, but disease did not occur in pigs inoculated with non-ET 1 isolates.

Further evidence that isolates of *B. bronchiseptica* of dissimilar ETs can differ dramatically in virulence for pigs was provided by data from pathogenicity tests of other isolates in our collection. Strain S-55 (ET 1, cluster A), from the pneumonic lung of a pig (15), and strain D-2 (ET 6, cluster B), from a dog with infectious tracheobronchitis, were assayed in mixed-breed dogs for the ability to induce infection of tracheal and bronchiole tissues (15). Following intranasal instillation, both strains were isolated from the animals throughout a 26-day test period, but D-2 was recovered much more often than S-55. Onset of clinical tracheobronchitis occurred 4 days after inoculation with strain D-2 and continued for 22 days, but strain S-55 did not produce bordetellosis. Furthermore, dogs inoculated with D-2 developed extensive histopathologic lesions in the trachea and bronchioles, whereas animals receiving strain S-55 did not experience such changes. These results suggest that strains of ET 1 and strains of ET 6, which have dissimilar alleles at two loci and belong to different clusters (Fig. 1), differ in virulence for dogs. However, not all strains of ET 1 are avirulent for dogs; in our collection, 19% of all dog isolates were of ET 1, and all ET-1 isolates from dogs were recovered from diseased animals. Additional evidence that strains of ET 1 are heterogeneous in pathogenicity is provided by the observation that 10 isolates of ET 1 in our collection were recovered from a herd of swine in Wisconsin that had no history of atrophic rhinitis.

The results of enzyme electrophoresis of four strains characterized for dermonecrotic toxin phenotype are consistent with evidence that the production of atrophic rhinitis and pneumonic lesions in pigs by *B. bronchiseptica* correlates with the amount of dermonecrotic toxin produced by a particular strain (39a). Strains B-205-BT, 1120A-83013, and CSU-P-1 were ET 1, and the fourth strain, Ross 64-CO-406 (D-1), was ET 8. B-205-BT and 1120A-83013 cause atrophic rhinitis and pneumonic lesions in pigs and produce dermonecrotic toxin, whereas strain CSU-P-1 neither causes disease in pigs nor produces toxin. Strain Ross 64-CO-406, which was the sole isolate of ET 8 identified in our study, colonizes neonatal pigs but does not produce dermonecrotic toxin (39a).

Although most isolates recovered from diseased pigs are of ET 1, strains of ET 1 have also been isolated from healthy pigs. Variation in virulence among porcine isolates of *B. bronchiseptica* also has been recorded under laboratory conditions (31, 48), and it is noteworthy that two groups of

TABLE 7. Estimates of host diversity (D_{hosts}) for three clone families of *B. bronchiseptica*

Clone family	No. of isolates[a]	No. of host species	D_{hosts}
A	138	8	0.516
B	37	5	0.306
D	57	10	0.821

[a] Isolates from Japan not included.

investigators have reported that in Great Britain the severity of atrophic rhinitis in pigs varies and that disease is less widespread than colonization by *B. bronchiseptica* (13, 41). Experiments are in progress to determine the genetic population structure of porcine isolates of *B. bronchiseptica* in Great Britain and elsewhere in Europe, with special reference to the spectrum of clinical illness.

In sum, our results, when interpreted together with data from in vivo pathogenicity experiments, indicate that multilocus enzyme genotypes mark certain clones that vary in virulence for particular host species. We do not discount the degree to which phenotypic states, including toxin production, hemolysin production, and phase variation, influence virulence and pathogenicity. Rather, we consider it likely that multilocus genotypes identify clones which, on average, differ in the ability to express or modulate phenotypes. This idea is a corollary of one consequence of the clonal population structure of *B. bronchiseptica*: with low rates of chromosomal recombination, individual lineages (clones) evolve more or less separately, and the extent of genetic dissimilarity between lines is primarily a function of the length of time since the lineages diverged from a common ancestor. One testable prediction of this thesis is that sequencing of virulence genes from strains of divergent ETs will reveal dissimilarity in structure that may in part account for some of the variation in their host specificities.

Host specificity of clones and clone families. Although one of us (D.A.B.) (3) has been pessimistic about the possibility of distinguishing *B. bronchiseptica* isolates from different host species, the results presented here clearly demonstrate a high degree of association of certain clones of *B. bronchiseptica* with particular host species. Thus, the distribution of numbers of isolates of the clone families A, B, and D among pigs, dogs, and a third category including all other host species is significantly nonrandom (χ^2 [4 df] = 233, $P < 0.001$). As a measure of this association, we have calculated coefficients of host diversity for the three families of clones A, B, and D, which include the three common ETs, ET 1, ET 6, and ET 16, respectively. The measure used was $D_{hosts} = (1 - \Sigma y_i^2)(n/n - 1)$, where y is the frequency of isolates from the ith host and n is the total number of host individuals (which is equivalent to the total number of isolates, since each isolate was recovered from a separate host individual). In this analysis, strains from Japan were excluded because no attempt was made to obtain isolates from host species other than the pig in that country.

As shown in Table 7, the degree of host diversity varied markedly among the clone families, being greatest for family D (D_{hosts} = 0.821) and smallest for family B (D_{hosts} = 0.306). Considered in both qualitative and quantitative terms of distribution among various species of hosts, ET 1 was clearly a pig "specialist" clone, although it was recovered from eight mammalian species. Similarly, ET 6 was a dog "specialist" clone, although it was also recovered from pigs and cats. ET 16 showed a lesser degree of host specificity than either of the other clones: the 38 isolates of ET 16 were recovered from a total of seven host species. Apparently this clone occurs most commonly in rabbits, but the data are perhaps biased, as explained in the Results.

Using serologic methods, Pedersen (35) could detect no antigenic variation among 80 isolates of *B. bronchiseptica* from swine in Denmark, and one isolate each from the United States and the Netherlands were serologically identical to the Danish isolates. This finding now makes sense in light of the very limited genetic diversity observed among isolates from pigs. The results of a serological examination of strains from rabbits and cats also led Pedersen (35) to conclude that cross-infection between pigs and other animals rarely occurs. However, our collection includes isolates of ET 1 recovered from pigs, rabbits, dogs, monkeys, and cats and isolates of ET 16 from the dog, cat, horse, rabbit, guinea pig, rat, and monkey.

Both rats and cats have been implicated as sources of infection in piggeries (49). We identified no instance of sharing of ETs between isolates from pigs and rats, but sharing of ETs between strains of cat and pig origin was observed. Two of the seven cat isolates were ET 1, two were ET 6, and the remainder were ET 16. Clearly an analysis of a much larger sample of isolates will be required to determine the extent to which rats, cats, and pigs share clones.

Diversity of clones associated with different host species. Inferences about the diversity of infecting clones can be drawn only for the three host species for which large numbers of isolates are available, the pig, dog, and rabbit. Estimates of clonal diversity for these three hosts are shown in Table 8. Clonal diversity was smallest for the pig, with only six clones represented among the 166 isolates and D_{clones} = 0.071. Twelve clones were represented by the 58 isolates from dogs, and diversity was an order of magnitude greater, D_{clones} = 0.732. Clonal diversity (0.572) among the 38 isolates from the rabbit was intermediate.

In sum, clones and clone families of *B. bronchiseptica* vary in the extent of their host ranges, and there is much less clonal diversity among isolates from pigs than among strains from dogs or rabbits. The propensity for host specificity as shown by ET 1 (pigs) and ET 6 (dogs) of *B. bronchiseptica* is a characteristic of the genus *Bordetella* that has been carried to an extreme in *B. parapertussis* and *B. pertussis*, for which humans are the only known natural host. On the grounds of overall genomic character and probable phylogenetic relationships, both *B. parapertussis* and *B. pertussis* can be considered clones of *B. bronchiseptica* that have become specialized human pathogens. ET 21 of *B. bronchiseptica* is a somewhat more divergent, nonpathogenic relative of the *B. bronchiseptica-B. parapertussis-B. pertussis* group that apparently is confined to pigs. Isolates currently classified as *Bordetella avium* (21) represent two distantly related families of clones that are associated predominantly with chickens and turkeys; both of these clone families are sufficiently divergent from the *B. bronchiseptica* complex to warrant specific recognition (Musser, unpublished data).

Value of the population genetic framework in studies of bordetellosis. Serologic analysis of *B. bronchiseptica* reflects

TABLE 8. Estimates of clonal diversity (D_{clones}) of *B. bronchiseptica* among isolates from three host species

Host	No. of isolates	No. of clones	D_{clones}
Pig	166	6	0.071
Dog	57	12	0.732
Rabbit	38	5	0.572

variation in poorly characterized somatic antigens and measures variation in an unknown but probably small number of structural gene loci for surface molecules or loci encoding enzymes involved in the synthesis of surface epitopes. Serotypes represent character states of bacterial isolates, and this is especially true for *B. bronchiseptica*, which during cultivation in vitro frequently expresses changes in surface components and in other biological properties commonly found in fresh clinical isolates (3, 9, 10, 28, 37). Musser et al. (33) demonstrated that phenotypic modulation of *B. bronchiseptica* by growth in different media has no effect on the electrophoretic mobilities of the metabolic enzymes studied herein. The ability of multilocus enzyme electrophoresis to stably mark strains provides a high-resolution technique for prospective epidemiological studies of *B. bronchiseptica*.

Serologic techniques and analysis of phenotypic characters, as applied to *B. bronchiseptica*, provide no information about the overall genetic relatedness of isolates and have not been proven to be of use in distinguishing clones in systematic and population genetics research. Thus, the conclusions that can be drawn from studies in which strains are classified solely or predominantly on serologic or other phenotypic characters are limited. Population genetic analysis provides reasons for discontinuing the practice of using phenotypic characteristics to categorize isolates of *B. bronchiseptica*.

Because experimental evidence indicates that most enzyme polymorphisms are selectively neutral or nearly so (17), it is unlikely that enzyme variants are virulence factors. However, under clonal population structure, genes whose products are causally related to pathogenicity may be in linkage disequilibrium (nonrandom association) with genes encoding "housekeeping" enzymes. At a genetic distance of approximately 0.15, *B. bronchiseptica* consists of five distinct lineages or clusters of lineages, and data from in vivo pathogenicity studies of some of the strains analyzed herein suggest that clones of the A, B, D, and E lines vary in host specificity or pathogenicity for pigs and dogs.

Much remains to be learned about the relationship between bacterial clonal diversity, disease, host affinity, and the clinical severity of symptoms. Adequate data are not available for any pathogen, although there is evidence that particular clones of *E. coli* are nonrandomly associated with either meningitis or pyelonephritis (1, 44). In vivo pathogenicity trials in pigs, utilizing strains of *B. bronchiseptica* from each of the three numerically dominant lines, ET 1, ET 6, and ET 16 of clusters A, B, and D, respectively, would permit prospective evaluation of pathogenicity in relation to the population genetic framework described herein. Similar experiments with strains of ETs 1, 4, 6, and 13 in dogs might be particularly fruitful, because these ETs apparently predominate in canine disease and previous in vivo pathogenicity trials used strains of ETs 1 and 6 only (15).

ACKNOWLEDGMENTS

We thank D. A. Caugant and T. S. Whittam for discussion and critical comments. Strains were provided by L. Backstrom, M. M. Chengappa, J. M. Cockrill, R. F. DiGiacomo, E. L. Hewlett, D. C. Hoefling, P. Novotny, M. S. Peppler, R. M. Roop II, R. F. Ross, L. K. Schlater, and R. Sonn. Technical laboratory assistance was provided by Avis C. James, Christine M. Sommers, and Lynn M. Tremblay. Patricia E. Pattison assisted in preparation of the manuscript.

This research was supported by Public Health Service grant AI-22144 from the National Institutes of Health to R.K.S.

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