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## Restriction enzyme analysis and ribotyping distinguish *Bordetella avium* and *Bordetella hinzii* isolates

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### SUMMARY

Fifty-seven bacterial isolates previously identified as *Bordetella avium* or *B. hinzii* were characterized by restriction enzyme analysis (REA) and/or ribotyping. Twenty restriction endonucleases were evaluated for REA. Digestion of chromosomal DNA from the 42 *B. avium* and 15 *B. hinzii* isolates with *Hinf*I produced 8 and 7 distinct fingerprint profiles, respectively. Digestion with *Dde*I further discriminated these *Bordetella* species and produced 12 fingerprint profiles for *B. avium* and 4 profiles of *B. hinzii*. In addition, *B. avium* isolates were clearly distinguishable from *B. hinzii* isolates by ribotyping with the restriction endonuclease *Pvu*II. The ribotype patterns of these two species of *Bordetella* were unique when compared to previously reported ribotype patterns for *B. bronchiseptica* isolates. Since it was possible to discern differences among isolates within each *Bordetella* species by REA analysis, we suggest that REA could be used in developing a typing system based on the fingerprint profiles generated.

### INTRODUCTION

The genus *Bordetella* is presently comprised of seven species. *B. pertussis* is the causative agent of whooping cough in humans, whereas *B. parapertussis* causes a milder form of whooping cough. *B. bronchiseptica* is a common respiratory pathogen in a number of animal species but is a rare cause of human infection. Two recently recognized members of the genus *Bordetella* include *B. holmesii*, which has been primarily isolated from immunocompromised adults with septicemia [1], and *B. trematum*, an organism isolated from wounds and ear infections in humans [2]. *B. avium* causes respiratory tract infections in poultry (coryza or rhinotracheitis). Organisms previously referred to as *B. avium*-like or *Alcaligenes faecalis* type II have been renamed *B. hinzii* [3]. Although these latter organisms are isolated from diseased birds, there is little evidence that they are pathogenic. Four isolations of *B. hinzii* from humans have been reported, including recent

isolations from blood of an AIDS patient [4] and from sputum of a patient with cystic fibrosis [5]. Distinguishing among *Bordetella* species has been based on biochemical and physiological characteristics, whole cell protein profiles, fatty acid analysis, DNA base ratio determinations, and/or DNA–DNA or DNA–rRNA hybridization [3, 6–10].

Methods such as REA of chromosomal DNA or DNA fingerprinting and analysis of restriction fragment length polymorphisms of rRNA genes, or ribotyping, may have power in discriminating among *Bordetella* strains for epidemiologic purposes. Indeed, ribotyping has recently been utilized to characterize *B. bronchiseptica* isolates from several animal species and was shown to provide a basis for grouping of these organisms into distinct types [11]. Moreover, REA and ribotyping have been utilized in molecular epidemiological studies of a number of bacterial species [12, 13]. The utility of DNA fingerprinting for distinguishing *Bordetella* species has been questioned since it was reported that RFLP analysis using

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frequently cutting restriction enzymes failed to discriminate among *Bordetella pertussis*, *B. parapertussis* or *B. bronchiseptica* isolates [14]. However, in that study chromosomal DNAs were digested only with *EcoRI* and, evidently, no other frequently cutting restriction enzymes were examined for use in RFLP analysis. In generating well-separated, easily distinguishable DNA fragments for fingerprint analysis, previous investigators have found it necessary to examine several restriction enzymes [15, 16]. Therefore, in the present study we have compared 20 restriction enzymes for use in REA of *B. avium* and *B. hinzii* isolates. In addition, since ribotyping has proven useful as a method for discriminating among *B. bronchiseptica* isolates, we have further examined ribotyping as a method of distinguishing *B. avium* isolates from *B. hinzii* isolates.

## METHODS

### Bacterial isolates

The avian isolates selected from the collection at the National Animal Disease Center and utilized in the present experiments are listed in Table 1. These isolates had been previously identified as *B. avium*, *B. avium*-like, *B. bronchiseptica*, *Alcaligenes faecalis* or *A. odorans* on the basis of biochemical and physiological characteristics [6]. Additional strains included reference strains of *Alcaligenes* from the Centers for Disease Control (Atlanta, GA), a vaccine strain (17) of *B. avium* (Art-Vax), type strains of *B. avium* (ATCC 35086<sup>T</sup>; originally isolated in Germany) and *B. hinzii* (ATCC 17583<sup>T</sup>; originally isolated in Australia), and the following human isolates: *B. hinzii* L60 (4), which was kindly provided by Dr Brad Cookson, University of Washington, Seattle, and *B. hinzii* DMMZ 1277 and DMMZ 1280 (5), which were kindly provided by Dr Reinhard Zbinden, Institute for Medical Microbiology, Zurich, Switzerland.

### Restriction enzyme analysis

Bacterial strains were grown on blood agar base slants (Difco, Detroit, MI) for 48 h at 37 °C. Bacterial cells were harvested and adjusted to a similar concentration in 0.85 M NaCl as previously described [15]. A 1.5 ml aliquot of the bacterial cells were centrifuged at 16000 g for 4 min. The supernatant was decanted; pellets were stored at –70 °C. DNA was isolated using

Table 1. *Geographic origin of isolates in the National Animal Disease Center collection used in the present study\**

Number of isolates	Designation	Geographic origin
7	P-4081 to P-4087	North Carolina
8	P-4088 to P-4089 P-4090 to P-4095	Germany
10	P-4134 to P-4143	Minnesota
23	P-4147 to P-4169	Ohio
7	P-4480 to P-4486	Iowa
4	P-4506 to P-4509	South Africa

\* These isolates were previously characterized as *B. avium*, *B. avium*-like, *B. bronchiseptica*, or *Alcaligenes* spp. based on biochemical characteristics.

a commercially available kit according to recommendations of the manufacturer (DNAzol<sup>®</sup>, Gibco–BRL, Gaithersburg, MD).

The following restriction enzymes (Gibco–BRL) were tested: *AluI*, *BglII*, *ClaI*, *DraI*, *DdeI*, *EcoRI*, *EcoRV*, *HaeIII*, *HhaI*, *HindIII*, *HinfI*, *HpaI*, *HpaII*, *MvaI*, *NciI*, *PvuII*, *PstI*, *RsaI*, *TaqI* and *XbaI*. Digestion of chromosomal DNA with each restriction enzyme was carried out via the recommendations of the manufacturer. The reactions were stopped by the addition of 5 µl of stop solution (0.25% bromophenol blue, 0.25% xylene cyanole, 25% Ficoll 400) to 21 µl of reaction mixture. The digested DNA fragments were electrophoresed in 0.7% agarose gels using TBE buffer (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA, pH 8.0). A *HindIII* digest of lambda phage DNA was used as a molecular size marker. Gels were stained and photographed as previously described [15]. Photographs were scanned for computer analysis using a Scanjet IICx with DeskScan software (Hewlett–Packard, Boise, ID). GelCompar software (Applied Maths, Kortrijk, Belgium) was used for comparison of fingerprint profiles. Similarity between all possible pairs of fingerprint profiles using the coefficient of Dice [18] was calculated by the cluster analysis module of the software. Dendrograms were derived from a matrix of similarity values by the unweighted pair group method using arithmetic averages.

### Ribotyping

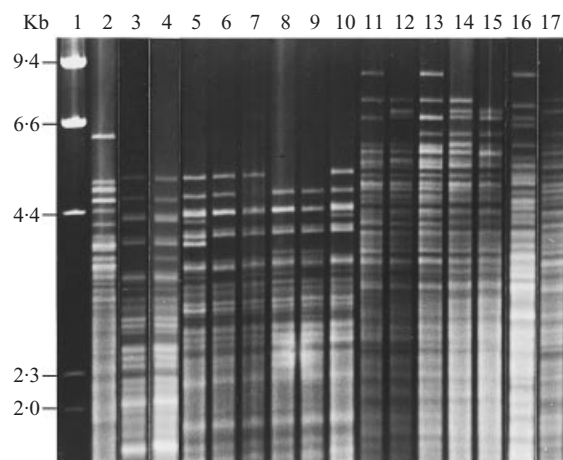
Methods for ribotyping were similar to those previously described for *B. bronchiseptica* isolates [11]. Genomic DNA was isolated using a commercially

available kit per recommendations of the manufacturer (Promega, Madison, WI). DNA precipitates were dissolved by overnight incubation at room temperature in 10 mM Tris-1 mM EDTA (pH 8.0). Concentration of DNA samples was determined spectrophotometrically. A 3- $\mu$ g sample of DNA was digested with 10 U of *Pvu*II at 37 °C overnight. Following incubation, loading dye (0.25% bromophenol blue, 30% glycerol) was added, and samples were electrophoresed in 0.6% agarose gels containing 0.5  $\mu$ g of ethidium bromide per ml in TBE buffer. A 1- to 12-kb DNA ladder (Boehringer-Mannheim, Indianapolis, IN) was included in each gel. Restriction fragments were transferred to charged nylon membranes by overnight capillary transfer in 10 $\times$ SSC (1 $\times$ SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0). Following transfer, DNA was fixed to the membranes by u.v. crosslinking with a u.v. Stratalinker (Stratagene, La Jolla, CA). Prehybridization and hybridization were carried out at 42 °C in maleic acid-based buffer, as described previously [11]. The hybridization solution contained 10 ng of digoxigenin-labelled pRRNB per ml in 5 $\times$ SSC-50% formamide-0.02% sodium dodecyl sulphate (SDS)-0.1% *N*-laroylsarcosine-2% blocking reagent-20 mM sodium maleate. Plasmid pRRNB contains a 5.4-kb fragment of the *Escherichia coli* rRNA operon *rrnB* [19]. Following removal from the hybridization solution, the membranes were washed twice for 5 min at room temperature in 2 $\times$ SSC containing 0.1% SDS and twice for 15 min at 65 °C in 0.5 $\times$ SSC containing 0.1% SDS. Bound probe was detected by using an anti-digoxigenin-alkaline phosphatase conjugate and LumiPhos as described (11).

## RESULTS

### Restriction enzyme analysis

Twenty restriction endonucleases were evaluated for use in REA of *B. avium* and *B. hinzii* isolates. Of the enzymes evaluated, *Hinf*I digestion of DNA resulted in well-separated and distinguishable bands in the 3–10 kb molecular size range. Digestion of DNA with *Dde*I produced fingerprint profiles with bands in the 3–23.1 kb molecular size range, which for certain isolates of *B. avium* were not as easily distinguished as bands generated by *Hinf*I digestion. However, we have included *Dde*I in our analyses since it has allowed the further discrimination of specific *B. avium*

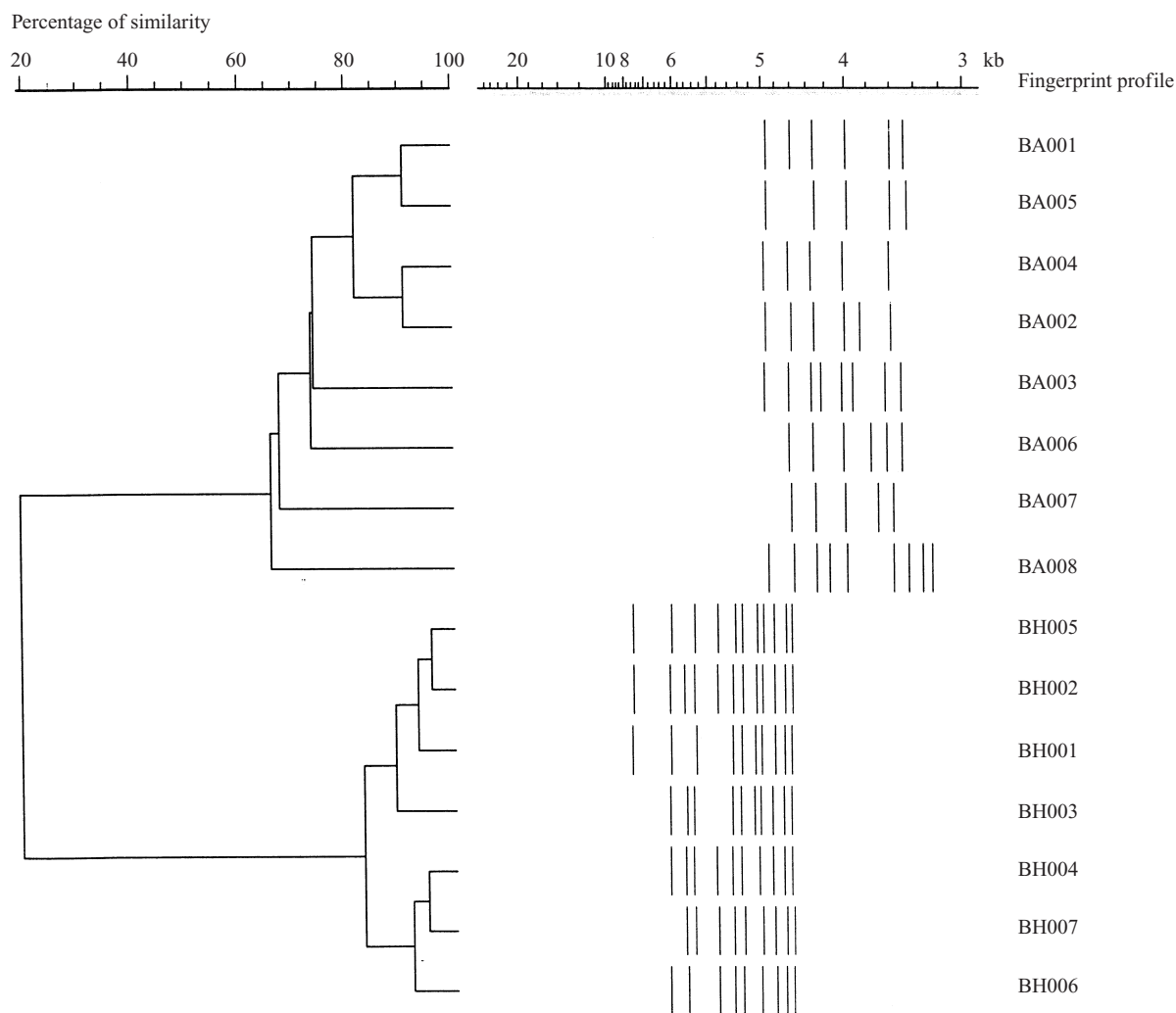


**Fig. 1.** Representative DNA fingerprint profiles of *B. avium* and *B. hinzii* isolates following *Hinf*I restriction enzyme digestion of chromosomal DNA. Lane 1, Molecular size marker, lambda phage *Hind*III digest; lane 2, *A. faecalis*; lanes 3–10, *B. avium* *Hinf*I profiles BA001 through BA008; lanes 11–17 *B. hinzii* *Hinf*I profiles BH001, BH003, BH005, BH004, BH007, BH002 and BH006. Fragments in the 3–10 kb molecular size range are readily distinguished for these isolates.

isolates. Use of the other endonucleases resulted in bands which could not be readily distinguished, especially in the 3–23.1 kb molecular size range, where optimum resolution occurs under the electrophoresis conditions used in this study.

Eight distinct DNA fingerprint profiles were found among the 42 *B. avium* isolates examined using *Hinf*I restriction endonuclease digestion. These profiles were assigned numbers BA001–BA008, and examples of each profile are shown in Figure 1 (lanes 3–10). Distinct bands were resolved in the 3–10 kb molecular size range. Isolates from each geographic region were represented in at least two *Hinf*I fingerprint profiles. Interestingly, the Art-Vax vaccine strain of *B. avium* (Fig. 1, lane 7) lacked a single band of 4.7 kb found in all other *B. avium* strains examined. Seven distinct profiles were observed following *Hinf*I restriction endonuclease digestion of DNA from the 15 *B. hinzii* isolates (Fig. 1, lanes 11–17). Furthermore, REA using *Hinf*I digestion readily distinguished the *Bordetella* species from *A. faecalis* (Fig. 1, lane 1). Genetic diversity among *B. avium* isolates was considerable, with similarity 65–90% (Fig. 2). Percent similarity among *B. hinzii* isolates was 80–95%. Between *B. avium* and *B. hinzii* isolates there was 20% similarity.

Twelve distinct profiles were observed following



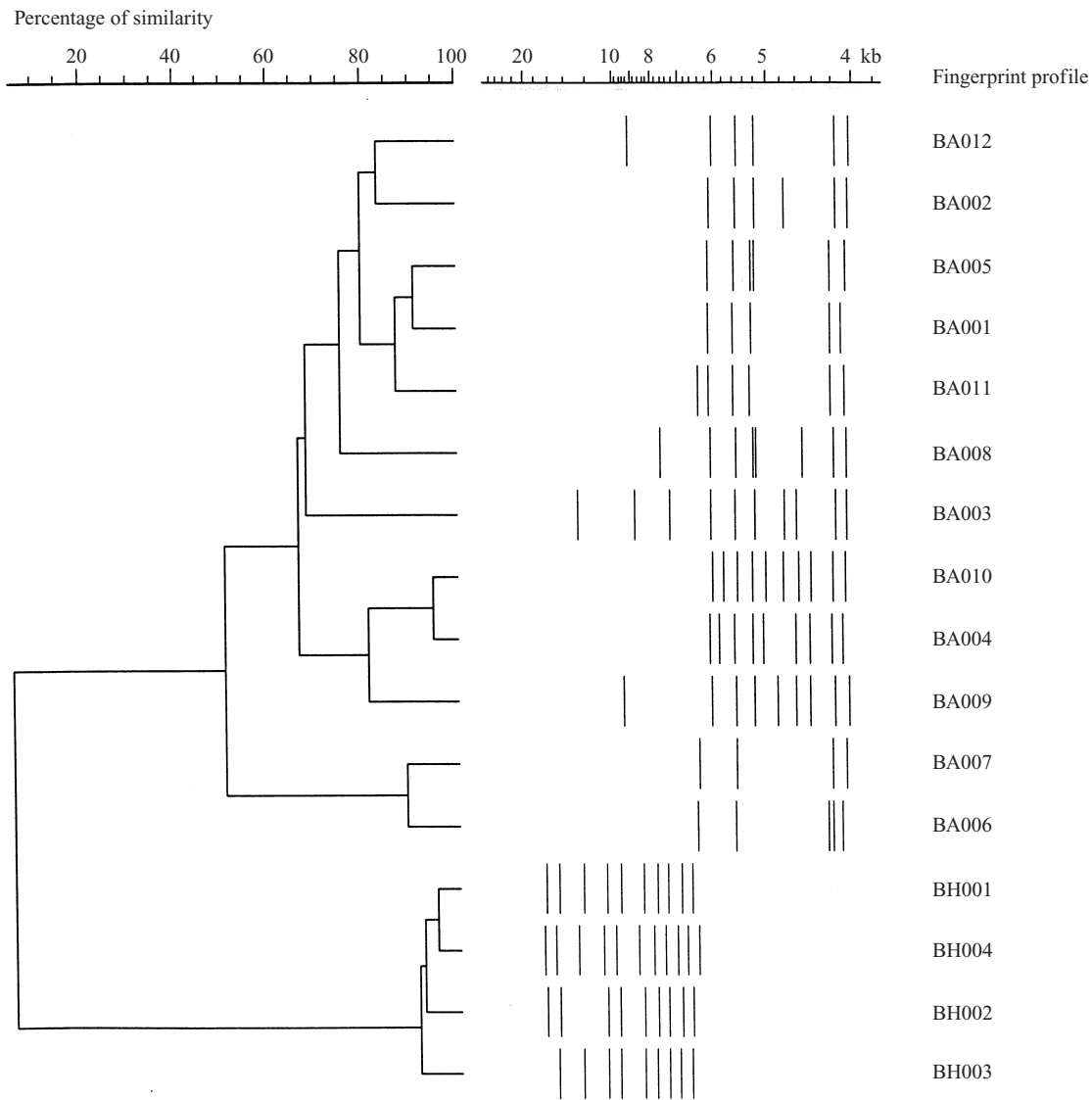
**Fig. 2.** Dendrogram showing percent similarity among *B. avium* and *B. hinzii* isolates using *Hinf*I restriction endonuclease digestion of chromosomal DNA. Each of the 8 profiles observed for 42 *B. avium* isolates and the 7 profiles observed for 12 *B. hinzii* isolates are shown. Similarity between fingerprint profiles using the coefficient of Dice was calculated by the cluster analysis module of GelCompar software.

*Dde*I digestion of DNA isolated from *B. avium* strains. The use of this restriction endonuclease allowed for further discrimination of specific *B. avium* isolates (Fig. 3). However, digestion with *Dde*I resulted in only four profiles for *B. hinzii* isolates and was, therefore, less discriminatory for this species of *Bordetella*. Percent similarity among *B. avium* isolates using *Dde*I restriction endonuclease was 50–95% and for *B. hinzii* isolates was 90–95% (Fig. 3). Similarity between *B. avium* and *B. hinzii* was 15%. Based on *Hinf*I and *Dde*I restriction enzyme analysis, the *B. avium* isolates were categorized into 16 distinct fingerprint profiles, whereas there were 7 distinct fingerprint profiles for *B. hinzii* isolates (Table 2). The human *B. hinzii* strain L60 originally isolated from an AIDS patient in Washington had the same REA

profile as the *B. hinzii* type strain originally isolated from a chicken in Australia. In contrast, the two human *B. hinzii* strains isolated from a patient in Switzerland had a unique REA profile.

### Ribotyping

Based on previous ribotyping experiments performed with *B. bronchiseptica* isolates [11], *Pvu*II restriction endonuclease digestion followed by Southern blotting and hybridization with pRRNB was utilized in the ribotyping of selected *B. avium* and *B. hinzii* isolates. There were two different ribotype patterns observed for seven *B. avium* isolates representing each geographic location and six different DNA fingerprint



**Fig. 3.** Dendrogram showing percent similarity among *B. avium* and *B. hinzii* isolates using *DdeI* restriction endonuclease digestion of chromosomal DNA. Each of the 12 profiles observed for *B. avium* and the 4 profiles observed for *B. hinzii* isolates are shown. Similarity between fingerprint profiles using the coefficient of Dice was calculated by the cluster analysis module of Gel Compar software.

profiles (Fig. 4). A distinguishing characteristic of ribotypes of *B. avium* was the cluster of four bands greater than 8 kb. The two *B. avium* ribotypes differed in the molecular size of a single fragment between 3 and 4 kb. Of the eight isolates of *B. hinzii* examined, there were two distinct ribotype patterns observed which were readily distinguished from those of *B. avium* (Fig. 5). Seven isolates (3 from humans, 3 from turkeys and the type strain) had the same ribotype pattern; the single isolate exhibiting a unique pattern was a *B. hinzii* isolate from a chicken in South Africa. There was no association between ribotype pattern and geographic location for either *B. avium* or *B. hinzii* isolates.

### DISCUSSION

REA is a highly discriminatory method for determining phylogenetic relationships and has been utilized by previous investigators in examining the molecular epidemiology of genetically diverse strains. In the present study, 20 restriction endonucleases were evaluated for use in REA of *B. avium* and *B. hinzii* isolates. Digestion of chromosomal DNA with *HinfI* or *DdeI* resulted in DNA fragments which were more readily distinguishable than fragments generated by digestion with the other restriction enzymes examined. Furthermore, we found that there is sufficient genetic diversity in *B. avium* and *B. hinzii* isolates such that

Table 2. REA fingerprint profiles of *Bordetella avium* and *Bordetella hinzii* isolates in the present study\*

Taxon	Number of isolates and origin	Fingerprint profile
<i>Bordetella avium</i>	14 OH, MN, NC, Germany	<i>Hinf</i> I BA001 <i>Dde</i> I BA001
	2 OH	<i>Hinf</i> I BA001 <i>Dde</i> I BA004
	1 South Africa	<i>Hinf</i> I BA001 <i>Dde</i> I BA006
	1 IA	<i>Hinf</i> I BA001 <i>Dde</i> I BA008
	1 IA	<i>Hinf</i> I BA001 <i>Dde</i> I BA009
	1 MN	<i>Hinf</i> I BA001 <i>Dde</i> I BA011
	9 OH, IA, NC	<i>Hinf</i> I BA002 <i>Dde</i> I BA001
	1 IA	<i>Hinf</i> I BA002 <i>Dde</i> I BA005
	3 IA, MN, Germany	<i>Hinf</i> I BA003 <i>Dde</i> I BA005
	1 South Africa	<i>Hinf</i> I BA004 <i>Dde</i> I BA006
	1 South Africa	<i>Hinf</i> I BA004 <i>Dde</i> I BA007
	1 Vaccine strain	<i>Hinf</i> I BA005 <i>Dde</i> I BA001
	1 NC	<i>Hinf</i> I BA006 <i>Dde</i> I BA010
	2 Germany	<i>Hinf</i> I BA007 <i>Dde</i> I BA002
	2 Germany	<i>Hinf</i> I BA007 <i>Dde</i> I BA003
	1 IA	<i>Hinf</i> I BA008 <i>Dde</i> I BA012
	<i>Bordetella hinzii</i>	1 MN
2 OH		<i>Hinf</i> I BH002 <i>Dde</i> I BH002
5 OH, MN, WA, Australia		<i>Hinf</i> I BH003 <i>Dde</i> I BH001
2 OH, MN		<i>Hinf</i> I BH004 <i>Dde</i> I BH001
2 MN		<i>Hinf</i> I BH005 <i>Dde</i> I BH003
1 South Africa		<i>Hinf</i> I BH006 <i>Dde</i> I BH001
2 Switzerland		<i>Hinf</i> I BH007 <i>Dde</i> I BH004

\* For assignment of fingerprint profiles, isolates characterized as *B. bronchiseptica* or *Alcaligenes* spp. were not included. Abbreviations for origin: IA, Iowa; OH, Ohio; MN, Minnesota; NC, North Carolina; WA, Washington.

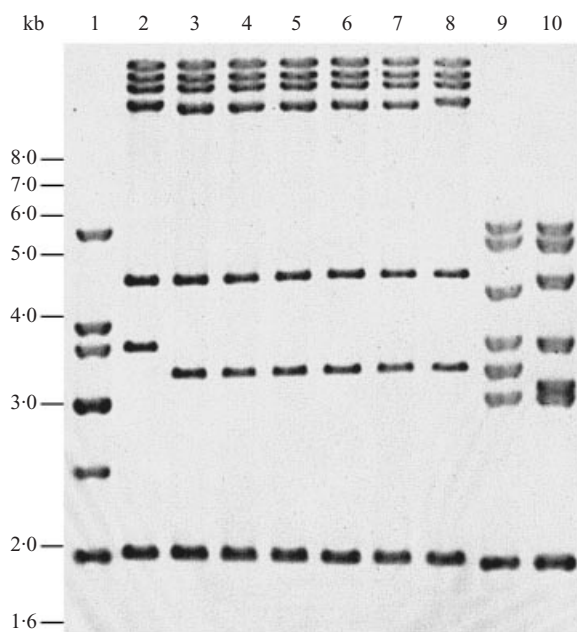


Fig. 4. Lumigraph showing ribotype profile comparison of *B. avium* and *B. bronchiseptica* isolates. Lanes 1, 9, 10: *B. bronchiseptica* isolates representing ribotypes 1, 9 and 10; lanes 2–8: representative *B. avium* isolates. Chromosomal DNAs were digested with *Pvu*II. Note 4 bands greater than 8 kb unique for *B. avium* isolates.

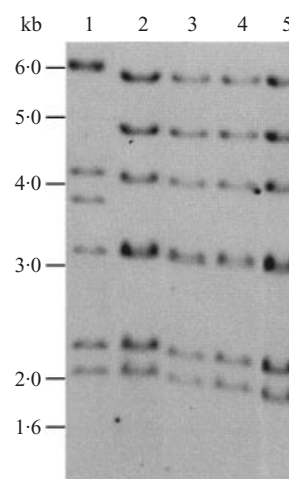


Fig. 5. Lumigraph showing ribotype profiles of representative *B. hinzii* isolates. Lane 1: chicken isolate; lanes 2, 3 and 5: turkey isolates; lane 4: human isolate. Chromosomal DNAs were digested with *Pvu*II.

REA has power in discriminating among isolates within these species. Restriction enzyme analysis using *Hinf*I and *Dde*I resulted in 16 distinct fingerprint profiles for 42 *B. avium* isolates from 6 different geographic locations. The majority of isolates were

classified into two fingerprint profiles, and interestingly, the only isolate found for *Hinf*I profile BA005 was the Art-Vax vaccine strain of *B. avium*, which lacks a single band of 4.7 kb found in all field isolates of *B. avium* examined in the present study. Thus, it is possible that *Hinf*I digestion of chromosomal DNA could be used to discriminate the vaccine strain from field isolates of *B. avium*. For *B. hinzii*, we found 7 distinct DNA fingerprint profiles among the 15 isolates examined.

Another widely used technique for discriminating among bacterial strains, ribotyping, was also employed in this study. The *B. avium* ribotype patterns were strikingly different from those previously observed for *B. bronchiseptica* isolates [11]. Most notably, there were four fragments greater than 8 kb observed in *B. avium* isolates not seen in numerous ribotype profiles of *B. bronchiseptica* isolates or in ribotype profiles of *B. hinzii* isolates examined in the present study. The ribotype patterns of *B. hinzii* were also distinct from those previously seen for *B. bronchiseptica* isolates. However, ribotyping did not appear to discriminate among *B. avium* or *B. hinzii* isolates as well as REA.

Previous techniques utilized to distinguish among *B. avium*, *B. hinzii* and *A. faecalis* isolates included biochemical and physiological characteristics, whole cell protein profiles, fatty acid analysis, DNA base ratio determinations, and/or DNA–DNA or DNA–rRNA hybridization. Methods which rely on stable genetic elements for classification of bacterial strains, such as REA and ribotyping, should be more reproducible than expression based methods. While ribotyping has been shown to be useful in discriminating among *B. bronchiseptica* isolates, the utility of DNA fingerprinting for *Bordetella* species has been questioned since it was previously reported that RFLP analysis using frequently cutting restriction enzymes failed to discriminate among *Bordetella pertussis*, *B. parapertussis* or *B. bronchiseptica* isolates when chromosomal DNAs were cut with *Eco*RI [14]. In our experiments, we found that restriction enzyme digestion of chromosomal DNA with *Eco*RI produced numerous bands in the 3–23.1 kb molecular size range such that discrimination among *Bordetella* isolates was not possible. However, the results presented herein demonstrate that REA using *Hinf*I or *Dde*I and ribotyping using *Pvu*II are useful in discriminating between *B. avium* and *B. hinzii* isolates. Since neither method is technically difficult, the combination of REA and ribotyping should prove useful in molecular epidemiological studies of *Bordetella* species.

There is presently no system available for typing of *B. avium* or *B. hinzii* strains. We propose that isolates could be assigned a descriptive identification epithet (DIE) based on fingerprint profiles generated by REA. For example, a *B. avium* isolate which has fingerprint profiles *Hinf*I BA001 and *Dde*I BA006 would be described as DIE code *B. avium Hinf*I BA001–*Dde*I BA006, whereas a *B. hinzii* isolate with profiles *Hinf*I BH007 *Dde*I BH004 would be described as DIE code *B. hinzii Hinf*I BH007–*Dde*I BH004. Numerous fingerprint profiles could be analysed and used to generate a DNA fingerprint data base from which individual isolates could be easily assigned a DIE code. Assignment of a DIE code would permit the orderly classification of *B. avium* and *B. hinzii* isolates not presently available.

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