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DNA Polymorphism in Strains of the Genus *Brucella*

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Preparations of DNA from 23 *Brucella* strains including 19 reference strains were compared by restriction endonuclease analysis. Pulsed-field gel electrophoresis resulted in optimal resolution of fragments generated by digestion with low-cleavage-frequency restriction enzymes such as *Xba*I. By this technique, five electrophoretypes were distinguished in five reference strains of the different species, i.e., *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*, and *B. ovis*. Minor profile differences allowed us to discriminate between most biovars within a species. However, the differences in the DNA patterns of different field strains of biovar 2 of *B. melitensis* were not sufficient to serve as markers for epidemiological studies. From the *Xba*I fragments, we were able to estimate the size of the genomes of *B. abortus* 544^T and *B. melitensis* 16 M^T. This method revealed a relationship between DNA fingerprints, species, and pathovars which could shed light on problems concerning the classification and evolution of members of the genus *Brucella*.

The taxonomy of the genus *Brucella* is still controversial. In *Bergey's Manual of Systematic Bacteriology* (5) the genus is divided into six species: *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* on the basis of growth and biochemical characteristics. However, by DNA-DNA hybridization, Hoyer and MacCullough (7, 8) found that six strains, one from each of the six species, showed very close homology. This work was later confirmed with 51 strains by Verger et al. (22), who used using a more modern DNA-DNA hybridization method (S1 nuclease method). Because of the DNA relatedness of 96 ± 5% found among all the strains studied, these authors proposed "that only one species, *B. melitensis*, be recognized in the genus" and "that other specific epithets. . . be used in a vernacular form for biovar designation."

Analysis by using restriction endonucleases, which is another method for studying DNA relationships, has been used to differentiate strains of viruses (21) and bacteria (2, 10, 11, 14, 16, 20) and to study plasmids (13). This technique was used in the present work for DNA analysis of 23 *Brucella* strains, including type strains. As previously demonstrated by O'Hara et al. (17), the commonly used restriction endonucleases (such *Eco*RI or *Hin*dIII) demonstrate only minor differences between *Brucella* strains. DNA digestion by low-cleavage-frequency restriction endonucleases, such as *Xba*I or *Not*I, gives rise to fewer fragments, which are well separated by pulsed-field gel electrophoresis, as described by Schwartz and Cantor (18); this presumably improves the sensitivity of the method. In the present study, this technique revealed genomic differences between species and biovar strains, confirming that at the DNA level the use of the natural host is a valid characteristic for classifying the members of the genus *Brucella*.

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MATERIALS AND METHODS

Bacterial strains. Table 1 lists the sources of *Brucella* strains used in this study. All strains came from the National Reference Center of Brucellosis, Montpellier, France. Species and biovars were characterized according to the recommendations of the World Health Organization (1). Cultures were grown on Brucella agar (Difco Laboratories) supplemented with 20% (vol/vol) glycerol. Cultures were checked for purity and kept frozen until DNA was prepared.

DNA preparation. Two methods were used to prepare the DNA. First, DNA was prepared in the liquid phase by a conventional method based on that of Brenner et al. (3). Second, DNA was prepared directly in a solid plug, called a gel insert, as described by Schwartz et al. (19), modified as follows. Agarose (1%: Appligene) in phosphate-buffered saline was prepared as described by Jackson and Cook (9). Equal volumes of agarose and a *Brucella* suspension of 5 × 10^9/ml were mixed in phosphate-buffered saline and dispensed in a slot former. Inserts were then incubated with a mixture of 0.5 M EDTA, 1% (wt/vol) sodium dodecyl sulfate, and 1 mg of pronase (Calbiochem) per ml for 48 h at 37°C. Protein digestion products were removed by washing the inserts twice for 1 h at 37°C in 10 mM Tris–0.1 mM EDTA (pH 7.5) (TE buffer) plus 1 mM phenylmethylsulfonyl fluoride and then three times in TE buffer alone for 1 h at room temperature.

Restriction enzyme digestion of DNA. DNA (5 μg) prepared by the first method was digested with 30 to 60 U of *Eco*RI or *Hin*dIII (New England BioLabs, Inc.) in a total volume of 50 μl of 2 h as recommended by the manufacturer. One insert of DNA was incubated overnight with 150 U of *Not*I, *Sfi*I, *Xba*I, or *Xho*I (New England BioLabs) in a total volume of 120 μl.

Gel electrophoresis. DNA digested with *Eco*RI or *Hin*dIII was separated by conventional electrophoresis (12). Fragments of DNA generated by *Not*I, *Sfi*I, *Xba*I, or *Xho*I were separated by pulsed-field gel electrophoresis, originally developed by Schwartz and Cantor (18), with an apparatus

TABLE 1. Strains used in this study

Species	Species biovar	Strain[a]	Host	Geographic origin
B. melitensis	Biovar 1	16M^T (ATCC 23456^T)	Goat	United States
	Biovar 2	63/9 (ATCC 23457)	Goat	Turkey
		1381	Cattle	France (Gap)
		1572	Human	France (Toulon)
		1574	Human	France (Marseille)
		1575	Sheep	France (Avignon)
		1577	Human	France (Paris)
		1578	Sheep	France (Draguignan)
		1590	Human	France (St. Dié)
	Biovar 3	Ether (ATCC 23458)	Goat	Italy
B. abortus	Biovar 1	544^T (ATCC 23448^T)	Cattle	England
	Biovar 2	86/8/59 (ATCC 23449)	Cattle	England
	Biovar 3	Tulya (ATCC 23450)	Human	Uganda
	Biovar 4	292 (ATCC 23451)	Cattle	England
	Biovar 5	B3196 (ATCC 23452)	Cattle	England
	Biovar 6	870 (ATCC 23453)	Cattle	Africa
	Biovar 9	C68 (ATCC 23455)	Cattle	England
B. suis	Biovar 1	1330^T (ATCC 23444^T)	Swine	United States
	Biovar 2	Thomsen (ATCC 23445)	Swine	Denmark
	Biovar 3	686 (ATCC 23446)	Swine	United States
	Biovar 4	40 (ATCC 23447)	Reindeer	USSR
B. ovis		63/290^T (ATCC 25840^T)	Sheep	Africa
B. canis		RM6/66^T (ATCC 23365^T)	Dog	United States

[a] Type strains and biovar reference strains are listed first by their Food and Agriculture Organization/World Health Organization designations and then by their American Type Culture Collection numbers. Wild-type strains are listed by the numbers of the *Brucella* culture collection of Montpellier, France.

locally constructed on the principle of the CHEF machine (4) and a Consort Bioblock power supply; a 1.5% agarose gel prepared and run in 0.0445 M Tris (pH 8)–0.0445 M boric acid–0.001 M EDTA (0.5× TBE) was used. The pulse times were 5 s for 24 h at 200 V and then 10 s for another 24 h at 200 V. Polymerized bacteriophage lambda DNA ladders and lambda DNA digested with *Hin*dIII were used as molecular weight markers. Lambda ladders were produced by the following method. Lambda DNA (18 μg; Appligene) was ligated with 24 U of T4 DNA ligase (New England BioLabs) for 48 h at 12°C. Polymerized DNA (1.4 μg) was loaded onto the gel after incubation for 15 min at 65°C. At the end of the run, the gels were immersed for 45 min in running buffer containing 0.5 μg of ethidium bromide per ml and photographed under UV light with a Polaroid CU camera.

RESULTS

Digestion of the DNA by high-cleavage-frequency restriction enzymes. When using high-cleavage-frequency restriction enzymes, we first tried enzymes that recognize 6-base-pair sites, such as *Hin*dIII and *Eco*RI. In the *Eco*RI digest (Fig. 1), the highest bands (10- to 20-kilobase (kb) fragments) were well separated. The patterns clearly showed great similarity. The majority of the bands were identical from strain to strain. Within the large fragments, some bands were missing in some strains and the highest bands migrated at slightly different positions, but these differences were not sufficient to separate one biovar or species from another. No differences could be seen with *Hin*dIII (data not shown).

Choice of the low-cleavage-frequency restriction enzyme. The 8-base-pair recognition site restriction enzymes *Not*I and *Sfi*I were used to cleave *Brucella* DNA. These enzymes were expected to have fewer recognition sites on the genome and thus to produce fewer and larger fragments than the endonucleases that recognize 6-base-pair sites. We obtained nearly 40 bands on the restriction pattern. The recognition sequences of these two enzymes contain G and C nucleotides and are relatively frequent in this DNA, which has a high G+C content (between 57.9 and 59.2 mol%) (6).

One of the restriction enzymes, *Xba*I, has 6-base-pair recognition sites that occur relatively rarely in various DNAs (15). This was the case with *Brucella* DNA, and after digestion of the DNAs with this nuclease we obtained large

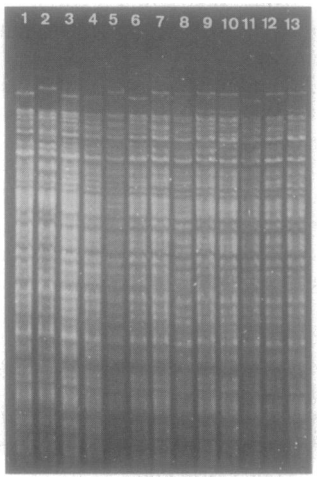

FIG. 1. *Eco*RI digestion of DNA from *Brucella* strains. Lanes: 1, *B. canis* RM6/66^T; 2, *B. ovis* 63/290^T; 3, *B. suis* 40; 4, *B. suis* 686; 5, *B. suis* Thomsen; 6, *B. suis* 1330^T; 7, *B. melitensis* Ether; 8, *B. melitensis* 16M^T; 9, *B. abortus* 870; 10, *B. abortus* B3196; 11, *B. abortus* Tulya; 12, *B. abortus* 86/8/59; 13, *B. abortus* 544^T.

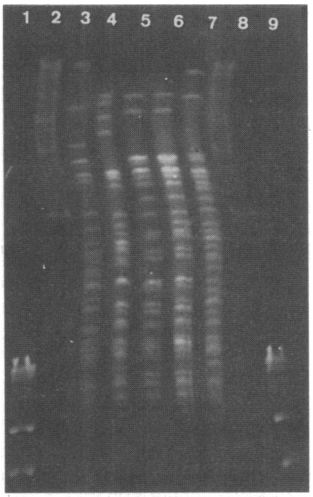

FIG. 2. *Xba*I digestion of DNA from strains of different *Brucella* species. Lanes: 3, *B. melitensis* 16M^T; 4, *B. abortus* 544^T; 5, *B. suis* 1330^T; 6, *B. canis* RM6/66^T; 7, *B. ovis* 63/290^T; 1 and 9, lambda DNA digested with *Hin*dIII; the bands represent (from above) DNA fragment sizes of 23.1, 9.4, and 6.6 kb, respectively. In lanes 2 and 8 are lambda concatemers (from above) of 350, 300, 250, 200, 150, 100, and 50 kb, respectively.

fragments and fewer bands, which could be separated by pulsed-field gel electrophoresis. We obtained the same result with another enzyme that has 6-base-pair recognition sites, *Xho*I (data not shown). Both enzymes provided 25 to 30 bands of various intensities, which were fairly well spread out along the electrophoretic lane.

***Xba*I restriction patterns of species and biovar strains.** Figure 2 shows the patterns of representative strains of the different species, *B. melitensis*, *B. abortus*, *B. suis*, *B. canis*, and *B. ovis*, each of which had a specific restriction pattern or fingerprint; however, only one band distinguished *B. suis* and *B. canis*.

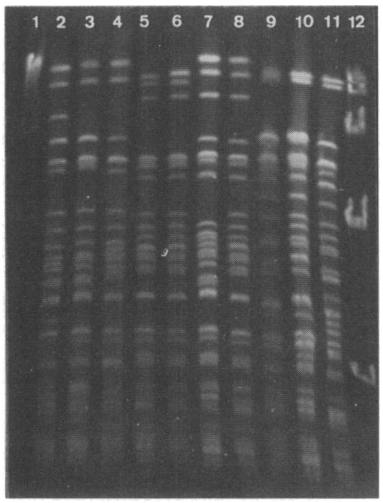

FIG. 3. *Xba*I digestion of DNA from strains of different *Brucella* biovars. Lanes: 2, *B. melitensis* 16M^T; 3, *B. melitensis* 63/9; 4, *B. melitensis* Ether; 5, *B. abortus* 544^T; 6, *B. abortus* 86/8/59; 7, *B. abortus* Tulya; 8, *B. abortus* 292; 9, *B. suis* 1330^T; 10, *B. suis* Thomsen; 11, *B. suis* 686; 1 and 12, lambda concatemers (from above) of 300, 250, 200, 150, 100, and 50 kb, respectively.

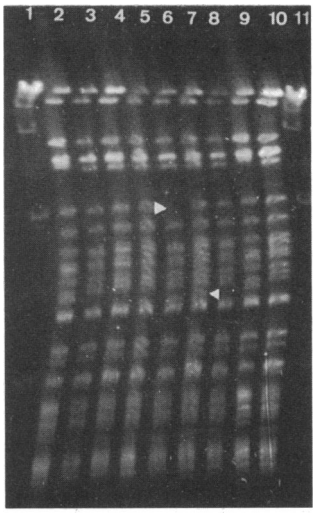

FIG. 4. *Xba*I digestion of DNA from strains of *B. melitensis* biovar 2 obtained from different geographic origins. Lanes: 2, 63/9 (biovar 2 reference strain); 3, 1381 (Gap); 4, 1572 (Toulon); 5, 1574 (Marseille); 6, 1575 (Avignon); 7, 1577 (Paris); 8, 1578 (Draguignan); 9, 1590 (St. Dié); 10, Ether (biovar 3 reference strain); 1, 11 lambda concatemers (from above) of 300, 250, 200, 150, and 50 kb, respectively. A band is missing on the fingerprint of 1575. There is an extra band on the fingerprint of 1577. These are denoted by white triangles.

Figure 3 shows the fingerprints of *B. melitensis* biovars 1, 2, and 3; *B. abortus* biovars 1, 2, 3, and 4; and *B. suis biovars* 1, 2, and 3. All the biovars in this figure differed within a given species, but biovars 5, 6, and 9 of *B. abortus* had fingerprints identical to that of biovar 4, and the fingerprint of biovar 4 of *B. suis* was the same as that of biovar 2 (data not shown).

It can be seen that a few minor differences occurred, but there were no systematic differences among the biovars of a given species. All the biovars in a single species showed a common profile that was, however, specific to the species. Thus, restriction analysis of the *Brucella* genome separated individual species but not biovars within species.

Figure 4 shows a comparison of different field strains of *B. melitensis* biovar 2 isolated in different parts of France, together with the reference strains of biovars 2 and 3. Four strains (1381, 1572, 1574, and 1578) had an identical pattern, and three strains (1575, 1577, and 1590) differed from this group and from each other. The fingerprint of strain 1590 is identical to the fingerprint of biovar 2. The other fingerprints are close to that of biovar 3. The same homogeneity between biovars of a given type was also observed with *B. melitensis* biovar 1 and *B. abortus* biovar 1 (data not shown).

DNA genome size. On the basis of the *Xba*I fragments of *B. abortus* 544^T and *B. melitensis* 16M^T, we estimated that these two genomes consist of about 2.6×10^6 bases. It was difficult to calculate precisely the size of *B. suis* 1330^T, since two groups of bands were not well separated. Nevertheless, the size of this genome appeared to be very similar.

DISCUSSION

Restriction endonuclease analysis of bacterial DNA has yielded interesting results for epidemiological purposes (2, 10, 11, 14, 16, 20). This method, based on DNA digestion by

high-cleavage-frequency enzymes (such as *Eco*RI or *Hin*dIII), has not allowed a clear distinction of *Brucella* species: only some high-molecular-weight bands differed. This could be the consequence of the excessive number of fragments obtained by *Eco*RI or *Hin*dIII digestion or of the strong homogeneity of the genus (22). For this reason, we tried to digest DNA with low-cleavage-frequency endonucleases such as *Xba*I, *Xho*I, *Not*I, or *Sfi*I. Like McClelland et al. (15), we found that in *Brucella* DNA, *Xba*I sites (recognizing a 6-nucleotide sequence) were rarer than *Not*I or *Sfi*I sites (with a cleavage specificity 8 base pairs in length).

Examination of electrophoretic gel patterns after *Xba*I digestion distinguished the five type strains studied. *B. melitensis*, *B. abortus*, and *B. suis* strains each had a species-specific DNA fingerprint. Differences between biovars within a given species or strains within a given biovar were not constant and consequently are not sufficient to serve as markers for accurate epidemiological studies.

B. canis has been proposed to be either a rough strain of *B. suis* or a separate species. The latter hypothesis has been retained because the strain was isolated from a particular host, the dog. The electrophoretic profile clearly places *B. canis* very near *B. suis*. On the other hand, DNA fingerprinting of *B. ovis* separated this species from all the others.

These results are not sufficient to invalidate the proposal of Verger et al. (22), confirmed by the results of De Ley et al. (6), that there is only one species in the *Brucella* genus. On the contrary, the homogeneity of genomic size found by us in three type species is another argument against the division of the genus into several species. Nevertheless, the differences in electrophoretic gel patterns unexpectedly confirm the use of the natural host as a phenotypic characteristic for classifying *Brucella*. From a phylogenetic point of view, four of the five species studied here seem to have arisen from a common ancestor, i.e., *B. melitensis*, *B. abortus*, *B. suis*, and *B. ovis*. *B. canis* can be considered to be an authentic strain of *B. suis* or a strain recently evolved from this species. This proposed evolution of the *Brucella* genus agrees with the fact that these bacteria, which survive almost exclusively in infected animals, show closely related DNA fingerprints when they are strains from the same pathovar. It is understandable that as long as the environment, i.e., the host, remains stable, the phenotype can persist (23).

The most important feature revealed by our results is the great heterogeneity in electrophoretic patterns among the so-called species. The observed differences were unexpected, since the DNA relatedness among all strains amounts to 96% ± 5% (22). The simplest explanation may be that these differences in restriction endonuclease site distribution are related to differences in DNA methylation, insofar as *Xba*I is indeed inhibited by *dam* or *dcm* methylation. Although this hypothesis cannot be completely ruled out, several facts indicate that it probably cannot account for all the observations. First, enzymes less sensitive to DNA methylation, such as *Not*I, showed similar heterogeneity (data not shown). Second, McClelland et al. (15) have demonstrated that the number of sites for a given endonuclease depends both on the nucleotide sequence of the recognized site and on the genomic G+C content. For the *Brucella* DNA with a G+C content of 59% (6) digested by *Xba*I, whose recognition site contains the rare tetranucleotide CTAG, the expected number of fragments is about 30. This is in good agreement with our results, indicating that methylation has a weak influence, if any, on site redistribution.

Point mutations may also affect the electrophoretic patterns, but this is not consistent with the very few differences observed in the numerous small fragments produced by high-cleavage-frequency enzymes compared with those observed in the few large fragments obtained by low-cleavage-frequency enzymes. Finally, there is an interesting possibility that the restriction site redistribution is due to significant chromosomal rearrangements, probably more translocations or inversions than large insertions or deletions, as indicated by the strong DNA homology among all the *Brucella* strains. Work is now in progress in our laboratory to test this possibility.

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