Restriction site polymorphism of the genes encoding the major 25 kDa and 36 kDa outer-membrane proteins of *Brucella*

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Seventy-seven *Brucella* reference and field strains from different geographic origins and hosts representing the six recognized species and their different biovars were analysed for diversity of their genes encoding the major 25 and 36 kDa outer-membrane proteins (OMPs) by PCR-RFLP. The 25 kDa OMP is encoded by a single gene (*omp25*) whereas two closely related genes (*omp2a* and *omp2b*) encode and potentially express the 36 kDa OMP. Analysis of PCR products of the *omp25* gene digested with nine restriction enzymes revealed two species-specific markers, i.e. the absence of the *EcoRV* site in all *Brucella melitensis* strains and an ~ 50 bp deletion at the 3' terminal end of the gene in all *Brucella ovis* strains. Analysis of PCR products of the *omp2a* and *omp2b* genes digested with 13 restriction enzymes indicated a greater diversity than the *omp25* gene among the six *Brucella* species and within the *Brucella abortus*, *Brucella suis*, *B. melitensis* and *B. ovis* species. Greater polymorphism was also detected for the *omp2b* than for the *omp2a* gene, especially in *B. ovis* which seemed to carry two similar (but not identical) copies of *omp2a* instead of one copy each of *omp2a* and *omp2b* for the other *Brucella* species as was previously suggested by Ficht *et al.* (1990; *Mol Microbiol* 4, 1135–1142). Results of PCR-RFLP indicated that distinction can be made between *Brucella* species and some of their biovars, except between *B. canis* and *B. suis* bv. 3 and 4, on the basis of the size and diversity of their major OMP genes, and that it could be of importance for diagnostic, epidemiological and evolutionary study purposes.

Keywords: *Brucella*, outer-membrane proteins, polymorphism

INTRODUCTION

Brucellae are Gram-negative facultative intracellular bacteria that can infect many species of animals and man. Six species are recognized within the genus *Brucella*: *B. abortus*, *B. melitensis*, *B. suis*, *B. ovis*, *B. canis* and *B. neotomae* (Corbel & Brinley-Morgan, 1984). This classification is mainly based on the difference in pathogenicity and in host preference (Corbel & Brinley-Morgan, 1984). Distinction between species and biovars is currently performed by differential tests based on phenotypic characterization of LPS antigens, phage typing, dye-sensitivity, CO₂ requirement, H₂S production, and metabolic properties (Alton *et al.*, 1988). It has been suggested on the basis of

DNA homology (> 90% for all species) that *Brucella* is a mono-specific genus (Verger *et al.*, 1985, 1987). However, Allardet-Servent *et al.* (1988) reported that RFLP can be used to distinguish species within the genus *Brucella* and supported the use of the preferred host for classifying the brucellae. More recently, Fekete *et al.* (1992) showed by arbitrarily primed PCR that *Brucella* strains can be distinguished according to the banding patterns of their amplified DNA on agarose gels. In addition, Grimont *et al.* (1992) showed by Southern blot analysis of *Bam*HI-digested *Brucella* DNA with random cloned *Brucella* DNA probes that the most frequently occurring biovars (*B. melitensis* bv. 1 and 3, *B. abortus* bv. 1 and 3, *B. suis* bv. 2 and *B. ovis*) could be distinguished from each other.

Two major proteins are expressed in the outer membrane of *Brucella*, the 25 and the 36 kDa OMPs (Cloeckaert *et al.*,

Abbreviation: OMP, outer-membrane protein.

Table 1. *Brucella* strains studied

No.	Species	Biovar	Strain*	Host/source	Geographic origin
1	<i>B. abortus</i>	1	544 (ATCC 23448; BCCN R4)†	Cattle	England
2		1	B19 (BCCN V1)‡	Cattle	USA
3		1	99S (BCCN R20)	Cattle	USA
4		2	86/8/59 (ATCC 23449; BCCN R5)	Cattle	England
5		3	Tulya (ATCC 23450; BCCN R6)	Human	Uganda
6		4	292 (ATCC 23451; BCCN R7)	Cattle	England
7		5	B3196 (ATCC 23452; BCCN R8)	Cattle	England
8		6	870 (ATCC 23453; BCCN R9)	Cattle	Africa
9		9	C68 (ATCC 23455; BCCN R11)	Cattle	England
10		R	45/20 (BCCN V2)‡	Cattle	England
11		R	RB51 (BCCN V5)	Cattle	USA
12	<i>B. melitensis</i>	1	16M (ATCC 23456; BCCN R1)†	Goat	USA
13		1	BCCN 93.4	Human	France
14		1	BCCN 92.106c	Unknown	Algeria
15		1	BCCN 93.2	Human	France
16		1	BCCN 88.41	Sheep	Israel
17		1	BCCN 88.42	Sheep	Israel
18		1	BCCN 88.43	Human	Israel
19		1	BCCN 88.39	Sheep	Israel
20		1	BCCN 75.477	Dog	Israel
21		1	BCCN 75.473	Cattle	Israel
22		1	BCCN 75.478	Cattle	Israel
23		1	BCCN 88.44	Human	Israel
24		1	BCCN 76.279	Cattle	Israel
25		1	BCCN 92.70	Human	France
26		1	Rev. 1 (BCCN V4a)‡	Goat	Mexico
27		1	H38 (BCCN V3)‡	Human	Mexico
28		1	BCCN 90.61	Sheep	South Africa
29		1	BCCN 87.92	Human	USA
30		2	63/9 (ATCC 23457; BCCN R2)	Goat	Turkey
31		3	Ether (ATCC 23458; BCCN R3)	Goat	Italy
32		3	BCCN 83.35	Human	Turkey
33		3	BCCN 83.198	Human	Spain
34		3	BCCN 90.112	Cattle	Greece
35		3	BCCN 90.128	Cattle	Greece
36		3	BCCN 75.472	Cattle	Israel
37		3	BCCN 75.479	Cattle	Israel
38		3	BCCN 92.42	Human	Tunisia
39		3	BCCN 92.80	Sheep	Spain
40		3	BCCN 92.83	Sheep	Spain
41		3	BCCN 92.106a	Unknown	Algeria
42		3	BCCN 92.118	Human	Tunisia
43		3	BCCN 92.120	Human	Tunisia
44		3	BCCN 92.121	Human	Tunisia
45		3	BCCN 93.3	Human	France
46		3	BCCN 93.1	Human	France
47		R	B115 (BCCN R19)	Goat	Malta
48		R	BCCN 92.119	Human	Tunisia
49		R	BCCN 92.116	Sheep	France
50		R	BCCN 92.117	Sheep	France
51		R	BCCN 93.5	Human	France
52	<i>B. suis</i>	1	1330 (ATCC 23444; BCCN R12)†	Swine	USA
53		1	S2 (BCCN V6)‡	Swine	China
54		2	Thomsen (ATCC 23445; BCCN R13)	Swine	Denmark
55		3	686 (ATCC 23446; BCCN R14)	Swine	USA
56		4	40 (ATCC 23447; BCCN R15)	Reindeer	Former Soviet Union
57		5	513 (BCCN R21)	Wild rodent	Former Soviet Union

Table 1. (cont.)

No.	Species	Biovar	Strain*	Host/source	Geographic origin
58	<i>B. ovis</i>	—	63/290 (ATCC 25840; BCCN R17)†	Sheep	Africa
59		—	Reo 198 (BCCN R22)	Sheep	USA
60		—	BCCN 76.247	Sheep	France
61		—	BCCN 76.250	Sheep	France
62		—	BCCN 91.66	Sheep	Spain
63		—	BCCN 91.70	Sheep	Spain
64		—	BCCN 91.208	Sheep	Spain
65		—	BCCN 91.212	Sheep	Spain
66		—	BCCN 91.217	Sheep	Spain
67		—	BCCN 91.264	Sheep	Argentina
68	<i>B. canis</i>	—	BCCN 91.266	Sheep	Argentina
69		—	BCCN 91.268	Sheep	Switzerland
70		—	RM6/66 (ATCC 23365; BCCN R18)†	Dog	USA
71		—	BCCN 87.62	Unknown	Mexico
72		—	BCCN 87.66	Dog	Canada
73	<i>B. canis</i>	—	BCCN C2	Dog	USA
74		—	BCCN C3	Dog	USA
75		—	BCCN C1	Dog	Madagascar
76		—	BCCN C4	Dog	USA
77	<i>B. neotomae</i>	—	5K33 (ATCC 23459; BCCN R16)†	Desert rat	USA

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

† Reference strain.

‡ Vaccine strain.

Table 2. Restriction patterns of the PCR-amplified *Brucella* 25 kDa OMP genes

<i>Brucella</i> species	Biovar	Pattern*	Pattern with restriction enzymes:								
			<i>Ava</i> II	<i>Ban</i> I	<i>Eco</i> RII	<i>Eco</i> RV	<i>Hind</i> III	<i>Sau</i> 3A	<i>Sty</i> I	<i>Taq</i> I	<i>Mae</i> III‡
<i>B. abortus</i> (strains 1–11)†	1–9, R	A	P1	P1	P1	P1	P1	P1	P1	P1	P1
<i>B. melitensis</i> (strains 12–51)†	1–3, R	B	P1	P1	P1	nc	P1	P1	P1	P1	P1
<i>B. suis</i> (strains 52–57)†	1–5	A	P1	P1	P1	P1	P1	P1	P1	P1	P1
<i>B. ovis</i> (strains 58–69)†		C	P2	P2	P2	P2	P2	P2	P2	P2	P2
<i>B. canis</i> (strains 70–76)†		A	P1	P1	P1	P1	P1	P1	P1	P1	P1
<i>B. neotomae</i> (strain 5K33)		A	P1	P1	P1	P1	P1	P1	P1	P1	P1

NC, Not cut.

* Overall pattern for each *Brucella* strain of the 25 kDa OMP gene cut with the different restriction enzymes.

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

‡ Only the reference strain of each *Brucella* species and the 12 *B. ovis* strains were tested for polymorphism of the 25 kDa OMP gene with *Mae*III.

1990; Douglas *et al.*, 1984; Dubray & Charriaut, 1983; Verstraete *et al.*, 1982), the latter having a porin activity (Douglas *et al.*, 1984; Marquis & Ficht, 1993). The gene encoding the major 25 kDa OMP (*omp25*) has been cloned from *B. abortus* strain 544 (bv. 1) genomic libraries constructed in lambda gt11 and lambda EMBL3 vectors by using monoclonal antibodies (mAbs) (Cloeckaert *et al.*, 1990) as probes, subcloned in plasmid pTZ18 and

sequenced (de Wergifosse *et al.*, 1995). By Southern blot analysis of restriction-digested *Brucella* DNA with the cloned gene as probe, we showed that *Brucella* probably contain a single copy of the gene in its DNA (Verger *et al.*, unpublished results). On the contrary, Ficht *et al.* (1988, 1989) demonstrated that two closely related genes (*omp2a* and *omp2b*), located in the *B. abortus omp2* locus, encode and potentially express the 36 kDa OMP. The genes

cloned from a *B. abortus* bv. 1 gene library share about 85% DNA homology. The two genes are separated by 900 bp and are oriented in opposite directions. Expression has been demonstrated only from the *omp2b* gene in *B. abortus*, or when the genes are cloned into *Escherichia coli* (Ficht *et al.*, 1989; Marquis & Ficht, 1993). In addition, Ficht *et al.* (1990) reported genetic variation at the *omp2* locus by Southern blot analysis with *omp2a*- or *omp2b*-specific probes and RFLP among the *Brucella* species. The

Fig. 1. Restriction map of the PCR-amplified *omp25* gene, according to the gene sequence determined from *B. abortus* bv. 1 (de Wergifosse *et al.*, 1995). The coding sequence is shown by a shaded rectangle under the restriction map. Restriction sites: Av, Avall; B, BamI; EII, EcoRII; EV, EcoRV; H, HindIII; M, MaeIII; S, Sau3A; St, Styl; T, TaqI.

Fig. 2. Electrophoretic analysis of PCR-amplified *omp25* genes of *B. abortus* (lane 2) and *B. ovis* (lane 3) and restriction patterns of the PCR-amplified *omp25* gene (lanes 4–21). Patterns P1 (*B. abortus*) and P2 (*B. ovis*) described in Table 2 are shown. Lanes: 4–5, patterns P1 and P2 Avall; 6–7, patterns P1 and P2 BamI; 8–9, patterns P1 and P2 EcoRII; 10–11, patterns P1 and P2 EcoRV; 12–13, patterns P1 and P2 HindIII; 14–15, patterns P1 and P2 Sau3A; 16–17, patterns P1 and P2 Styl; 18–19, patterns P1 and P2 TaqI; 20–21, patterns P1 and P2 MaeIII; 1, molecular mass markers (kit VI, Boehringer Mannheim) 2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234, 220 and 154 bp.

Table 3. Restriction patterns of the PCR-amplified *Brucella* 36 kDa *omp2a* genes

Brucella species	Biovar	Pattern*	Pattern with restriction enzymes:													
			AluI	BanI	BglII	Clal	EcoRI	HaeIII	HinfI	KpnI	NcoI	PstI	PvuII	StyI	TaqI	
B. abortus																
Strains 1–4, 6, 10, 11†	1, 2, 4, R	A	P1	P1	NC	NC	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1
Strains 5, 7–9†	3, 5, 6, 9	B	P2	P2	NC	NC	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2
B. melitensis																
Strains 12, 13, 26–29, 47†	1, R	C	P2	P2	NC	NC	P2	P2	P2	P2	P2	P3	P2	P2	P2	P2
Strains 14–25, 30–46, 48–51†	1, 2, 3, R	B	P2	P2	NC	NC	P2	P2	P2	P2	P2	P2	P2	P2	P2	P2
Strains 48–51†																
B. suis																
Strains 1330, S2, 686, 40	1, 3, 4	D	P2	P3	NC	NC	P2	P2	P3	NC	P2	P2	P2	P2	P2	P2
Strain Thomsen	2	E	P2	P3	NC	NC	P2	P2	P3	NC	NC	P2	P2	NC	NC	P2
Strain 513	5	F	P2	P3	NC	NC	P2	P2	P4‡	NC	NC	P2	P2	NC	NC	P2
B. ovis																
Strains 58–69†		G	P3	P3	NC	NC	P2	P2	P2	NC	NC	P2	P2	NC	NC	P2
B. canis																
Strains 70–76†		D	P2	P3	NC	NC	P2	P2	P3	NC	P2	P2	P2	P2	P2	P2
B. neotomae																
Strain 5K33		H	P4‡	P3	NC	NC	P2	P2	P4‡	NC	NC	P2	P2	NC	NC	P2

NC, Not cut.

* Overall pattern for each *Brucella* strain of the 36 kDa *omp2a* genes.

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

‡ Pattern similar to that of the *omp2b* gene of *B. ovis* cut by the same restriction enzymes.

Table 4. Restriction patterns of the PCR-amplified *Brucella* 36 kDa *omp2b* genes

Brucella species	Biovar	Pattern*	Pattern with restriction enzymes:												
			AluI	BanI	BglII	Clal	EcoRI	HaeIII	HinfI	KpnI	NcoI	PstI	PvuII	StyI	TaqI
B. abortus															
Strains 1–4, 11†	1, 2, R	A	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1	NC	P1	P1
Strains 5–9†	3, 4, 5, 6, 9	B	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1	NC	P1	P2
Strain 45/20	R	C	P1	P1	P2	P2	P2	P1	P2	P1	P1	P1	NC	P2	P1
B. melitensis															
Strains 12–15, 26–29, 31, 35, 36, 38, 41–51†	1, 3, R	B	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1	NC	P1	P2
Strains 16–24, 34, 37†	1, 3	D	P1	P1	NC	P1	P1	P1	P1	P1	P1	P1	NC	P1	P2
Strains 25, 30, 32, 33, 39, 40†	1, 2, 3	E	P1	P1	NC	P1	P1	P1	P3	P1	P1	P1	NC	P1	P2
B. suis															
Strains 1330, S2, 513	1, 5	B	P1	P1	P1	P1	P1	P1	P1	P1	P1	P1	NC	P1	P2
Strain Thomsen	2	F	P1	P2	P1	P1	P3	P1	P1	NC	P1	P1	NC	P1	P2
Strains 686, 40	3, 4	G	P1	P2	P1	P1	P1	P1	P1	NC	P1	P1	NC	P1	P2
B. ovis															
Strain 63/290		H	P2	P3‡	NC‡	NC‡	P4‡	P2‡	P4	NC‡	NC‡	P2‡	P2‡	NC‡	P3
Strain Reo 198		I	P2	P3‡	NC‡	NC‡	P4‡	P3	P4	NC‡	NC‡	P2‡	P2‡	NC‡	P3
Strains 60–69†		J	P2	P3‡	P3	NC‡	P4‡	P3	P5	NC‡	NC‡	P3	P2‡	P3	P3
B. canis															
Strains 70–76†		G	P1	P2	P1	P1	P1	P1	P1	NC	P1	P1	NC	P1	P2
B. neotomae															
Strain 5K33		K	P1	P1	P1	P1	P1	P4	P1	P1	P1	P1	NC	P1	P2

NC, Not cut.

* Overall pattern for each *Brucella* strain of the 36 kDa *omp2b* genes.

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

‡ Pattern similar to that of the corresponding strain *omp2a* gene.

different patterns they observed could be used to distinguish the type strains of the six *Brucella* species. Five of the six species were shown to contain single copies of *omp2a* and *omp2b*, whereas *B. ovis* appeared to have two copies of *omp2a*.

In the present study, we analysed by PCR-RFLP the polymorphism of the *omp25*, *omp2a* and *omp2b* genes among 77 *Brucella* reference and field strains representing all species with their different biovars. Studying polymorphism by PCR-RFLP has the advantage over Southern blotting because it is easier to set up and is less time-consuming for application to large numbers of samples. In addition, PCR combined with use of species-specific markers to identify the causative agent of disease could be used for diagnostic purposes (Fekete *et al.*, 1990; Herman & De Ridder, 1992).

METHODS

Bacterial strains. The 77 strains used in this study are listed in Table 1. All strains were kept freeze-dried in the *Brucella* Culture

Collection, INRA, Nouzilly, France. Cultures were grown on Trypcase Soy Agar (BioMérieux) supplemented with 0.1% (w/v) yeast extract (Difco) (TSAYE). For fastidious strains (*B. abortus* bv. 2 and *B. ovis*), sterile equine serum (BioMérieux) was added to TSAYE to a final concentration of 5% (v/v) (TSAYES). The strains were checked for purity and species and biovar characterization by standard procedures (Alton *et al.*, 1988).

DNA preparation. For each strain, a 1-d-old 37 °C culture on a TSAYE or TSAYES slope was harvested with 3 ml sterile distilled water, and centrifuged at 4500 r.p.m. for 10 min. The pellet was suspended in 567 µl TE/sodium buffer (50 mM Tris, 50 mM EDTA, 100 mM NaCl, pH 8.0). SDS solution (30 µl 10%, w/v) and 3 µl 2% (w/v) proteinase K solution were then added and held at 37 °C for 1 h. The lysed cell suspension was extracted twice with phenol then chloroform (v/v). Nucleic acids were precipitated by gently mixing the resultant aqueous phase with 2 volumes cold ethanol. The precipitate was dissolved in 100 µl 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 DNA standards.

Primers. Two 20-mer primers for each gene were obtained from Appligene. Primers were chosen to amplify the entire gene with its Shine–Dalgarno and putative transcription terminator sequences according to the *B. abortus* bv. 1 determined nucleotide sequences of *omp25* (de Wergifosse *et al.*, 1995), and *omp2a* and *omp2b* (Ficht *et al.*, 1989). Primers used for amplification of the *omp25* gene were 25A (5'-GGACCGCG-CAAAACGTAATT-3') and 25B (5'-ACCGGATGCCTGAA-ATCCTT-3'), for amplification of the *omp2a* gene, 2aA (5'-GGCTATTCAAAATCTGGCG-3') and 2aB (5'-ATCGAT-TCTCAGCTTTCGT-3'), and for amplification of the *omp2b* gene, 2bA (5'-CCTTCAGCCAAATCAGAATG-3') and 2bB (5'-GGTCAGCATAAAAAGCAAGC-3').

Amplification conditions. Amplification reactions were prepared in 100 µl volumes 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 × PCR buffer; Appligene), 200 µM (each) dNTPs, 1 µM primer, 100 ng genomic DNA, and 2.5 U *Taq* DNA polymerase (Appligene). To reduce evaporation, the mixture was overlaid with a drop of mineral oil. The temperature cycling for the amplification was performed in a Techne PHC-2 thermocycler as follows. The first cycle was 95 °C for 5 min (denaturation), 58 °C for 2 min (annealing) and 70 °C for 3 min (extension). The next 30 cycles were 95 °C for 1 min (denaturation), 58 °C for 2 min (annealing) and 70 °C for 3 min (extension). The last cycle was 95 °C for 1 min (denaturation), 58 °C for 2 min (annealing) and 70 °C for 10 min (extension).

Restriction digestion. Restriction enzymes were chosen according to the *B. abortus* bv. 1 determined gene sequences. Figs 1, 5 and 7 show the restriction map of each gene with the enzymes used. Restriction enzymes *AluI*, *AvaII*, *BglII*, *ClaI*, *EcoRI*, *HaeIII*, *HindIII*, *HinfI*, *KpnI*, *NcoI*, *PstI*, *PvuII* and *TaqI* were purchased from Appligene, and *BanI*, *EcoRII*, *EcoRV*, *MaeIII*, *Sau3A* and *SylI* were purchased from Boehringer Mannheim. PCR product (5 µl) was cleaved by 1–5 U of each restriction enzyme in a 20 µl reaction volume; the manufacturer's recommended buffer and incubation temperature were used.

Agarose gel electrophoresis. After amplification of the genes, 5 µl reaction mixture or 20 µl each restriction digest were mixed with 1 and 2 µl, respectively, of glycerol-dye loading buffer, and the mixture was electrophoresed on agarose gel consisting of 1 or 1.5 % (w/v) standard quality agarose (Appligene) or 2.5 % NuSieve 3:1 agarose (TEBU) and 0.5 µg ethidium bromide ml⁻¹ (Sigma) in TBE buffer (89 mM Tris/HCl, 89 mM boric acid, 2 mM EDTA, pH 8.0). The samples were electrophoresed at 20 V overnight on an Apex ST 1006 electrophoresis unit. Molecular mass DNA marker kit VI (Boehringer Mannheim) was used as standard. A 254 nm UV transilluminator was used to detect the bands.

RESULTS

Polymorphism of the *Brucella* gene encoding the 25 kDa OMP

All *Brucella* strains had the same restriction pattern for the amplified fragment carrying the *omp25* gene, except that *B. melitensis* (all 40 strains) lacked the *EcoRV* site, and *B. ovis* (all 12 strains) had a short deletion (Table 2, Fig. 2). The deletion in the *B. ovis* gene was about 50 bp in length and was located between the *MaeIII* and *EcoRV* sites, including the *Sau3A* site at position 605 (see Fig. 1).

Fig. 3. Electrophoretic analysis of PCR-amplified *omp2a* (lanes 3, 5, 7, 9, 11, 13, 15 and 17) and *omp2b* (lanes 2, 4, 6, 8, 10, 12, 14 and 16) genes of *B. abortus* bv. 1, 2 or 4 (lanes 2 and 3), *B. abortus* bv. 3, 5, 6 or 9 (lanes 4 and 5), *B. abortus* strain 45/20 (lanes 6 and 7), *B. melitensis* (lanes 8 and 9), *B. suis* (lanes 10 and 11), *B. ovis* (lanes 12 and 13), *B. canis* (lanes 14 and 15), and *B. neotomae* (lanes 16 and 17); lane 1, molecular mass markers (VI).

Fig. 4. Restriction patterns of the PCR-amplified *omp2a* gene. The patterns P1–P4 of Table 3 are shown, in numerical order, for each of the following enzymes: (a) lanes: 2–5, patterns P1–P4 *AluI*; 6–8, patterns P1–P3 *BanI*; 9–10, patterns P1 and P2 *EcoRI*; 11–12, patterns P1 and P2 *HaeIII*; 13–16, patterns P1–P4 *HinfI*; 17–18, patterns P1 and P2 *KpnI*; 1, molecular mass markers (VI). (b) lanes: 2–3, patterns P1 and P2 *NcoI*; 4, pattern P1 *PstI*; 5, pattern P3 *PstI*; 6, pattern P2 *PstI*; 7–8, patterns P1 and P2 *PvuII*; 9–10, patterns P1 and P2 *SylI*; 11–12, patterns P1 and P2 *TaqI*; 1, molecular mass markers (VI).

Polymorphism of the *Brucella* genes encoding 36 kDa OMPs

Compared to the 25 kDa OMP gene, the 36 kDa OMPs genes (*omp2a* and *omp2b*) showed a greater degree of diversity among the different *Brucella* species, biovars and strains (Tables 3 and 4). The sizes of the amplified *omp2a* and *omp2b* genes were similar for all *Brucella* species except

Fig. 5. Restriction maps of the PCR-amplified *omp2a* gene of *Brucella* species and biovars. Restriction map of pattern A was deduced from the nucleotide sequence of the *omp2a* gene of *B. abortus* bv. 1 (Ficht *et al.*, 1989). The corresponding patterns B–H of Table 3 are compared with pattern A (*B. abortus* bv. 1, 2, 4 and R). Only differences compared with the restriction map of strains showing pattern A are indicated in the restriction maps of strains showing patterns B–H. Missing sites in the *omp2a* gene of strains showing patterns B–H are barred. Additional sites and their most probable location according to restriction patterns and single nucleotide substitutions in the *omp2a* gene sequence of *B. abortus* bv. 1 are indicated by the restriction enzyme name (e.g. Hf) and the most probable location when possible by the position number and an asterisk. The 120 bp deletion in the *omp2a* gene of the *B. abortus* biovars showing pattern A is indicated by a hatched box located between the *TaqI* (517) and *TaqI* (676) sites of restriction map A. Restriction sites: A, *AluI*; B, *BanI*; C, *Clai*; E, *EcoRI*; Ha, *HaeIII*; Hf, *HinfI*; K, *KpnI*; N, *NcoI*; P, *PstI*; Pv, *PvuII*; St, *StyI*; T, *TaqI*.

those of the *omp2a* genes of *B. abortus* bv. 1, 2, 4 and R which were shorter compared to the other *B. abortus* biovars and other *Brucella* species (Fig. 3). This observation confirms the results published by Ficht *et al.* (1990) concerning the absence of a 120 bp segment in the *omp2a* genes of *B. abortus* bv. 1, 2 and 4.

omp2a. On the basis of restriction patterns (from 13 restriction enzymes) of the PCR-amplified *omp2a* genes (Fig. 4), *Brucella* strains could be classified into eight different groups (Table 3). Some overall patterns were specific to *Brucella* species and/or biovars. The restriction maps corresponding to these patterns are shown in Fig. 5. Pattern A was specific to *B. abortus* bv. 1, 2, 4 and R and corresponds to the *omp2a* nucleotide sequence published by Ficht *et al.* (1989). It was characterized, compared to the other *Brucella* species and biovars, by a 120 bp deletion probably located between the *TaqI* (517) and *TaqI* (676) sites (Fig. 4).

omp2b. On the basis of restriction patterns (from 13 restriction enzymes) of the PCR-amplified *omp2b* genes (Fig. 6), *Brucella* strains could be classified into 11 different groups (Table 4), indicating a greater diversity than the *omp2a* genes. Like *omp2a* restriction patterns, some overall *omp2b* patterns were specific to *Brucella* species and/or biovars. The restriction maps corresponding to these patterns are shown in Fig. 7. Pattern A was specific to *B. abortus* bv. 1, 2 and R, and corresponds to the *omp2b* nucleotide sequence published by Ficht *et al.* (1989). Pattern C was specific to *B. abortus* strain 45/20. It was characterized, compared to the other *B. abortus* strains, by a short deletion (~20 bp) located between the *BglII* (589) and *HinfI* (774) sites (Figs 6 and 7). The shorter size of the *omp2b* gene of *B. abortus* 45/20 was not apparent with the undigested amplified *omp2b* gene (Fig. 3). Pattern D was characteristic of 11 strains of *B. melitensis* bv. 1 and 3, and it should be noted that 10 of the 11 strains isolated from different hosts (Table 1), including men, were from Israel,

Fig. 6. Restriction patterns of the PCR-amplified *omp2b* gene. The patterns P1–P5 of Table 4 are shown, in numerical order, for each of the following enzymes: (a) lanes: 2–3, patterns P1 and P2 *AluI*; 4–6, patterns P1–P3 *BlnI*; 7–9, patterns P1–P3 *BglII*; 10–11, patterns P1 and P2 *ClaI*; 12–15, patterns P1–P4 *EcoRI*; 16–19, patterns P1–P4 *HaeIII*; 1, molecular mass markers (VI). (b) lanes: 2–6, patterns P1–P5 *HinfI*; 7, pattern P1 *KpnI*; 8, pattern P1 *NcoI*; 9–11, patterns P1–P3 *PstI*; 12, pattern P2 *PvuII*; 13–15, patterns P1–P3 *StyI*; 16–18, patterns P1–P3 *TaqI*; 1, molecular mass markers (VI).

suggesting that particular patterns may be found depending on the geographic region.

***B. ovis* contains two similar copies of *omp2a* instead of one copy of *omp2a* and one copy of *omp2b*.** Patterns H, I and J for *omp2b* were specific to *B. ovis* strains. With each restriction enzyme tested, a different pattern was observed compared to *omp2b* patterns of all other *Brucella* strains (Fig. 6). Pattern H was specific to *B. ovis* strain 63/290 (reference strain), pattern I was specific to *B. ovis* strain Reo 198, and pattern J was specific to *B. ovis* field strains isolated from different distant geographic regions (Table 1).

Ficht *et al.* (1990) reported that *B. ovis* contains two copies of *omp2a* instead of the one copy of *omp2a* and one copy of *omp2b* found in the other *Brucella* species. Our results are in agreement with this observation in that the PCR-amplified *omp2b* genes of all *B. ovis* strains tested lacked, like the *omp2a* genes, the *ClaI*, *KpnI* and *NcoI* sites, but contained the *PvuII* site absent in the *omp2b* genes of the other *Brucella* species. Moreover, the *BlnI*, *EcoRI* and *PvuII* restriction patterns of the PCR-amplified *omp2b* genes from *B. ovis* were similar to those of the amplified *omp2a* genes from the corresponding strain (Table 4). However, restriction patterns with *AluI*, *HinfI* and *TaqI* indicated that the two genes of *B. ovis* are not identical.

Species-specific markers. Tables 3 and 4 show that PCR-RFLP of *omp2a* and *omp2b* can be used to distinguish *Brucella* species and some of their biovars. Accordingly, based on the diversity of both genes, distinction can be

made between all *Brucella* species except between *B. abortus* bv. 3, 5, 6, 9 and a number of strains of *B. melitensis* bv. 1 and 3, and between *B. canis* strains and *B. suis* bv. 3 and 4 (strains 686 and 40, respectively). Taking into account polymorphism of the *omp25* gene, distinction can be made between all *Brucella* species except between *B. canis* and *B. suis* bv. 3 and 4.

DISCUSSION

On the basis of DNA–DNA hybridization studies (Hoyer & McCullough, 1968a, b; Verger *et al.*, 1985), it has been shown that the genus *Brucella* is a highly homogeneous group and it has been proposed that this genus should comprise only one genomic species (Verger *et al.*, 1985, 1987). This proposal was also supported by rRNA–DNA hybridization data (De Ley *et al.*, 1987) and total DNA restriction analysis (McGillivray *et al.*, 1988; O'Hara *et al.*, 1985). However, for epidemiological and diagnostic purposes *Brucella* strains need to be distinguished. Thus, current research on the taxonomy and identification of *Brucella* strains is focused on molecular typing systems which would provide greater objectivity and safety (less handling of live *Brucella*) than phenotypic characterization.

The most significant observations in the present study were: (i) identification of species and biovar-specific markers on the basis of gene diversity of the 25 kDa and 36 kDa major OMPs by use of PCR-RFLP which could be applied in contrast to other studies on a large number of *Brucella* strains from different geographic origins and different hosts; (ii) that intra-species polymorphisms of *omp2a* and *omp2b* genes exist especially within the *B. melitensis* and *B. ovis* species; (iii) that *B. ovis* represents the most distant species of the others, as was suggested by other studies (Ficht *et al.*, 1990; Halling & Zehr, 1990; Meyer, 1990; O'Hara *et al.*, 1985).

The major polymorphism found for *omp25* was an ~ 50 bp deletion between the *MaeIII* and *EcoRV* sites at the 3' terminal end of the gene for all *B. ovis* strains studied. The consequences of this deletion on exposure of B-cell surface-exposed epitopes and on the topology of the 25 kDa *B. ovis* OMP in the outer membrane are currently being studied with mAbs specific for the 25 kDa OMP (Cloeckaert *et al.*, 1990). The potential different topology of this OMP might contribute to specific pathogenesis of *B. ovis* infection in rams. Nucleotide sequence analysis should also provide further information on the polymorphism of *omp25* within *Brucella* species in addition to that detected by PCR-RFLP.

Ficht *et al.* (1990) reported genetic variation at the *omp2* porin locus of the brucellae by Southern blot and RFLP analysis. Results of PCR-RFLP that are in agreement with theirs include: (i) the subdivision of *B. abortus* biovars into two groups based on the size of their *omp2a* genes, i.e. bv. 1, 2 and 4 contained *omp2a* genes of shorter size compared to the other *B. abortus* biovars and to the other *Brucella* species; (ii) the presence of species-specific markers for reference strains *B. suis* bv. 1 strain 1330, *B. canis* strain RM6/66 and *B. neotomae* strain 5K33; (iii) the presence of

Fig. 7. Restriction maps of the PCR-amplified *omp2b* gene of *Brucella* species and biovars. Restriction map of pattern A was deduced from the nucleotide sequence of the *omp2b* gene of *B. abortus* bv. 1 (Ficht *et al.*, 1989). The corresponding patterns B–K of Table 4 are compared with pattern A (*B. abortus* bv. 1, 2 and R). Only differences compared with the restriction map of strains showing pattern A are indicated in the restriction maps of strains showing patterns B–K. Missing sites in the *omp2b* gene of strains showing patterns B–K are barred. Additional sites and their most probable location according to restriction patterns and single nucleotide substitutions in the *omp2b* gene sequence of *B. abortus* bv. 1 are indicated by the restriction enzyme name (e.g. Hf) and the most probable location when possible by the position number and an asterisk. The short deletion (~20 bp) in the *omp2b* gene of *B. abortus* strain 45/20 (pattern C) is indicated by a hatched box located between the *Bgl*II (589) and *Hinf*I (774) sites of restriction map A. Restriction sites abbreviations are as in Fig. 5, plus *Bgl*II.

two similar but not identical copies of *omp2a* in *B. ovis* reference strain 63/290. PCR-RFLP used in the present study showed a greater diversity in the *omp2a* and *omp2b* genes among the *Brucella* species, biovars and strains. It included the shorter size of *omp2b* of the *B. abortus* 45/20 vaccine strain, the diversity of both *omp2a* and *omp2b* among *B. melitensis* strains, biovar-specific markers for *B. suis* biovars, and the intra-species diversity of the *omp2b* genes of *B. ovis* reference and field strains.

Some polymorphisms found for the *omp2a* and *omp2b* genes among *B. melitensis* strains, like the absence of the *Bgl*II site in *omp2b*, seemed to be characteristic of *B. melitensis* strains from a defined geographic region, i.e. Israel. Depending on the host from which the *Brucella* strains were isolated, differences in porin genes might also be expected. However, strains isolated from Israel were from different hosts including man. Recently, *B. melitensis* bv. 1 atypical strains, susceptible to dyes and penicillin, were isolated in Israel and characterized; this suggested

the evolution of a new variant (Banai *et al.*, 1990). Susceptibility to dyes and penicillin is probably directly related to porin structure and the polymorphism we found for the *omp2b* genes might be related to these susceptibilities. Therefore, it should be interesting to analyse *omp2a* and *omp2b* genes of these atypical strains for other polymorphisms. In addition, these results show the usefulness of PCR-RFLP in identifying epidemiological markers for brucellosis outbreaks.

Intra-species polymorphism was also found for the porin genes of *B. ovis* strains. The hypothesis of the presence of two similar copies of *omp2a* in *B. ovis*, instead of one copy of *omp2a* and one copy of *omp2b* for the other *Brucella* species (Ficht *et al.*, 1990), is supported by our PCR-RFLP results. However, the similarity between the two copies is more divergent in *B. ovis* field strains than in *B. ovis* reference strains 63/290 and Reo198. Interestingly, in contrast to *B. ovis* reference strain 63/290 and strain Reo198, the amplified *omp2b* genes of the *B. ovis* field strains

Fig. 8. Hypothetical *Brucella* phylogenetic networks based on restriction fragment differences of the *omp2a* and *omp2b* genes. Open circles represent hypothetical intermediates that were not found in the present study. Each unit length represents a single mutational event. *B. ovis* is not shown on the *omp2b* diagram as its gene belongs on the *omp2a* tree. Letters indicated at each node correspond to the patterns described in Tables 3 and 4.

contained *Bgl*II and *Sty*I sites like the *omp2b* genes of the other *Brucella* species. It might be expected that *B. ovis* will evolve to possess one copy of *omp2a* and one copy of *omp2b* like the other *Brucella* species. These observations together with the presence of a deletion in the *omp25* gene, which probably occurred from an entire gene, are in favour of *B. ovis* being a recently emerged species, as has been previously proposed (Meyer, 1990). Nevertheless, it may also be that *B. ovis* branched off or diverged from the group at an earlier time. An alternative explanation is that a gene conversion recently homogenized the two gene copies or that the *omp2* gene duplication only recently occurred in this organism. However, it seems more probable that the event in the *B. ovis* lineage was a gene conversion rather than an *omp2b* deletion and new *omp2a* duplication, because the successful PCR amplification implies that the sequences surrounding the *B. ovis* '*omp2b*' gene are similar to those that surround the *omp2b* gene in the other species, and different from those around the *omp2a* gene.

Meyer (1990) has proposed a model for evolutionary derivation of *Brucella* organisms on the basis of phenotypic characteristics. PCR-RFLP analysis of the porin genes which are in agreement with this model include (i) the close relationship between *B. abortus* bv. 3, 5, 6, 9 and *B. melitensis*; (ii) the presence of specific markers for *B. suis* bv. 2 and *B. neotomae*; (iii) the close relationship between *B. suis* bv. 3 and 4 and *B. canis* which were indistinguishable on the basis of PCR-RFLP results of both the *omp2a* and *omp2b* genes; and finally (iv) the fact that *B. ovis* represents the most widely diverged lineage. However, it seems

unlikely that *B. abortus* bv. 2 represents the progenitor species since its *omp2a* gene has a deletion of 120 bp like *B. abortus* bv. 1 and 4. The progenitor species logically carried two entire *omp2* genes. Hypothetical phylogenetic networks based on restriction fragment differences of the *omp2a* and *omp2b* genes are shown in Fig. 8.

In addition to study of the fundamental evolutionary aspects of the *Brucella omp25* and porin genes, PCR-RFLP of the major OMP genes could also be useful for diagnostic and identification purposes as was proposed for other *Brucella* genes (Fekete *et al.*, 1990; Herman & De Ridder, 1992).

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