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Polymorphism in *Brucella* spp. Due to Highly Repeated DNA

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The species of *Brucella* are very closely related, but *Brucella ovis* does not express detectable amounts of a protein, designated BCSP31, that is common to the other species. We studied the lack of expression of BCSP31 by Southern analysis. DNAs from the *B. ovis* culture collection strains and field isolates were probed with a 1.3-kb *Hin*dIII fragment encoding BCSP31 of *Brucella abortus*. The probe hybridized to a 1.6-kb *Hin*dIII fragment of all *B. ovis* strains tested, showing that the gene is present in *B. ovis* but occurs on a larger restriction fragment. DNA linkage studies and restriction mapping of the cloned polymorphic region of *B. ovis* showed that the polymorphism was due to a DNA insertion of approximately 0.9 kb at a site downstream of the BCSP31-coding region. When the 1.6-kb polymorphic *B. ovis* fragment was used to probe a *Hin*dIII Southern blot of cellular DNA of strains of *B. ovis* and of *B. abortus*, at least 24 fragments of *B. ovis* and 6 fragments of *B. abortus* hybridized to the inserted DNA. Specimens of *B. ovis* collected over a 30-year period on two continents had similar hybridization patterns. The large difference between *B. ovis* and *B. abortus* in the number of copies of the repeated DNA is interesting in the context of the closeness of the *Brucella* species.

Brucella species infect a variety of warm-blooded animals and, in some cases, cause substantial economic livestock losses. The natural host has been used as a characteristic for classifying species of *Brucella*. Humans are a secondary host.

The six species of the genus *Brucella* are similar when they are compared by DNA fingerprinting (4, 13, 15, 17), DNA-DNA hybridization studies (22, 23), pulsed-field electrophoresis of genomic DNA (1), sodium dodecyl sulfate-polyacrylamide gel electrophoresis of cellular proteins (14, 19, 24; E. S. Zehr and S. M. Halling, Abstr. Advances in Brucellosis Research: An International Symposium, p. 477–478, 1990), and Western immunoblot analysis with convalescent sera (24). As an example, a salt-extractable immunogenic protein with a molecular mass of 31 kDa, BCSP31 (21), is highly conserved in *Brucella* species. Only *Brucella ovis* could not be shown to express detectable amounts of this protein when whole-cell lysates of more than 30 strains of the six *Brucella* species were analyzed by Western analysis (5). This apparent lack of expression of BCSP31 suggested to us that it was a nonessential gene that could be utilized in the development of technology to genetically engineer *Brucella* species for construction of a vaccine by gene replacement.

Here we report Southern analyses, cloning, and restriction endonuclease maps showing that *B. ovis* contains sequences homologous to the BCSP31 gene of *Brucella abortus* and repeated DNA. Repeated DNA is of interest because it can cause genetic instability and genomic rearrangements. The variation in the number of copies found among *B. ovis* strains and between the two *Brucella* species suggests that we have discovered a mobile element (9, 10) in *Brucella* species which may cause genetic instability. Furthermore, differences in the number of copies between *B. ovis* and *B. abortus* make it possible to distinguish these species from each other.

(A preliminary report of this work has been presented [S. M. Halling and E. S. Zehr, Abstr. Annu. Meet. Am. Soc. Microbiol. 1990, H248, p. 195].)

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MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from New England BioLabs, Inc., or Bethesda Research Laboratories. T4 DNA ligase was obtained from Bethesda Research Laboratories. Agarose was purchased from FMC Bioproducts, Inc. Molecular biology-grade cesium chloride was obtained from International Biotechnologies, Inc., and ultrapure redistilled phenol was purchased from either International Biotechnologies or Bethesda Research Laboratories. Calf alkaline phosphatase, proteinase K, RNase A, and a random hexamer priming kit were purchased from Boehringer-Mannheim. The electroporator and avian myeloblastosis virus reverse transcriptase were purchased from Bio-Rad. Radiolabeled [α-^{32}P]dCTP (3,000 Ci/mmol) was obtained from ICN Biomedicals, Inc. A modified nylon membrane for blotting was purchased from Schleicher & Schuell, Inc. Zwittergent 3-14 was obtained from Calbiochem, and *N*-lauroylsarcosine was obtained from Sigma.

Microorganisms and media. The *B. abortus* type and reference strain for biovar 1 (ATTC 23448), supplied by Billy Deyoe, was used as a control. The *B. ovis* strains were either preserved culture collection strains isolated on two continents and stored at low temperature for 20 to 30 years or recent field isolates from different Colorado sheep herds. The sources, geographic locations, and dates of isolation of the *B. ovis* strains used in this work are listed in Table 1. The *B. ovis* 25840 reference strain (no. 1) was obtained from the American Type Culture Collection (6). National Animal Disease Center culture collection strains (no. 2 through 7) were supplied by Billy Deyoe, National Animal Disease Center, Ames, Iowa. The cultures were low passage, having been subcultured two or three times, freeze-dried, and stored at low temperature. The field isolates (no. 8 through 12) were supplied by Robert Jones, Colorado State University. These cultures were recently isolated from rams with epididimyitis. Animals belonged to separate flocks. In transformation and cloning experiments, *Escherichia coli* JM101 [*supE44 thi* Δ(*lac-proAB*) *gyrA96 endA1 hsdR17 relA* (F′*traD36 proAB*$^+$ *lacI*^{q} (*lacZ*ΔM15)] was used as the host.

Brucella species were cultured beneath an atmosphere of

TABLE 1. Strains of *B. ovis*

No.	Strain	Geographic origin	Year isolated
1	ATCC 2540	New Zealand	1956
2	ANH3572	Idaho	1968
3	ANH3512-S[a]	Idaho	1968
4	51-265	New Zealand	1957
5	53-287	New Zealand	1957
6	53-37	New Zealand	1957
7	1922	California	1965
8	L-80-891	Colorado	1984
9	L-745	Colorado	1984
10	21466	Colorado	1988
11	10546	Colorado	1986
12	12576	Colorado	1984

[a] S denotes subculture after an additional passage on medium. The New Zealand cultures were originally isolated by M. Buddle. The Idaho cultures were isolated by G. Brown. The California culture was isolated by M. Meyer.

carbon dioxide (5%) on tryptose agar (2) supplemented with heat-inactivated calf serum (5%) and agar (0.5%). Several plates were streaked from a single colony and incubated for 3 to 4 days before cells were collected by suspension in sterile saline (0.85% sodium chloride).

Plasmids and plasmid construction. The plasmid pBA31-R7 was described previously (12). Briefly, pBA31-R7 has an *Eco*RI fragment of 4.2 kb containing a 1.3-kb *Hin*dIII fragment encoding BCSP31 (Fig. 1).

The vector pUC12, from J. Messing, was used in the construction of pBO31-I1. Before use, the vector was modified by destroying the unique *Ava*I restriction endonuclease site. pBO31-I1 was constructed for this work by cloning *Eco*RI restriction fragments of *B. ovis*. *Eco*RI-cut *B. ovis* genomic DNA was electrophoresed through 0.85% agarose. Fragments of approximately 5 kb were electroeluted (microelectroeluter; Amicon) and concentrated (microconcentrater; Amicon) before ligation into appropriately cut and phosphatase-treated vector. *E. coli* cells were transformed as specified by the manufacturer of the electroporator. pBO31-I1 was identified by colony hybridization with the 1.3-kb BCSP31 probe of *B. abortus*. Ligations, treatment with restriction enzymes, and colony lifts were essentially as described by Maniatis et al. (11), except that excess restriction enzyme was used.

Preparation of genomic DNA. DNA was isolated from methanol-killed cells essentially as described by Anderson and colleagues (3), except that the cells were incubated in the presence of Zwittergent 3-14 (1%) and citric acid (0.1 M) at 50°C for 1 h before being lysed. Briefly, detergent-treated cells were washed and then lysed by sequential addition of lysozyme, proteinase K, EDTA, and sarcosine. The lysate was treated with RNase A, extracted with phenol, precipitated from ethanol, dissolved in TE (10 mM Tris hydrochloride, 1 mM disodium EDTA [pH 8.0]), and centrifuged in the presence of CsCl.

Probes and Southern blot analysis. To generate probes, the 1.3- and 2.2-kb *Hin*dIII fragments of pBA31 and the 1.6-kb *Hin*dIII fragment of pBO31-I1 were recovered by electroelution from preparative agarose gels and precipitated with ethanol as described above. Radioactively labeled probes were generated by random hexamer priming (7, 8) or by treatment with reverse transcriptase in the presence of radiolabeled dCTP. Before treatment with reverse transcriptase (11), the *Hin*dIII DNA fragment was cut with an excess of both *Hin*PI and *Hpa*II restriction endonucleases to generate 5′ overhangs.

For Southern analysis, genomic DNA (0.2 to 1 μg) was treated for 4 h with 10 to 20 U of restriction endonuclease at 37°C before electrophoresis through a 0.75 to 1.0% agarose gel for 15 to 17 h at 25 V. The gels were 11 by 14 cm or 20 by 25 cm. DNA was transferred to a nylon membrane by the technique of Southern (20) after denaturation in 0.5 M sodium hydroxide–1.5 M sodium chloride, neutralization in 1 M Tris hydrochloride–1.5 M sodium chloride, and capillary blotting with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Membrane-bound DNA was hybridized at 42°C with a radioactively labeled probe in 50% formamide and washed with decreasing concentrations of salt at increasing temperatures, essentially as described by Maniatis et al. (11).

Blots were stripped of hybridized probe for reprobing by washing membranes twice for 45 min at 65°C in 50% formamide–6× SSPE (1× SSPE is 1 mM disodium EDTA, 0.01 M NaH_2PO_4, 0.15 M NaCl) and then boiling once in 0.01× SSPE–0.5% sodium dodecyl sulfate (Schleicher & Schuell insert). To verify that the label was removed from stripped membranes, the autoradiograms were exposed overnight at −70°C with intensifying screens.

RESULTS

Southern analysis of *Brucella* genomic DNA. A 1.3-kb *Hin*dIII fragment encoding BCSP31 (Fig. 1) of *B. abortus* was used to probe a *Hin*dIII Southern blot of DNAs from 12 strains of *B. ovis*. The probe hybridized to a 1.6-kb *Hin*dIII fragment of each *B. ovis* strain tested (Fig. 2), showing that the BCSP31 locus is present in *B. ovis*. We describe this larger fragment as a polymorphic fragment.

Southern analysis to determine nature of polymorphism. A Southern blot of *Eco*RI-cut *B. ovis* DNA was probed successively with two contiguous *B. abortus* *Hin*dIII fragments, a 1.3-kb fragment encoding BCSP31 and the downstream 2.2-kb fragment (Fig. 1). The two *Hin*dIII probes hybridized

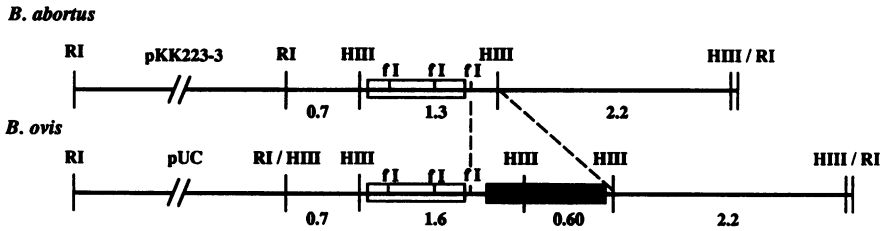

FIG. 1. Structure of pBA31-R7, which encodes *B. abortus* BCSP31, and of pBO31-I1, which has the insertion sequence found near the BCSP31 locus of *B. ovis*. pBA31-R7 was constructed previously (15), and pBO31-I1 was constructed for this work. The numbers are *Hin*dIII fragment sizes in kilobase pairs. Restriction endonuclease sites: RI, *Eco*RI; H3, *Hin*dIII; fI, *Hin*fI. The open box represents the BCSP31-coding region or its homolog. The black box represents the downstream insertion of the BCSP31 homolog of *B. ovis*. The dotted lines link identical BCSP31 *Hin*dIII sites. The vector is indicated by either pKK223-3 or pUC.

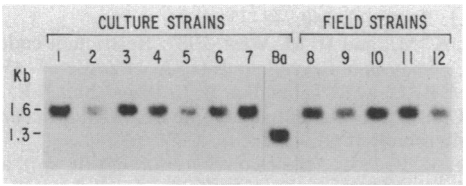

FIG. 2. *B. ovis* DNA is polymorphic at the BCSP31 locus. DNA was electrophoresed through a 20- by 25-cm 1.0% agarose gel. A *Hin*dIII Southern blot of DNAs from *B. ovis* culture collection strains (lanes 1 through 7) and field isolates (lanes 8 through 12) was probed with the 1.3-kb *B. abortus Hin*dIII BCSP31 fragment. Lane numbers correspond to the strains listed in Table 1. *B. abortus* DNA is shown in lane Ba. Fragment sizes are indicated in kilobase pairs. We only show the portion of the autoradiogram that had bands.

to a 5.1-kb *Eco*RI fragment of *B. ovis* (Fig. 3, lanes 1). *B. abortus* DNA digested with *Eco*RI served as the control; both probes hybridized to a 4.2-kb *Eco*RI fragment of *B. abortus* (Fig. 3, lanes 2). The *B. ovis* 5.1-kb *Eco*RI fragment was 0.9 kb longer than the corresponding *B. abortus Eco*RI fragment.

Cloning and mapping the polymorphic region. A 5.1-kb *Eco*RI fragment of *B. ovis* (Fig. 3) was cloned in pUC12 and designated pBO31-I1. A map of pBO31-I1 was constructed and found to be similar to that of pBA31-R7, except that the former contained an insertion of 0.9 kb (Fig. 1). The inserted sequence has a *Hin*dIII site.

Southern analysis with the *B. ovis* polymorphic fragment. When Southern blots of *Hin*dIII-cut *B. ovis* cellular DNA were probed for homology with the 1.6-kb *B. ovis* polymorphic *Hin*dIII fragment of pBO31-I1, at least 24 *B. ovis* fragments hybridized, as did at least 7 fragments of *B. abortus* (Fig. 4). The intense 1.3-kb band that appears in the *B. abortus* lane of the autoradiogram contains the BCSP31 locus. The culture collection strains gave identical hybridization patterns. Three hybridization patterns were noted among the field isolates: two of the field isolates, *B. ovis* L-80-891 (lane 8) and *B. ovis* L745 (lane 9), had hybridization patterns that were identical to those of the culture collection strains; two isolates, *B. ovis* 10546 (lane 11) and *B. ovis* 12576 (lane 12), had hybridization patterns that differed from those of the culture collection strains in that there was an extra band at 5.2 kb and the band at 1.3 kb was missing; and the fifth field isolate, *B. ovis* 21466 (lane 10), had a hybridization pattern that differed from that of the culture collection strains in that two fragments of 1.3 and 4.2 kb were missing.

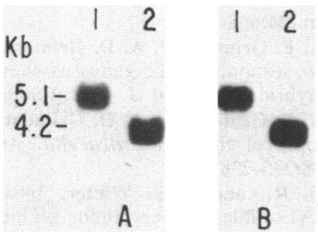

FIG. 3. The polymorphism at the BCSP31 locus of *B. ovis* is due to an insertion. An *Eco*RI Southern blot of *B. ovis* and *B. abortus* DNA was probed successively for homology with (A) the 1.3-kb *Hin*dIII fragment and (B) the linked 2.2-kb *Hin*dIII fragment of the *B. abortus*-derived plasmid pBA31-R7 (Fig. 1). *B. ovis* DNA (lane 1) was hybridized with a radiolabeled 1.3-kb *Hin*dIII probe (A) and with a radiolabeled 2.2-kb *Hin*dIII probe (B). *B. abortus* DNA (lanes 2) served as the control. Fragment sizes are indicated in kilobase pairs. No other bands were seen on the autoradiogram.

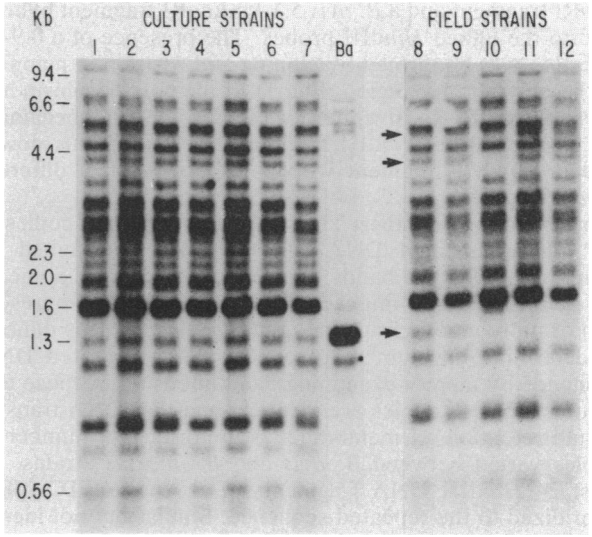

FIG. 4. The sequences inserted at the BCSP31 locus of *B. ovis* are highly repeated in *Brucella* species. DNA was electrophoresed through a 20- by 25-cm 1.0% agarose gel. A *Hin*dIII Southern blot of DNAs from *B. ovis* culture collection strains (lanes 1 through 7) and field isolates (lanes 8 through 12) was probed with the 1.6-kb *B. ovis* polymorphic *Hin*dIII fragment. Lane numbers correspond to the strains listed in Table 1. *B. abortus* DNA is shown in lane Ba. Fragment sizes are indicated in kilobase pairs. Arrows show differences found among *B. ovis* strains.

DISCUSSION

Discovery of polymorphism in *Brucella* species. Differences in restriction fragment patterns were found when Southern blots of DNA from both *B. ovis* and *B. abortus* isolates were hybridized to a 1.6-kb *Hin*dIII fragment of *B. ovis* containing repeated DNA and to a 1.3-kb *Hin*dIII fragment of *B. abortus* encoding BCSP31. When DNAs from the other *Brucella* species were hybridized with a BCSP31 probe, only *B. ovis* was different (S. M. Halling and E. S. Zehr, Abstr. Advances in Brucellosis Research: An International Symposium, p. 476–477, 1990).

The 0.6-kb *Hin*dIII fragment of *B. ovis* was not detected in hybridization studies with the 1.3-kb *Hin*dIII fragment of *B. abortus*, even though the 0.6-kb *Hin*dIII fragment of *B. ovis* contains approximately 40 bp of the 1.3-kb *Hin*dIII fragment of *B. abortus* (Fig. 1). The lack of detection of the smaller fragment is probably the result of it having only a short stretch of homology to the probe and of the reduced efficiency of recovering small fragments during blotting.

Why *B. ovis* does not express BCSP31 was not determined. Hybridization of the *B. abortus* BCSP31 probe to *B. ovis* DNA shows that the lack of expression of BCSP31 is not due to the absence of the locus. Restriction maps constructed for this work showed that the BCSP31-coding sequences are upstream of the insertion (Fig. 1). Of course, a single-base-pair change in the coding region would not be detected by hybridization that could result in the loss of expression of BCSP31. It is also possible that BCSP31 is not expressed by *B. ovis* because the gene encoding BCSP31 is part of an operon and expression is dependent on a downstream gene product.

DNA polymorphism is due to an insertion. Linkage studies utilizing probes to *B. abortus* BCSP31 and to the contiguous downstream 2.2-kb *Hin*dIII fragment showed that the polymorphism is the result of an insertion. A *B. abortus* 4.2-kb

*Eco*RI fragment and a *B. ovis* 5.1-kb *Eco*RI fragment hybridized to the linked *Hin*dIII probes. The presence of a 0.9-kb insertion was confirmed by cloning and restriction mapping of the polymorphic region (Fig. 1). If the polymorphism had been caused by an inversion of the chromosome beginning within the 1.3-kb *Hin*dIII fragment, the contiguous downstream *Hin*dIII fragment would have been on a different *Eco*RI fragment.

Insertion copy number. The minimum number of copies of the highly repeated DNA sequence was determined by simply counting the bands on the autoradiogram. Some of the bands are more intense than others and may represent two or more unique fragments that hybridized. It is difficult to determine the number of copies of the repeated DNA sequence by merely examining band intensity, because this assumes that all copies are identical and that DNA transfer to and retention on membranes is uniform. The number of copies varied between *B. ovis* and *B. abortus* strains. At least 24 *Hin*dIII DNA fragments of *B. ovis* ATTC 25840 hybridized to the repeated sequence. Similar but not identical hybridization patterns were seen with the other *B. ovis* strains. The few differences in hybridization patterns among *B. ovis* strains isolated over a 30-year time span suggest that the *Brucella* genome is relatively stable. Fewer fragments hybridized when *B. abortus* DNA was examined. At least six *B. abortus* fragments hybridized to the repeated DNA; a seventh fragment hybridized because of its homology to the gene encoding BCSP31. DNAs from *B. abortus* S19 and a field isolate gave the same hybridization pattern (B. Bricker, personal communication; S. M. Halling, data not shown).

Large segments of repeated DNA in bacteria are usually due to discrete mobile DNA sequences referred to as insertion elements or insertion sequences (9). These sequences range in size from 800 to 2,500 bp and are often present in more than one copy per genome. The insertional element IS*1* is found in 0 to 50 copies in different strains of *E. coli* (16, 18). This insertion element is not the only one found in *E. coli*; there are several other insertion sequences in *E. coli*, and some of them are also multicopy sequences (9). Repeated DNA is not found only in *E. coli* and other closely related bacteria. Two families of highly repeated DNA sequences, subsequently shown to be insertion sequences (25), were reported in *Thiobacillus ferrooxidans* (26). Sequences from both families are repeated 20 to 30 times in the genome. We are characterizing the family of repeated DNA described in this report.

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