DNA polymorphism at the *omp-31* locus of *Brucella* spp.: evidence for a large deletion in *Brucella abortus*, and other species-specific markers

Nieves Vizcaíno,† Jean-Michel Verger, Maggy Grayon,
Michel S. Zygmunt and Axel Cloeckaert

Author for correspondence: Axel Cloeckaert. Tel: +33 2 47 42 78 72. Fax: +33 2 47 42 77 79.
e-mail: Axel.Cloeckaert@tours.inra.fr

Laboratoire de Pathologie
Infectieuse et
Immunologie, Centre de
Recherches de Tours,
Institut National de la
Recherche Agronomique,
37380 Nouzilly, France

The *omp-31* gene, encoding a major outer-membrane protein in *Brucella melitensis*, was PCR-amplified from *Brucella* strains representing all species and known biovars by using primers selected according to the *B. melitensis* 16M *omp-31* published sequence. Amplification of *omp-31* was achieved from DNA of all *Brucella* species with the exception of *Brucella abortus*, the only *Brucella* species where expression of *omp-31* was not detected by reactivity with an mAb specific for an epitope located in Omp-31. Southern blot hybridization of plasmid probes, bearing inserts (4.4–17 kb) containing *B. melitensis* 16M *omp-31* and adjacent DNA of different sizes, with *HindIII*-digested total DNA showed that a large fragment, comprising the entire *omp-31* gene and flanking DNA, was actually absent in *B. abortus* strains. The size of this DNA fragment has been determined to be about 10 kb. Southern blot hybridization with the different plasmid probes identified species-specific markers for *B. abortus* and *B. melitensis*. At the biovar level, a specific marker for *B. melitensis* bv. 1 was also identified. Additionally, PCR-RFLP studies of *omp-31* revealed specific markers for *Brucella ovis*, *Brucella canis* and *Brucella suis* bv. 2. Using a combination of *omp-31* PCR-RFLP patterns and Southern blot hybridization profiles *Brucella* species were differentiated with the sole exception of *Brucella neotomae* which was not differentiated from *B. suis* bv. 1, 3, 4 and 5. Results presented in this paper demonstrate the potential of *omp-31* for differentiating the brucellae and show that *B. abortus* lacks a large DNA fragment of about 10 kb containing *omp-31* and flanking DNA. In such a large deletion, other genes in addition to *omp-31* are probably involved. Sequencing of this DNA fragment will help to identify the missing genes in *B. abortus* which could possibly be involved in the differences of pathogenicity and host preference seen in *Brucella* species.

Keywords: *Brucella*, *omp-31*, DNA polymorphism, outer-membrane protein

INTRODUCTION

Brucella strains are the causative agents of brucellosis, a widespread infectious disease affecting many animal species and man. Six species, classified on the basis of differences in pathogenicity and host preference, are

recognized within the genus *Brucella*: *B. melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (Corbel & Brinley-Morgan, 1984). Differentiation between these species and their different biovars is currently based on serotyping, phage typing, dye-sensitivities, CO₂ requirement, H₂S production and metabolic properties (Alton *et al.*, 1988). However, reported instability for some of these phenotypic characteristics (Meyer, 1976) sometimes makes it difficult to identify particular isolates. Therefore, the identification of stable DNA-specific markers is considered a high

†Present address: Dpto Microbiología y Genética, Edificio Departamental, Universidad de Salamanca, Avda. Campo Charro s/n, 37007 Salamanca, Spain.

Abbreviation: OMP, outer-membrane protein.

Table 1. *Brucella* strains used in this study

No.	Species and strain*	Biovar	Host/source	Geographic origin
<i>B. abortus</i>				
1	544 (ATCC 23448; BCCN R4)†	1	Cattle	England
2	86/8/59 (ATCC 23449; BCCN R5)	2	Cattle	England
3	Tulya (ATCC 23450; BCCN R6)	3	Human	Uganda
4	292 (ATCC 23451; BCCN R7)	4	Cattle	England
5	B3196 (ATCC 23452; BCCN R8)	5	Cattle	England
6	870 (ATCC 23453; BCCN R9)	6	Cattle	Africa
7	C68 (ATCC 23455; BCCN R11)	9	Cattle	England
8	45/20 (BCCN V2)‡	R	Cattle	England
9	RB51 (BCCN V5)‡	R	Cattle	USA
<i>B. melitensis</i>				
10	16M (ATCC 23456; BCCN R1)†	1	Goat	USA
11	H38 (BCCN V3)‡	1	Human	Mexico
12	Rev.1 (BCCN V4a)‡	1	Goat	Mexico
13	BCCN 90.61	1	Sheep	South Africa
14	BCCN 92.70	1	Human	France
15	BCCN 94.37	1	Human	France
16	BCCN 87.92	1	Human	USA
17	BCCN 92.106c	1	Unknown	Algeria
18	BCCN 75.477	1	Dog	Israel
19	BCCN 88.42	1	Sheep	Israel
20	63/9 (ATCC 23457; BCCN R2)	2	Goat	Turkey
21	BCCN 95.33	2	Sheep	Italy
22	Ether (ATCC 23458; BCCN R3)	3	Goat	Italy
23	BCCN 95.30	3	Sheep	Italy
24	BCCN 94.16	3	Cattle	France
25	BCCN 92.106a	3	Unknown	Algeria
26	BCCN 92.42	3	Human	Tunisia
27	BCCN 92.118	3	Human	Tunisia
28	BCCN 92.80	3	Sheep	Spain
29	BCCN 92.83	3	Sheep	Spain
30	B115 (BCCN R19)	R	Goat	Malta
31	BCCN 92.116	R	Sheep	France
32	BCCN 94.62	R	Human	France
<i>B. suis</i>				
33	1330 (ATCC 23444; BCCN R12)†	1	Swine	USA
34	S2 (BCCN V6)‡	1	Swine	China
35	Thomsen (ATCC 23445; BCCN R13)	2	Swine	Denmark
36	BCCN 94.9	2	Hare	France
37	BCCN 94.10	2	Hare	France
38	BCCN 94.11	2	Wild boar	France
39	BCCN 93.60	2	Swine	France
40	BCCN 93.21	2	Hare	France
41	BCCN 93.72	2	Swine	Spain
42	BCCN 93.74	2	Swine	Spain
43	BCCN 93.77	2	Swine	Spain
44	BCCN 93.78	2	Swine	Spain
45	BCCN 96.122	2	Wild boar	France
46	BCCN 96.123	2	Swine	France
47	686 (ATCC 23446; BCCN R14)	3	Swine	USA
48	40 (ATCC 23447; BCCN R15)	4	Reindeer	Former Soviet Union
49	513 (BCCN R21)	5	Wild rodent	Former Soviet Union

Table 1 (cont.)

No.	Species and strain*	Biovar	Host/source	Geographic origin
<i>B. ovis</i>				
50	63/290 (ATCC 25840; BCCN R17)†	—	Sheep	Africa
51	Reo 198 (BCCN R22)	—	Sheep	USA
52	BCCN 76.247	—	Sheep	France
53	BCCN 76.250	—	Sheep	France
54	BCCN 91.70	—	Sheep	Spain
55	BCCN 91.208	—	Sheep	Spain
56	BCCN 91.212	—	Sheep	Spain
57	BCCN 91.217	—	Sheep	Spain
58	BCCN 91.266	—	Sheep	Argentina
<i>B. canis</i>				
59	RM6/66 (ATCC 23365; BCCN R18)†	—	Dog	USA
60	BCCN C1	—	Dog	Madagascar
61	BCCN C2	—	Dog	USA
62	BCCN C3	—	Dog	USA
63	BCCN C4	—	Dog	USA
64	BCCN 87.62	—	Unknown	Mexico
65	BCCN 87.66	—	Dog	Canada
66	BCCN 87.65	—	Dog	Canada
67	BCCN 96.103	—	Dog	Rumania
68	BCCN 96.104	—	Dog	Rumania
69	BCCN 96.105	—	Dog	Rumania
70	BCCN 96.106	—	Dog	Rumania
71	BCCN 96.107	—	Dog	Rumania
72	BCCN 96.121	—	Dog	France
<i>B. neotomae</i>				
73	5K33 (ATCC 23459; BCCN R16)†	—	Desert rat	USA

* ATCC, American Type Culture Collection; BCCN, *Brucella* Culture Collection, Nouzilly, France.

† Reference strain.

‡ Vaccine strain.

priority for diagnostic, epidemiological and taxonomic purposes.

Since the brucellae share a high degree of DNA homology (>90% for all species) and *Brucella* has been proposed as a mono-specific genus (Verger *et al.*, 1985, 1987), several techniques have been employed to find DNA polymorphisms which would enable the molecular typing of the *Brucella* species and their different biovars (Allardet-Servent *et al.*, 1988; Bricker & Halling, 1994, 1995; Cloeckaert *et al.*, 1995, 1996b; Fekete *et al.*, 1992; Ficht *et al.*, 1990, 1996; Grimont *et al.*, 1992; Halling & Zehr, 1990; Mercier *et al.*, 1996; Ouahrani *et al.*, 1993). Among these, detection of RFLPs is considered a reproducible and easily performed method.

Major outer-membrane protein (OMP) genes of *Brucella* have recently attracted attention as they exhibit sufficient polymorphism to allow differentiation between *Brucella* species and some of their biovars (reviewed by Cloeckaert *et al.*, 1996c). Studies of the RFLP profiles of two closely related genes: *omp2a* and *omp2b*, encoding and potentially expressing the *Brucella* spp. major OMP of 36 kDa (Ficht *et al.*, 1988, 1989), showed that the type

strains of the six *Brucella* species could be differentiated on this basis (Ficht *et al.*, 1990). More recently using PCR-RFLP and a greater number of restriction enzymes, *Brucella* species-, biovar-, or strain-specific markers have been detected for the *omp25* gene, encoding the *Brucella* 25 kDa major OMP (de Wergifosse *et al.*, 1995), and for the *omp2a* and *omp2b* genes. However, *B. canis* remained undistinguishable from *B. suis* bv. 3 and 4 (Cloeckaert *et al.*, 1995).

The 31–34 kDa OMP has been identified as a minor OMP in *B. abortus* strains but as a major OMP in the other *Brucella* species (Dubray, 1981). The *B. melitensis* 16M gene encoding this protein (*omp-31*) has recently been cloned and sequenced (Vizcaino *et al.*, 1996). In the present work, we studied polymorphism at the *omp-31* locus, by either Southern blot hybridization or PCR-RFLP, of 73 *Brucella* reference and field strains representing the six species and their different biovars searching for DNA markers that could improve differentiation among the brucellae.

Plasmid	Partial restriction profile of the <i>B. melitensis</i> 16M DNA insert	<i>B. melitensis</i> 16M DNA insert cloned into plasmid	Cloning site	Insert size (kb)
pNV3102		pGEM-7Zf	EcoRI	4.4
pNV3104		pGEM-7Zf	SacI	7.0
pNV3125		pGEM-7Zf	BamHI	8.0
pNV3126		pGEM-7Zf	BamHI-SacI	1.8
pNV3103		pGEM-5Zf	NotI	17

Fig. 1. Relevant characteristics of the plasmids used in this study. Plasmids were obtained by subcloning, into pGEM-5Zf or pGEM-7Zf, the DNA of a phage, bearing *omp-31*, from a *B. melitensis* 16M genomic library constructed in λ GEM-12 (Vizcaíno *et al.*, 1996). The darkest region corresponds to location of *omp-31* within the insert. Only the restriction map with some enzymes is shown and the EcoRI restriction map is not complete in pNV3103, pNV3104, pNV3125 and pNV3126. Sites marked with an asterisk probably correspond to the multiple cloning site of phage λ GEM-12 and are absent in the *Brucella* DNA. The HindIII site marked by † could be symmetrically located between the two adjacent HindIII sites, closer to the HindIII site on its left. Restriction sites: B, BamHI; E, EcoRI; H, HindIII; N, NotI; S, SacI.

METHODS

Bacterial strains and plasmids. The 73 *Brucella* strains used in this study are listed in Table 1. All strains were maintained freeze-dried in the INRA *Brucella* Culture Collection, Nouzilly (BCCN), France. Cultures were grown on tryptic soy agar (Gibco-BRL) supplemented with 0.1% (w/v) yeast extract (Difco) (TSAYE). For fastidious strains (*B. abortus* bv. 2 and *B. ovis*), 5% sterile horse serum (Gibco-BRL) was added to TSAYE (TSAYES medium). The strains were checked for purity and species and biovar characterization by standard procedures (Alton *et al.*, 1988). *Escherichia coli* JM109 cells bearing recombinant plasmids pNV3102, pNV3103, pNV3104, pNV3125 or pNV3126 were cultured overnight on LB medium containing 50 μ g ampicillin ml⁻¹.

Plasmids pNV3102, pNV3103, pNV3104 and pNV3125 contain *B. melitensis* 16M DNA inserts bearing *omp-31* and were obtained by subcloning, into pGEM-5Zf or pGEM-7Zf (Promega), the DNA insert of a phage from a *B. melitensis* 16M genomic library constructed in λ GEM-12 *Xho*I half-site arms (Promega) as described previously (Vizcaíno *et al.*, 1996). Plasmid pNV3126 was obtained by subcloning a BamHI-SacI fragment of pNV3125 into pGEM-7Zf. The relevant characteristics of the five plasmids are shown in Fig. 1.

Immunoblotting. SDS-PAGE was performed as described by Laemmli (1970) with bacterial suspensions diluted in Laemmli sample buffer and heated for 10 min at 100 °C. After electrophoresis, proteins were transferred at 0.8 mA cm⁻² for 75 min to a nitrocellulose membrane. Detection of protein bands reacting with the anti-Omp-31 mAb A59/10F09/G10 (IgG2a) was accomplished as described previously (Vizcaíno *et al.*, 1996).

DNA preparation. The strains were cultured for 24 h at 37 °C on TSAYE or TSAYES slopes and harvested, in 3 ml sterile distilled water, by centrifugation at 2000 *g* for 10 min. The pellet was suspended in 567 μ l TE/sodium buffer (50 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0). Then, 30 μ l 10% (w/v) SDS solution and 3 μ l 2% (w/v) proteinase K solution were added and the mixture was kept at 37 °C for 1 h. The lysed cell suspension was extracted twice with phenol-chloroform and nucleic acids were precipitated by gently mixing the aqueous phase with 2 vols cold ethanol. The precipitate was dissolved in 100 μ l TE (10 mM Tris, 1 mM EDTA, pH 8.0). The amount of DNA was measured by electrophoresis of an aliquot of each sample through 0.8% agarose gels and comparison with standard DNA solutions.

Southern blot hybridization. DNA from *Brucella* strains was digested with HindIII and fragments resolved by agarose gel electrophoresis and transferred onto Immobilon-S nylon membranes (Millipore) by vacuum transfer with the VacuGene System (Pharmacia LKB Biotechnology).

pNV3102, pNV3103, pNV3104, pNV3125, pNV3126 and PCR amplified *B. melitensis* 16M *omp-31* were biotin-labelled with the NEBlot Phototope kit (New England Biolabs), following the instructions of the manufacturer, and used as probes for hybridization with the transferred HindIII-digested *Brucella* spp. DNA. Detection of HindIII *Brucella* spp. DNA fragments hybridizing with the biotinylated probes was performed with the Phototope-Star Detection kit for Nucleic Acids (New England Biolabs), according to the manufacturer's instructions, and exposure of the hybridized membranes to an X-ray film (Dupont).

A mixture of biotinylated HindIII digest of λ DNA and HaeIII

digest of ϕ X174 DNA (Biolabs) was used as molecular size markers.

Primers and amplification conditions. Two 20-mer primers (Isoprimer) were selected according to the reported *omp-31* nucleotide sequence (Vizcaino *et al.*, 1996) to amplify the entire *omp-31* with its putative Shine-Dalgarno and transcription terminator sequences. These primers were 31sd (5'-TGACAGACTTTTTCGCCGAA-3') and 31ter (5'-CATTC-AGGACAATTCCCGCC-3').

Amplification reaction mixtures were prepared in volumes of 100 μ l containing 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mg gelatin ml⁻¹ (1 $\times$ PCR buffer; Appligene), 200 μ M each dNTP, 1 μ M each primer, 100 ng genomic DNA, and 2.5 U *Taq* DNA polymerase (Appligene). The temperature cycling for the amplification was performed in a GeneAmp PCR System 9600 thermocycler (Perkin Elmer) as follows: cycle 1 was 94 °C for 5 min (denaturation); the next 30 cycles were 58 °C for 30 s (annealing), 70 °C for 30 s (extension) and 94 °C for 30 s (denaturation); the last cycle was 58 °C for 30 s (annealing) and 70 °C for 10 min (extension).

Restriction digestion. For PCR-RFLP, the following restriction enzymes were used: *Ava*II, *Kpn*I and *Sau*3AI (Appligene), *Ban*I and *Hae*II (Promega), *Hae*III and *Pvu*II (New England Biolabs), *Rsa*I and *Sal*I (Gibco-BRL) and *Sty*I (Boehringer Mannheim).

Restriction enzymes were chosen according to the *B. melitensis* 16M *omp-31* determined nucleotide sequence (Vizcaino *et al.*, 1996). The *omp-31* restriction map for the selected enzymes is shown in Fig. 5. Five microlitres of each PCR product were cleaved with 1–5 U each restriction enzyme in a 20 μ l volume. Buffer and temperature conditions recommended by the manufacturers were used. Restriction fragments from each reaction were separated in gels with 1.5% (w/v) agarose (Appligene) and 0.5 μ g ml⁻¹ ethidium bromide (Sigma). The 100 bp DNA ladder (Gibco-BRL) was used as molecular size markers.

RESULTS

Expression of *omp-31* in *Brucella* spp.

To verify expression of *omp-31* in *Brucella* spp., *Brucella* strains representing all species and known biovars were submitted to SDS-PAGE followed by immunoblotting against the anti-Omp-31 mAb A59/10F09/G10.

All biovars of *B. melitensis* and *B. suis*, and *B. ovis*, *B. canis*, and *B. neotomae* reference strains reacted in immunoblotting with the anti-Omp-31 mAb (Fig. 2), showing a multiple banding pattern ranging from 28 to 34 kDa according to molecular mass protein standards, which is characteristic for Omp-31 (Vizcaino *et al.*, 1996).

In contrast, no reactivity of the anti-Omp-31 mAb was detected with any of the *B. abortus* biovars (Fig. 2), suggesting that (1) the specific epitope recognized by the mAb is not present in *B. abortus* Omp-31, or (2) the *omp-31* gene is not expressed or (3) it is absent in *B. abortus* strains.

Southern blot hybridization

Biotin-labelled pNV3102, pNV3103, pNV3104 and pNV3125, and PCR-amplified *B. melitensis* 16M *omp-*

Fig. 2. Occurrence of Omp-31 in *Brucella* species and biovars. Immunoblotting with anti-Omp-31 mAb A59/10F09/G10 after SDS-PAGE of whole-cell lysates from *B. abortus* strains 544 (bv. 1) (lane 1), 86/8/59 (bv. 2) (lane 2), Tulya (bv. 3) (lane 3), 292 (bv. 4) (lane 4), 83196 (bv. 5) (lane 5), 870 (bv. 6) (lane 6), C68 (bv. 9) (lane 7); *B. melitensis* strains 16M (bv. 1) (lane 8), 63/9 (bv. 2) (lane 9), Ether (bv. 3) (lane 10); *B. suis* strains 1330 (bv. 1) (lane 11), Thomsen (bv. 2) (lane 12), 686 (bv. 3) (lane 13), 40 (bv. 4) (lane 14), 513 (bv. 5) (lane 15); *B. ovis* strain 63/290 (lane 16); *B. canis* strain RM6/66 (lane 17); *B. neotomae* strain 5K33 (lane 18).

31 were used as probes to detect hybridization with *Hind*III-digested DNA from *Brucella* strains representing the six species and their biovars.

The PCR-amplified *omp-31* probe hybridized with one 1.6 kb *Hind*III fragment in genomic DNA from *B. ovis*, *B. canis*, *B. neotomae* and all biovars of *B. suis* (see arrowed band in Fig. 3a, lanes 6–9) and with one 0.9 kb *Hind*III fragment in DNA from the three biovars of *B. melitensis* (see arrowed band in Fig. 3a, lanes 3–5). No hybridization was detected with *B. abortus* DNA showing that *omp-31* is absent in *B. abortus* strains. According to these results, the *omp-31* probe differentiated *B. melitensis* from the other *Brucella* species.

The pNV3102 probe, which contains a 4.4 kb *B. melitensis* 16M DNA insert bearing *omp-31* (Fig. 1), developed three bands (1.6, 2.4 and 4 kb) with DNA from *B. ovis*, *B. canis*, *B. neotomae* and all biovars of *B. suis* (Fig. 3a, lanes 6–9). With this probe *Hind*III fragments of 0.9, 2.4 and 4.3 kb were detected with *B. melitensis* 16M (bv. 1) DNA (Fig. 3a, lane 3) and fragments of 0.6, 0.9, 2.4 and 4 kb with *B. melitensis* 63/9 (bv. 2) DNA (Fig. 3a, lane 4) and *B. melitensis* Ether (bv. 3) DNA (Fig. 3a, lane 5). The pNV3102 probe did not hybridize with DNA of the *B. abortus* biovars (Fig. 3a, lane 2). Thus, the pNV3102 probe distinguished *B. melitensis* bv. 1 from bv. 2 and 3 of the same species and also differentiated the three *B. melitensis* biovars from the other *Brucella* species.

The pNV3104 and pNV3125 (Fig. 1) probes containing inserts of respectively 7.0 and 8.0 kb did not hybridize with *B. abortus* DNA and the same banding pattern as that detected with pNV3102, with an additional 5 kb band (see arrowed band in Fig. 3b), was observed with the other *Brucella* species (data not shown).

However, when pNV3103 containing an insert of 17 kb (Fig. 1) was used as a probe, hybridization with three *Hind*III fragments (0.7, 2 and 6 kb) was detected in DNA from all biovars of *B. abortus* (Fig. 3b, lane 2) showing that DNA sequence from at least one side of the

Fig. 3. Southern blot hybridization with biotinylated pNV3102 (a) and pNV3103 (b) probes. Lane 1, molecular size markers; lanes 2–9, *Hind*III digest of DNA from *B. abortus* 544 (bv. 1) (lane 2), *B. melitensis* 16M (bv. 1) (lane 3), *B. melitensis* 63/9 (bv. 2) (lane 4), *B. melitensis* Ether (bv. 3) (lane 5), *B. suis* 1330 (bv. 1) (lane 6), *B. ovis* 63/290 (lane 7), *B. canis* RM6/66 (lane 8) and *B. neotomae* 5K33 (lane 9). All *B. abortus* and *B. suis* biovars gave the same profile as shown in lanes 2 and 6, respectively. Arrows show the band observed with the biotinylated PCR-amplified *omp-31* probe (a) and the 5 kb additional band, as compared with the biotinylated pNV3102 probe profile, of the profile observed with biotinylated pNV3104 and pNV3125 probes (b).

B. abortus deletion is present in the *B. melitensis* 16M DNA insert of pNV3103. The 0.7 and 2 kb *Hind*III fragments were also detected in the other *Brucella* species (Fig. 3b, lanes 3–9), not revealing additional polymorphism to that detected with the pNV3102 probe (Fig. 3a, lanes 3–9). The use of the pNV3103 probe thus differentiated *B. abortus* and *B. melitensis* from each other and from the other *Brucella* species (Fig. 3b). Moreover, *B. melitensis* bv. 1 was distinguishable from the two other biovars of the same species (Fig. 3b, lanes 3–5).

To determine whether the DNA delimiting both sides of the *B. abortus* deletion was contained in the *B. melitensis* 16M DNA insert of pNV3103, the biotinylated insert of plasmid pNV3126 (Fig. 1) was used as a probe to hybridize with *Hind*III-digested *B. abortus* 544 DNA. Only one band, located between the 9.4 and 23.1 kb DNA markers, was weakly detected. This band did not appear with probes pNV3125 or pNV3103, both containing the pNV3126 insert (Fig. 1), which could be explained by the greater molecular size of these probes. As three additional bands were detected after hybridization of the pNV3103 probe with *B. abortus* DNA, these results suggest that the entire DNA fragment deleted in *B. abortus* is contained in the *B. melitensis* 16M insert of pNV3103. Nucleotide sequence at both ends of the pNV3103 insert was determined, as previously described (Vizcaíno *et al.*, 1996), by using the pGEM-5Zf M13 reverse and forward primers (data not shown) and PCR amplification from *B. abortus* DNA was performed with primers selected according to the identified sequences. A DNA band of about 7 kb was detected on agarose gels (data not shown) thus delimiting the missing DNA in *B. abortus* to a fragment of about 10 kb of the pNV3103 *B. melitensis* 16M DNA insert.

RFLP of the *omp-31* gene

Ten restriction enzymes, chosen according to the restriction map deduced from the *B. melitensis* 16M *omp-31* nucleotide sequence (Vizcaíno *et al.*, 1996), were used

Fig. 4. Restriction patterns of the PCR-amplified *omp-31* gene. The patterns P1–P3 of Table 2 are shown for each enzyme. Lanes: 1, molecular size markers; 2–4, patterns P1, P2 and P3 with *Avall*; 5 and 6, patterns P1 and P2 with *Hae*III; 7 and 8, patterns P1 and P2 with *Sal*I; 9 and 10, patterns P1 and P2 with *Sau*3AI.

to study the RFLP in the PCR-amplified *omp-31* genes from the different brucellae. *Brucella* reference and field strains representing all species and their biovars were included in this study, with the exception of *B. abortus* strains where PCR-amplification of *omp-31* was unsuccessful. For each restriction enzyme, pattern P1 was assigned to strains showing the same restriction pattern to that deduced from the *B. melitensis* 16M *omp-31* nucleotide sequence (Vizcaíno *et al.*, 1996).

Six restriction enzymes (*Ban*I, *Hae*II, *Kpn*I, *Pvu*II, *Rsa*I and *Sty*I) did not reveal any *omp-31* polymorphism among the brucellae, giving the same restriction pattern with all the strains tested (data not shown).

Avall gave three different restriction patterns (Fig. 4, lanes 2–4; Table 2). Restriction pattern P1 (Fig. 4, lane

Table 2. Restriction patterns of the PCR-amplified *omp-31* gene of the different *Brucella* strains

Species	Biovar	Pattern*	Pattern with restriction enzymes†			
			<i>Ava</i> II	<i>Hae</i> III	<i>Sal</i> I	<i>Sau</i> 3AI
<i>B. abortus</i> (strains 1–9)‡	1–9, R	Not amplified				
<i>B. melitensis</i> (strains 10–32)‡	1–3, R	A	P1	P1	P1	P1
<i>B. suis</i> (strains 33, 34, 47–49)‡	1, 3–5	A	P1	P1	P1	P1
<i>B. suis</i> (strains 36, 40)‡	2	B	P2	P1	P1	P1
<i>B. suis</i> (strains 35, 37–39, 41–46)‡	2	C	P2	P2	P1	P1
<i>B. ovis</i> (strains 50–58)‡		D	P1	P1	P1	P2
<i>B. canis</i> (strains 59–63, 65, 67–72)‡		E	P3	P1	P1	P1
<i>B. canis</i> (strains 64, 66)‡		F	P3	P1	P2	P1
<i>B. neotomae</i> (strain 73)‡		A	P1	P1	P1	P1

* Overall pattern of *omp-31*, cut with the different restriction enzymes, for each *Brucella* strain.

† *Ban*I, *Hae*II, *Kpn*I, *Pvu*II, *Rsa*I and *Sty*I were also used. For each of these enzymes pattern P1 was obtained with all *Brucella* strains tested.

‡ Strain numbers as referred to in first column of Table 1.

Fig. 5. Restriction maps of the PCR-amplified *omp-31* gene of *Brucella* species and biovars. Restriction map of pattern A was deduced from the nucleotide sequence of the *B. melitensis* 16M *omp-31* gene (Vizcalno et al., 1996). Patterns B–F of Table 2 were established by comparison with pattern A and only differences with pattern A are shown. Missing restriction sites in the *omp-31* gene of strains showing patterns B–F are barred. Additional restriction sites and their most probable location according to restriction patterns and single nucleotide substitutions in the *omp-31* gene sequence of *B. melitensis* 16M are indicated by the enzyme name and the most probable location by the position number and an asterisk. Restriction sites: A, *Ava*II; B, *Ban*I; H, *Hae*II; Ha, *Hae*III; K, *Kpn*I; P, *Pvu*II; R, *Rsa*I; S, *Sal*I; Sa, *Sau*3AI; St, *Sty*I.

2) was observed with all strains of *B. melitensis*, *B. ovis* and *B. neotomae*, and with all *B. suis* strains belonging to bv. 1, 3, 4 and 5 (Table 2). Restriction pattern P2 was characteristic to *B. suis* bv. 2 strains (Fig. 4, lane 9), and pattern P3 to *B. canis* strains (Fig. 4, lane 10). Thus, *omp-31* restriction with *Ava*II allowed the distinction of *B. suis* bv. 2 and *B. canis* from each other and from the other brucellae.

*Hae*III allowed the differentiation of *B. suis* bv. 2 strains (pattern P2) (Fig. 4, lane 6; Table 2) from the other *Brucella* strains (pattern P1) (Fig. 4, lane 5; Table 2), with the exception of strains numbered 36 and 40 which showed pattern P1 as the other *Brucella* strains (Table 2). *Sal*I showed a different pattern with only two strains (strains numbered 64 and 66) of *B. canis* (Fig. 4, lanes 7 and 8; Table 2). Finally, *Sau*3AI gave a species-specific marker to *B. ovis* strains showing pattern P2 (Fig. 4, lane

10) distinguishable from pattern P1 (Fig. 4, lane 9) of the other *Brucella* strains (Table 2).

According to the RFLP detected in the *omp-31* gene with the ten restriction enzymes used in this study, *Brucella* strains could be classified into seven groups (Table 2) including *B. abortus* strains where *omp-31* was not amplified. The six *omp-31* restriction maps obtained with the ten enzymes used are represented in Fig. 5. and are classified into overall patterns A–F (see also Table 2). Pattern A was specific for *B. melitensis*, *B. neotomae* and *B. suis* bv. 1, 3, 4 and 5. Pattern B, characterized by the absence of the *Ava*II site located at position 592, was detected with two strains of *B. suis* bv. 2 (strains numbered 36 and 40) while most of *B. suis* bv. 2 strains showed pattern C which corresponds to pattern B with an additional *Hae*III site probably located at the same 592 position. All *B. ovis* strains tested showed pattern D,

characterized by the absence of the *Sau3AI* (385) site. *B. canis* strains showed pattern E (pattern A with an additional *AvaII* site probably located at position 399) or pattern F (two *B. canis* strains) which corresponds to pattern E lacking the *Sall* (708) site.

DISCUSSION

In the present study, expression of *omp-31* in the brucellae has been examined with an mAb specific for an epitope located in Omp-31. Reactivity was detected with all *Brucella* species and biovars with the exception of *B. abortus* strains. The reasons for this may be that (1) the epitope recognized by the mAb is not present in *B. abortus* Omp-31 or that (2) *omp-31* is not expressed or that (3) *omp-31* is absent in this species. PCR amplification of *omp-31* from *B. abortus* DNA was unsuccessful with the selected primers and, furthermore, no hybridization was detected between PCR-amplified *B. melitensis* 16M *omp-31* probe and *HindIII*-digested *B. abortus* total DNA. These results demonstrate that the entire *omp-31* gene is absent in *B. abortus* strains and therefore that Omp-31 cannot be present among the *B. abortus* OMPs. Deletions in genes blocking or not the expression of the encoded protein have been reported in some *Brucella* species or strains (Cloeckart *et al.*, 1996a, c, d; Sangari *et al.*, 1994), and in some cases lack of expression of an existing gene has been demonstrated (Ficht *et al.*, 1989; Halling & Zehr, 1990). However, this is the first report describing the deletion of an entire gene in one of the *Brucella* species. Moreover, hybridization of *HindIII*-digested *Brucella* spp. DNA with plasmid probes containing *B. melitensis* 16M DNA inserts of several sizes and bearing *omp-31*, revealed that a large DNA fragment, with a size of about 10 kb, is deleted in *B. abortus* strains. In such a large fragment other genes, in addition to *omp-31*, are probably included. In spite of the high degree of DNA homology detected in the *Brucella* genus (Hoyer & McCullough, 1968a, b; Verger *et al.*, 1985), here we report significant differences at the genetic level in one *Brucella* species which could be involved in some of the phenotypic and/or pathogenic characteristics that differentiate the *Brucella* species.

Results presented in this paper do not allow us to explain the mechanism responsible for the deletion of this 10 kb DNA fragment in *B. abortus*. However, a *B. melitensis* 16M DNA fragment of 1.8 kb (cloned into plasmid pNV3126), which only contains one *HindIII* restriction site and bears a portion of DNA flanking one side of the *B. abortus* deletion, hybridized with more than two fragments of *HindIII*-digested DNA of the other *Brucella* species (data not shown). Indeed, this hybridization pattern was observed with all *Brucella* spp. biovars with the exception of the seven *B. abortus* biovars which developed only one band. This result suggests that the 1.8 kb insert of pNV3126 contains a DNA element which is repeated along the chromosome in all brucellae except *B. abortus*. Moreover, according to the *HindIII* restriction profile and the Southern blot pattern of pNV3103, a plasmid bearing a 17 kb *B.*

melitensis 16M insert covering the 10 kb region deleted in *B. abortus* and flanking DNA, it seems probable that the repeated element is present in more than one copy in the pNV3103 DNA insert. As repeated DNA elements, such as insertion sequences, have been suggested to be involved in the mechanism of deletion process of large chromosomal DNA segments (Fetherston & Perry, 1994), location of this repeated element flanking both sides of the 10 kb DNA fragment deleted in *B. abortus* would provide a possible explanation for the origin of this deletion. Studies to determine the nucleotide sequence of this fragment and adjacent DNA are currently being performed in our laboratory and these will help both to identify the genes located in the 10 kb fragment and to determine the nature and possible role of the repeated element in the deletion process.

Several techniques have been employed in an attempt to find suitable DNA markers for the molecular typing of the *Brucella* genus. Some of these methods do not involve known *Brucella* gene sequences (Allardet-Servent *et al.*, 1988; Fekete *et al.*, 1992; Grimont *et al.*, 1992; Mercier *et al.*, 1996) and others take advantage of identified genes or insertion sequences (Bricker & Halling, 1994, 1995; Cloeckart *et al.*, 1995, 1996b, c; Ficht *et al.*, 1990, 1996; Halling & Zehr, 1990; Ouahrani *et al.*, 1993). Among them, PCR-RFLP with *Brucella* spp. OMP genes has been shown to be an interesting method for the differentiation of the brucellae (Cloeckart *et al.*, 1995). PCR-RFLP studies with *omp2a*, *omp2b* and *omp25* from all *Brucella* species and biovars have allowed the differentiation of all *Brucella* species and some of their biovars, with the sole exception of *B. canis* which remained indistinguishable from *B. suis* bv. 3 and 4 (Cloeckart *et al.*, 1995). *B. canis* and *B. suis* bv. 3 and 4 have been reported to be closely related on the basis of phenotypic characteristics (Meyer, 1990) which can explain the difficulties found in their differentiation at the molecular level. In the present work, PCR-RFLP analysis with *Brucella* spp. *omp-31* has allowed the identification of a *B. canis*-specific marker that makes this species distinguishable from the other *Brucella* species and their biovars, including *B. suis* bv. 3 and 4. Moreover, specific markers for *B. ovis* and *B. suis* bv. 2 have also been found.

In addition to DNA markers detected by PCR-RFLP, other markers have been found by Southern blot hybridization with plasmids probes containing *omp-31* and adjacent DNA, identifying *omp-31* as an interesting gene for the differentiation of *Brucella* strains. By combining PCR-RFLP patterns and Southern blot hybridization results, differentiation of all *Brucella* species can be accomplished with the exception of *B. neotomae* which remains indistinguishable from *B. suis* bv. 1, 3, 4 and 5. Moreover, biovar-specific markers have been found for *B. melitensis* bv. 1 and *B. suis* bv. 2.

In summary, work presented in this paper, regarding polymorphism at the *omp-31* locus, has allowed the identification of useful DNA markers for the differentiation among the brucellae and has provided the first evidence for the absence of at least one gene in one

Brucella species. Further work is necessary to identify the other missing genes in *B. abortus* and to determine their possible roles in the development of the infectious process. This may lead to a better understanding of differences in pathogenicity and host preference in *Brucella* species.

REFERENCES

- Allardet-Servent, A., Bourg, G., Ramuz, M., Pages, M., Bellis, M. & Roizes, G. (1988). DNA polymorphism in strains of the genus *Brucella*. *J Bacteriol* 170, 4603–4607.
- Alton, G. G., Jones, L. M., Angus, R. D. & Verger, J. M. (1988). *Techniques for the Brucellosis Laboratory*. Paris: Institut National de la Recherche Agronomique.
- Bricker, B. J. & Halling, S. M. (1994). Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv. 1 by PCR. *J Clin Microbiol* 32, 2660–2666.
- Bricker, B. J. & Halling, S. M. (1995). Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. *J Clin Microbiol* 33, 1640–1642.
- Cloekaert, A., Verger, J.-M., Grayon, M. & Grépinet, O. (1995). Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*. *Microbiology* 141, 2111–2121.
- Cloekaert, A., Debbarh, H. S. A., Vizcaíno, N., Saman, E., Dubray, G. & Zygmunt, M. S. (1996a). Cloning, nucleotide sequence, and expression of the *Brucella melitensis* *bp26* gene coding for a protein immunogenic in infected sheep. *FEMS Microbiol Lett* 140, 139–144.
- Cloekaert, A., Verger, J. M., Grayon, M. & Grépinet, O. (1996b). Polymorphism at the *dnaK* locus of *Brucella* species and identification of a *Brucella melitensis* species-specific marker. *J Med Microbiol* 45, 200–205.
- Cloekaert, A., Verger, J. M., Grayon, M. & Vizcaíno, N. (1996c). Molecular and immunological characterization of the major outer membrane proteins of *Brucella*. *FEMS Microbiol Lett* 145, 1–8.
- Cloekaert, A., Verger, J. M., Grayon, M., Zygmunt, M. S. & Grépinet, O. (1996d). Nucleotide sequence and expression of the gene encoding the major 25-kilodalton outer membrane protein of *Brucella ovis*: evidence for antigenic shift, compared with the other *Brucella* species, due to a deletion in the gene. *Infect Immun* 64, 2047–2055.
- Corbel, M. J. & Brinley-Morgan, W. J. (1984). Genus *Brucella* Meyer and Shaw 1920, 173AL. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 377–388. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Dubray, G. (1981). *Etude ultrastructurale et biochimique des enveloppes des bactéries du genre Brucella*. PhD thesis. Université de Paris-Sud, Paris.
- Fekete, A., Bantle, J. A., Halling, S. M. & Stich, R. W. (1992). Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. *J Bacteriol* 174, 7778–7783.
- Fetherston, J. D. & Perry, R. D. (1994). The pigmentation locus of *Yersinia pestis* KIM6+ is flanked by an insertion sequence and includes the structural genes for pesticin sensitivity and HMWP2. *Mol Microbiol* 13, 697–708.
- Ficht, T. A., Bearden, S. W., Sowa, B. A. & Adams, L. G. (1988). A 36-kilodalton *Brucella abortus* cell-envelope protein is encoded by repeated sequences closely linked in the genomic DNA. *Infect Immun* 56, 2036–2046.
- Ficht, T. A., Bearden, S. W., Sowa, B. A. & Adams, L. G. (1989). DNA sequence and expression of the 36-kilodalton outer membrane protein gene of *Brucella abortus*. *Infect Immun* 57, 3281–3291.
- Ficht, T. A., Bearden, S. W., Sowa, B. A. & Marquis, H. (1990). Genetic variation at the *omp2* porin locus of the brucellae: species-specific markers. *Mol Microbiol* 4, 1135–1142.
- Ficht, T. A., Hussein, H. S., Derr, J. & Bearden, S. W. (1996). Species-specific sequences at the *omp2* locus of *Brucella* type strains. *Int J Syst Bacteriol* 46, 329–331.
- Grimont, F., Verger, J. M., Cornelis, P., Limet, J., Lefèvre, M., Grayon, M., Régnault, B., Van Broeck, J. & Grimont, P. A. D. (1992). Molecular typing of *Brucella* with cloned DNA probes. *Res Microbiol* 143, 55–65.
- Halling, S. M. & Zehr, E. S. (1990). Polymorphism in *Brucella* spp. due to highly repeated DNA. *J Bacteriol* 172, 6637–6640.
- Hoyer, B. H. & McCullough, N. B. (1968a). Polynucleotide homologies of *Brucella* deoxyribonucleic acids. *J Bacteriol* 95, 444–448.
- Hoyer, B. H. & McCullough, N. B. (1968b). Homologies of deoxyribonucleic acids from *Brucella ovis*, canine abortion organisms, and other *Brucella*. *J Bacteriol* 96, 1783–1790.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Mercier, E., Jumas-Bilak, E., Allardet-Servent, A., O'Callaghan, D. & Ramuz, M. (1996). Polymorphism in *Brucella* strains detected by studying distribution of two short repetitive DNA elements. *J Clin Microbiol* 34, 1299–1302.
- Meyer, M. E. (1976). Evolution and taxonomy in the genus *Brucella*: concepts on the origins of the contemporary species. *Am J Vet Res* 37, 199–202.
- Meyer, M. E. (1990). Current concepts in the taxonomy of the genus *Brucella*. In *Animal Brucellosis*, pp. 2–13. Edited by K. Nielsen & J. R. Duncan. Boca Raton, FL: CRC Press.
- Ouahrani, S., Michaux, S., Widada, J. S., Bourg, G., Tournebize, R., Ramuz, M. & Liautard, J. P. (1993). Identification and sequence analysis of IS6501, an insertion sequence in *Brucella* spp.: relationship between genomic structure and the number of IS6501 copies. *J Gen Microbiol* 139, 3265–3273.
- Sangari, F. J., García-Lobo, J. M. & Agüero, J. (1994). The *Brucella abortus* vaccine strain B19 carries a deletion in the erythritol catabolic genes. *FEMS Microbiol Lett* 121, 337–342.
- Verger, J. M., Grimont, F., Grimont, P. A. D. & Grayon, M. (1985). *Brucella*, a monospecific genus as shown by deoxyribonucleic acid hybridization. *Int J Syst Bacteriol* 35, 292–295.
- Verger, J. M., Grimont, F., Grimont, P. A. D. & Grayon, M. (1987). Taxonomy of the genus *Brucella*. *Ann Inst Pasteur Microbiol* 138, 235–238.
- Vizcaíno, N., Cloekaert, A., Zygmunt, M. S. & Dubray, G. (1996). Cloning, nucleotide sequence, and expression of the *Brucella melitensis* *omp31* gene coding for an immunogenic major outer membrane protein. *Infect Immun* 64, 3744–3751.
- de Wergifosse, P., Lintermans, P., Limet, J. N. & Cloekaert, A. (1995). Cloning and nucleotide sequence of the gene coding for the major 25-kilodalton outer membrane protein of *Brucella abortus*. *J Bacteriol* 177, 1911–1914.

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