Deoxyribonucleotide Sequence Relationships Among *Bordetella* Species†

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Deoxyribonucleotide sequence relationships among currently recognized *Bordetella* species (*Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica*) were examined by deoxyribonucleic acid (DNA) hybridization involving the hydroxyapatite batch procedures of Brenner and co-workers. The results indicated that strains from all species tested were highly related. At the stringent criterion (80°C), the relative binding of *B. pertussis* DNA to *B. parapertussis* DNA was $75 \pm 9\%$, and to *B. bronchiseptica* DNA it was $73 \pm 8\%$. Intraspecies binding was $93 \pm 8\%$. Under similar conditions, the relative binding of *B. parapertussis* DNA to *B. bronchiseptica* DNA was $85 \pm 9\%$. The various so-called *Bordetella* species may be reconsidered as representing different sub-species belonging to a single species.

The genus *Bordetella* is currently composed of three phenotypically distinguishable species: *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* (8). Recognition of the species *B. pertussis* is especially important in human medicine as it is the major causative agent of whooping cough, or pertussis (5). The severity of pertussis is usually much less with *B. parapertussis* infection. *B. bronchiseptica* has been isolated most frequently from the respiratory tracts of a variety of other mammals and may produce such respiratory diseases as bronchopneumonia (8) and infectious atrophic rhinitis (9).

In this investigation, we examined the deoxyribonucleotide sequence relationships among *Bordetella* species. We also considered the question of whether or not separation of the organisms on different host species might have promoted speciation. Preliminary observations on the deoxyribonucleic acid relatedness of *Bordetella* species have been reported previously (3; W. E. Kloos, W. J. Dobrogosz, J. W. Ezzell, and B. R. Kimbro, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, H122, p. 124).

MATERIALS AND METHODS

Bacterial strains. The 16 strains studied and their sources are listed in Table 1. All of the *Bordetella* cultures were maintained on Bordet-Gengou agar slants (supplemented with 15% sheep blood) at 4°C. Each of the strains obtained for this study conformed to its original species description (3, 8).

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Cultivation and lysis of *Bordetella* species for DNA isolation. For the isolation of unlabeled deoxyribonucleic acid (DNA), *B. pertussis* strains were grown in a modified Stainer-Scholte broth medium containing 1% enzymatically hydrolyzed casein (3, 11). For the growth of cells to be used as inocula, the above-mentioned medium was supplemented with 1.5% agar (Difco Laboratories) and 1% activated charcoal (neutralized). *B. parapertussis* and *B. bronchiseptica* were grown in brain heart infusion broth (Difco Laboratories). Cells for lysis were produced as follows. Cells from a 24- to 36-h-old agar slant culture were suspended in 1 ml of saline ($\approx 1 \times 10^8$ to 5×10^8 colony-forming units) and were used to inoculate 500 ml of the appropriate broth medium (above) contained in a 2-liter Erlenmeyer flask. The inoculated cultures were shaken at 250 rpm at 34°C. *B. pertussis* cultures were shaken for a period of 36 to 48 h, and *B. parapertussis* and *B. bronchiseptica* cultures were shaken for 18 to 24 h, until the cell density reached approximately 5×10^8 to 5×10^9 colony-forming units per ml. Cultures were then centrifuged at 4°C, and the cell pellet was suspended in 50 to 100 ml of saline-ethylenediamine-tetraacetate buffer (0.1 M NaCl-0.05 M ethylenediaminetetraacetic acid, pH 7.0), containing 100 μ g of lysozyme per ml. Pronase B (Calbiochem) (500 μ g/ml) may be substituted for lysozyme with equally satisfactory results. The cell suspension was frozen overnight at -20°C. After it was thawed, the cell suspension was incubated at 37°C until lysis was clearly evident (usually within 20 min), at which time sodium lauryl sulfate was added to a final concentration of 2%.

For the isolation of labeled DNA, all *Bordetella* strains were grown in 750 ml of modified Stainer-Scholte broth medium, containing a mixture of 11 amino acids instead of casein hydrolysate (3), a reduced level of KH_2PO_4 (15 mg/750 ml), and carrier-free [^{32}P]orthophosphate (10 mCi/750 ml), in a 2-liter Erlenmeyer flask. Cultures were shaken at 250 rpm for

TABLE 1. *Strains used in this study*

Strain	Source	Obtained from ^a
<i>Bordetella pertussis</i>		
ATCC 10380	Human	ATCC
ATCC 12741	Human	ATCC
NCTC 10902	Human	NCTC
Tohama L5	Human	BB
BB-185	Human	BB
BB-134	Human	BB
<i>Bordetella parapertussis</i>		
NCTC 5952	Human	NCTC
ATCC 15237	Human	ATCC
ATCC 15989	Human	ATCC
ATCC 21449	Human	BB
<i>Bordetella bronchiseptica</i>		
ATCC 4617	Unknown	ATCC
ATCC 19395	Dog	ATCC
ATCC 31124	Dog	R. A. Goodnow ^b
K-1011	Unknown	M. J. Pickett ^c
K-1012	Guinea Pig	M. J. Pickett
<i>Rhizobium japonicum</i> (DNA)		
61A76	Soybean	G. H. Elkan ^d

^a ATCC, American Type Culture Collection, Rockville, Md.; NCTC, National Collection of Type Cultures, London, England; BB, Bureau of Biologics, Food and Drug Administration, Bethesda, Md.

^b Burns-Biotec, Omaha, Neb.

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24 to 48 h at 34°C. Cell lysis procedures were similar to those described above for unlabeled DNA.

Isolation of DNA. *Bordetella* species DNA was isolated according to a modification of the procedures of Brenner and co-workers (1; D. J. Brenner and A. G. Steigerwalt, personal communication) as reported by Kloos and Wolfshohl (4). DNA was fragmented by sonication with a Branson Sonifier cell disruptor (model 185) to a molecular size of about 0.5×10^5 to 1.0×10^5 . Labeled DNA fragments were further purified by passing them through a hydroxyapatite column at 60°C in 0.14 M phosphate buffer plus 0.4% sodium lauryl sulfate, after dissociation by heating at 100°C for 6 min. Preparations with a relatively high zero binding time value (>8%) were passed through the hydroxyapatite column once more.

DNA reassociation reactions. A 0.1- μ g amount of sheared, heat-denatured, [³²P]DNA was allowed to react with 120 μ g of sheared, heat-denatured, unlabeled DNA in 1 ml of 0.28 M phosphate buffer for 20 h at either 65 or 80°C (optimal and stringent reassociation criteria, respectively). The actual binding of labeled DNA fragments to unlabeled DNA from the same source (homologous reactions) was $76 \pm 9\%$. The concentration of labeled DNA was small enough to prevent significant reassociation of labeled DNA fragments with one another ($4 \pm 2\%$).

Separations of single- and double-stranded DNA. At the conclusion of reassociation reactions, double-stranded (hybridized) DNA was separated from unreacted, single-stranded DNA on hydroxyapatite by the batch procedure described by Brenner and co-workers (1). The percent relative binding was de-

termined by dividing the fraction of labeled DNA bound to hydroxyapatite in heterologous reactions by that bound in the homologous reaction and multiplying the quotient by 100.

RESULTS AND DISCUSSION

The results of reassociation reactions with [³²P]DNA from reference and type strains of *Bordetella* species are shown in Table 2. As shown in the data, the DNA relatedness among these so-called species is so close that all strains tested could be considered as belonging to a single species, although a formal proposal to this effect will not be made at this time. There is a suggestion of a very slight divergence among DNAs from *B. pertussis* and the other species, but certainly not to the extent generally accepted for different genospecies. At the stringent criterion, the relative binding of *B. pertussis* DNA to *B. parapertussis* was $75 \pm 9\%$, and to *B. bronchiseptica* DNA it was $73 \pm 8\%$; on the other hand, intraspecies binding was $93 \pm 8\%$. Under similar conditions the relative binding of *B. parapertussis* DNA to *B. bronchiseptica* DNA was $85 \pm 9\%$. In earlier preliminary studies (3), we determined that the thermal stability of heterologous DNA duplexes formed by the reassociation of DNAs between a selected strain of each of the *Bordetella* species was very high

TABLE 2. Reassociation reactions with [³²P]DNA from reference and type strains of *Bordetella* species

Source of unlabeled DNA	% relative binding of labeled DNA from:									
	<i>B. pertussis</i>				<i>B. parapertussis</i> NCTC 5952 ^a		<i>B. bronchiseptica</i>			
	ATCC 10380		BB-185				ATCC 19395 ^a		ATCC 31124	
	65°C	80°C	65°C	80°C	65°C	80°C	65°C	80°C	65°C	80°C
<i>B. pertussis</i>										
ATCC 10380	100 ^b	100			71	80	61	72		
ATCC 12741	98	92			74	76	62	67		
BB-185	104	84	100	100	60	69	63	62	80	74
BB-134	82	101	88	78	87	66	66	60	87	71
NCTC 10902			92	78					77	71
Tohama L5			96	83					79	76
<i>B. parapertussis</i>										
NCTC 5952 ^a	78	68			100	100	78	78		
ATCC 15237	81	74			103		78	86		
ATCC 15989	70	89	87	87	99	89	108	70	96	86
ATCC 21449	81	78	77	60	101	105	77	85	91	82
<i>B. bronchiseptica</i>										
ATCC 19395 ^a	87	84			94	94	100	100		
ATCC 4617	80	73			88	99	85	92		
K-1011	71	87	73	73	96	76	84	85	87	90
K-1012	71	72	79	75	72	98	75	93	93	97
ATCC 31124	69		85	85					100	100
<i>Rhizobium japonicum</i> 61A76	0	0	0	0	0	0	<1	<1	0	0

^a Type strain. ATCC 10380 was the proposed reference strain for *B. pertussis* at the time of this investigation. More recently, ATCC 9797 has been designated the type strain of *B. pertussis* (10) although additional study will be required to determine whether it is suitable for this purpose.

^b Relative binding data represent the average of duplicate reassociation reactions.

($\Delta T_{m(e)} = 0.7$ to 1.6). These results support our present observations on the relative binding of DNAs between various *Bordetella* species.

Since there are still some practical reasons for making a distinction on phenotypic grounds (3, 8), the three groups might be best considered as separate subspecies. *B. bronchiseptica* strains usually grow more rapidly and produce larger colonies than the others, utilize citrate, reduce nitrates, demonstrate strong urease activity, possess the specific heat-labile antigen factor 12, and are motile. The distinction between *B. pertussis* and *B. parapertussis* strains is not always clear, but usually *B. parapertussis* grows well on blood-free peptone agar and produces a browning of this medium (phase 4 *B. pertussis* strains also grow well on this medium, but the characteristically more virulent phase 1 strains do not). *B. parapertussis* strains also usually grow more rapidly on Bordet-Gengou agar than do *B. pertussis* strains, utilize citrate, demonstrate moderate urease activity, and possess the specific heat-labile antigen factor 14.

In contrast to accumulating evidence that certain cutaneous staphylococci have evolved concurrently with their hosts (2, 4), the very close

DNA relatedness of *Bordetella* species isolated from distantly related hosts (e.g., humans, dogs, and guinea pigs) would suggest that the genome of these bacteria has not diverged much with changes in host range. Perhaps the major habitat (i.e., mucosal surfaces of the trachea and bronchi and associated ciliated epithelial cells) of *Bordetella* species (6, 7) has not changed much with the evolution of certain mammals. If selective niche properties have not changed significantly, divergence and ultimate speciation of the inhabiting bacteria might be severely limited. An alternative explanation may be that the spread of *Bordetella* species to certain hosts (e.g., from lower mammals to humans) may be relatively recent in biological time.

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REPRINT REQUESTS

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