Identification and sequence analysis of IS6501, an insertion sequence in *Brucella* spp.: relationship between genomic structure and the number of IS6501 copies

SAFIA OUAHRANI, SYLVIE MICHAUX, JOANNES SRI WIDADA, GISELE BOURG, REGIS TOURNEBIZE, MICHEL RAMUZ and JEAN-PIERRE LIAUTARD*

INSERM U-65, Département Biologie Santé, C.P. 100, Université de Montpellier II, Place E. Bataillon, 34095 Montpellier, France

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An insertion sequence (IS) element of *Brucella ovis*, named IS6501, was isolated and its complete nucleotide sequence determined. IS6501 is 836 bp in length and occurs 20–35 times in the *B. ovis* genome and 5–15 times in other *Brucella* species. Analysis of the junctions at the sites of insertion revealed a small target site duplication of four bases and inverted repeats of 17 bp with one mismatch. IS6501 presents significant similarity (53.4%) with IS427 identified in *Agrobacterium tumefaciens*, suggesting a common ancestral sequence. A long ORF of 708 bp was identified encoding a protein with a predicted molecular mass of 26 kDa and sharing sequence identity with the hypothetical protein 1 of *A. tumefaciens* and with the transposase of *Mycobacterium tuberculosis*. IS6501 is present in all *Brucella* strains we have tested. Restriction fragment length polymorphism of reference and field strains of two species (*B. melitensis* and *B. ovis*) was studied using either pulsed field gel electrophoresis (PFGE) on *Xba*I-digested DNA or hybridization of *Eco*RI-digested DNA using IS6501 as a probe. The genome of *B. melitensis* biovar 3 contains about 10 IS copies per genome and field strains of the same species could not be distinguished either by IS hybridization or by *Xba*I (PFGE) restriction patterns. In contrast, the number of IS copies in the *B. ovis* genome is around 30 and the different field strains can be differentiated by both methods. As ISs have been shown to be implicated in chromosomal rearrangement, we propose that the chromosomal polymorphism revealed by PFGE and high copy number of IS6501 observed in *B. ovis* may be related to the presence of an active IS in this species.

Introduction

Bacteria of the genus *Brucella* are Gram-negative facultative intracellular pathogens that cause infections in animals and humans. The genus comprises six individualized groups, often considered as species (*B. abortus*, *B. canis*, *B. melitensis*, *B. neotomae*, *B. ovis* and *B. suis*), named according to their preferential hosts

(Corbel & Brinley-Morgan, 1984). The different nomen species are classified according to their metabolic phenotypes and dye and phage sensitivity (Meyer & Cameron, 1961). However, on the basis of DNA analysis, measured by hybridization, Verger *et al.* (1985) have proposed that the genus is comprised of only one species *B. melitensis*. This proposition is strengthened by the rarity of genes that show restriction fragment length polymorphism (RFLP) and the very high conservation of the sequence of *hsp60* in different species (unpublished results). In contrast, we have shown (Allardet-Servent *et al.*, 1988) that a substantial polymorphism can be detected when the genome of *Brucella* species is analysed by pulsed field gel electrophoresis (PFGE) using several rare cutting restriction endonucleases.

Using PCR on the gene encoding the BCSP31 protein, characterized by Mayfield *et al.* (1988) for its antigenic character, an insertion sequence (IS6501) was observed

* Author for correspondence. Tel. +33 67 14 32 09; fax +33 67 14 33 38.

Abbreviations: IHF, integration host factor; IS, insertion sequence; PFGE, pulsed field gel electrophoresis; RFLP, restriction fragment length polymorphism.

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Table 1. *Brucella* strains

Species	Biovar	Strain*	Host	Geographic origin
<i>B. melitensis</i>	M1	16M ^T (ATCC 23456)	Goat	USA
	M2	63/9 (ATCC 23457)	Goat	Turkey
	M3	Ether (ATCC 23458)	Goat	Italy
		1381	Cattle	France (Gap)
		1572	Human	France (Toulon)
		1574	Human	France (Marseille)
		1575	Sheep	France (Avignon)
		1577	Human	France (Paris)
		1578	Sheep	France (Draguignan)
		1590	Human	France (St Dié)
<i>B. abortus</i>	A1	544 ^T (ATCC 23448)	Cattle	England
	A2	86/8/59 (ATCC 23449)	Cattle	England
	A3	Tulya (ATCC 23450)	Human	Uganda
	A4	292 (ATCC 23451)	Cattle	England
	A5	B3196 (ATCC 23452)	Cattle	England
	A6	870 (ATCC 23453)	Cattle	Africa
	A9	C68 (ATCC 23455)	Cattle	England
<i>B. suis</i>	S1	1330 ^T (ATCC 23444)	Swine	USA
	S2	Thomsen (ATCC 23445)	Swine	Denmark
	S3	686 (ATCC 23446)	Swine	USA
	S4	40 (ATCC 23447)	Reindeer	USSR
<i>B. canis</i>		63/290 ^T (ATCC 23365)	Dog	USA
<i>B. ovis</i>		RM6/66 ^T (ATCC 25840)	Sheep	Africa
		76/247		France (Toulon)
		76/250		France (Biarritz)
		91/66		Spain (Aragon)
		91/70		Spain (Aragon)
		91/208		Spain (Bilbao)
		91/212		Spain (Bilbao)
		91/217		Spain (Bilbao)
		91/264		Argentina
		91/266		Argentina
		91/268		Switzerland

* Type strains and biovar reference strains are listed by their FAO/WHO designation and their American Type Culture Collection numbers. Field strains of *B. melitensis* are listed by the numbers of the culture collection of Montpellier (France) and those of *B. ovis* by the number of INRA-Nouzilly collection (Tours, France).

downstream of the *B. ovis* BCSP31 gene. However, this IS is absent downstream of the BCSP31 gene in other species of *Brucella*. Such a sequence was also observed by Halling & Zehr (1990). This IS was isolated, its nucleotide sequence determined and its distribution analysed in the different nomen species of *Brucella*. Diversity found by PFGE correlates well with the polymorphism revealed by RFLP using IS6501 as a probe.

Methods

Bacterial strains and culture conditions. *Brucella* strains used in this study are listed in Table 1. Reference strains of *B. melitensis* biovar 3 and *B. ovis* were obtained from the American Type Culture Collection, Rockville, MD, USA. *B. ovis* field strains were provided by Dr J. P. Verger, INRA, Nouzilly-les-Tours, France. The other strains originate from the reference laboratory of Montpellier (France). Cultures were grown on *Brucella* agar (Difco) at 37 °C for 72 h. Cells were harvested

by centrifugation, then washed twice with TE buffer and stored at –20 °C until extraction of DNA.

Preparation of genomic DNA and plasmid vector. Total DNA was extracted from the different strains of *Brucella* using the procedure described for Gram-negative bacteria by Ausubel *et al.* (1987). *Escherichia coli* XL-1 Blue competent cells and plating cultures were prepared as described by Sambrook *et al.* (1989) for use as a host for plasmid pBSIIKS+ purchased from Stratagene (pBluescriptII Exo/mung Kit). They were processed as described previously (Cellier *et al.*, 1992).

Polymerase chain reaction (PCR) and oligonucleotide selection. Starting with the sequence of the BCSP31 gene published by Mayfield *et al.* (1988), three oligonucleotides were selected to generate fragments of either 350 bp or 710 bp by using oligonucleotides I and II or I and III, respectively. Oligonucleotide I (5' GTATCGTTCCTTGAAGCCT-AC 3') is 20 bases in length and starts at base 1055; oligonucleotide II (5' CCTTGGTGATGTTATAGATG 3'), complementary to the coding sequence, is 20 bases in length and starts at base 1405; oligonucleotide III (5' GTGCATTTCATAGGCTAGAG 3'), complementary to the coding sequence, is 21 bases in length and starts at

base 1765. Amplification of DNA was performed in a total volume of 50 µl in 1 × *Taq*-DNA-Polymerase buffer (Promega) with 50 pmol of each primer, 0.2 mM of each deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 2.5 U of *Taq* DNA polymerase (Promega) and 100 ng of target DNA. The mixture was overlaid with 50 µl of mineral oil and subjected to 30 cycles of amplification as follows: denaturation at 95 °C for 35 s, annealing at 56 °C for 45 s and extension at 72 °C for 45 s. Thermal cycling was performed in a PCR processor trio thermo bloc (Biometra). Five percent of amplified DNA was analysed by 1.5% agarose gel electrophoresis.

Molecular cloning and DNA sequence analysis. Molecular cloning techniques were carried out as described by Sambrook *et al.* (1989). The 1600 bp fragment amplified by PCR was cloned in pBSIISK+ and sequenced using a sequencing kit (USB) according to the manufacturer's instructions using a commercially available primer (Stratagene). Sequencing gels were 8% (w/v) polyacrylamide containing 8 M-urea. The sequence data were compiled and analysed using the DNA analysis program MBS (Macro/Bio/Soft) purchased from Prolabo (France).

Cloning and screening of other copies of the IS. A genomic library of *B. ovis* DNA was constructed in pBSIISK+ to isolate ISs. Total DNA was digested by *Hind*III and ligated into *Hind*III sites of pBS. *E. coli* cells were transformed by electroporation with a Cellject system (Eurogentec) and spread on LB plates containing (ml⁻¹) 100 µg ampicillin, 25 µg tetracycline, 2 mM-IPTG and 400 µM-XGal, and grown overnight at 37 °C. White colonies were picked to 96-well plates (Falcon), grown overnight in LB plus ampicillin and stored in 50% (v/v) glycerol at -20 °C. Colony blots were carried out using GeneScreen Plus nylon membranes as recommended by the supplier (New England Biolabs). IS6501 was radiolabelled *in vitro* using a random priming kit (Boehringer Mannheim) with [α -³²P]dCTP, according to the supplier's protocol. Prehybridization and hybridization were performed at 42 °C in 50% (v/v) formamide. The membranes were washed briefly twice in 2 × SSC at room temperature (1 × SSC is 150 mM-NaCl, 15 mM-sodium citrate dihydrate, pH 7.0), twice in 2 × SSC with 1% (w/v) SDS and 50% formamide for 30 min at 37 °C, and twice with 0.1 × SSC for 30 min at room temperature followed by autoradiography on Fuji X-ray medical films. Positive clones were analysed by restriction analysis. They were also sequenced, using pBS primers and primers calculated from IS6501.

RFLP. Preparation, digestion by *Xba*I and PFGE of genomic *Brucella* DNA were performed as described previously (Allardet-Servent *et al.*, 1991; Michaux *et al.*, 1993). *Eco*RI-digested DNA was separated by conventional electrophoresis. The IS6501 probe was labelled by a non-isotopic method (Boehringer Mannheim) and hybridized as recommended by the manufacturer.

Computer analysis. The Macro/Bio/Soft package (Prolabo, France) was used for routine sequence analysis. BISANCE (Dessen *et al.*, 1990) was used for on-line analysis.

Results

Evidence for an IS in the PCR products

The nucleotide sequence of the BCSP31 gene, which was cloned and sequenced by Mayfield *et al.* (1988), was used to develop a specific diagnostic test based on PCR. Amplifications performed with oligonucleotides I and II (see Methods) generated a unique fragment of 350 bp in all five species of *Brucella* tested. However, when

oligonucleotides I and III were used, a fragment of 750 bp was amplified for all species of *Brucella* except for *B. ovis* where a fragment of 1600 bp was observed (Fig. 1). A DNA fragment of about 900 bp is inserted at this location in the genome of *B. ovis*. Southern blot analysis of the genomic DNA, using the *B. ovis* amplified fragment as a probe, revealed more than 20 bands (see Fig. 5). This implied that the DNA inserted in the genome of *B. ovis* is analogous to an IS. To confirm this finding it was necessary to sequence it.

Characterization of the IS

Whilst we were studying these PCR products, Halling & Zehr (1990) described a similar insertion element of approximately 0.9 kb at a site downstream of the BCSP31 coding region in strains of *B. ovis*. However, they gave no details about its nature, sequence analysis, site of insertion, or its distribution in other species of *Brucella*.

To characterize the 1600 bp fragment amplified by PCR, a restriction map was made. No sites were found for *Bam*HI, *Cla*I, *Eco*RI, *Eco*RV or *Pst*I (data not shown). *Hind*III generated two fragments of 1000 and 600 bp. Digestion by *Hin*fI gave a profile similar to that described by Halling & Zehr (1990). The PCR products whose sizes were 350 and 710 bp, respectively, were sequenced directly using the amplification primers. As the 1600 bp fragment amplified with *B. ovis* was too long to be sequenced using this method, we cloned it in pBSIISK+. Firstly, *Hind*III linkers were added to its extremities by ligation. *Hind*III digestion gave rise to two fragments of 1000 and 600 bp which were then cloned separately in the *Hind*III site of pBSIISK+. The resultant clones were sequenced in both directions using the primers T3, KS, SK and T7 of the pBS vector and the PCR oligonucleotides (I, II, III). The sequences obtained were aligned and compared with the BCSP31 gene nucleotide sequence using the DNA analysis program MBS. The deduced nucleotide sequence of the IS element, named IS6501, is presented in Fig. 2.

To confirm the sequence, to identify the exact duplication target site, to determine the flanking regions, and to know if the sequenced element is the same in the *B. ovis* genome, other copies were cloned and sequenced. A DNA library was screened with the IS6501 probe. The restriction enzyme site profiles of positive clones were characterized and Southern blot hybridizations were used to confirm the presence of IS6501 (data not shown). Twenty isolates were identified and sequenced. The data were aligned for comparison. In these clones, the sequences of IS6501 with its inverted repeats and its direct repeats were the same. However, in the flanking regions of IS6501 (Fig. 3), no specific consensus sequence between these clones was found.

Fig. 1. Agarose gel electrophoresis of DNA fragments obtained by PCR with oligonucleotides I and III. Lanes: 1, molecular size standard (1 kb ladder, BRL); 2, *B. abortus*; 3, *B. canis*; 4, *B. melitensis* 16M; 5, *B. ovis*; 6, *B. suis*; 7, negative control. The size of amplified fragments is indicated on the right. No amplification was observed with the negative control.

Sequence analysis of IS6501

The nucleotide sequence of IS6501 is 836 bp long and is flanked by two 17 bp inverted repeats with one mismatch (Fig. 2). At the site of insertion, 4 bp target DNA duplications are generated (CTAG). The sequence of four flanking genes is presented in Fig. 3. It appears that no specific sequence or base composition is obvious around the insertion site. However, at the 5' end some bases are shared by the four sequences. The significance of this observation is difficult to assess.

Three consensus IHF (integration host factor) sites (AANNNTTGAT) are present in IS6501. Five Dam-methylase sites (GATC) are also present (Fig. 2).

A computer search in the Genbank/EMBL database revealed 53.8% sequence identity with IS427 of *Agrobacterium tumefaciens* over 742 bp (De Meirsmen *et al.*, 1990) (data not shown). No other significant sequence homology has been found with other sequenced ISs present in the database. Unexpectedly, although a high degree of similarity was found with IS427 of *A. tumefaciens*, the ORFs are localized differently and no real homology was detected at the protein level. Numerous deletions (or insertions) of one or two nucleotides appear between the two sequences breaking the reading frame in *A. tumefaciens*.

Analysis of the putative transposase

A major ORF of 236 amino acids is revealed by computer analysis with a putative Shine–Dalgarno sequence and a slightly degenerated Pribnow-box. To confirm that the ORF delineated by computer really represents a protein, the protein NBRF database was searched for similarity. Using the FASTA program, 36.4% identity over 165 aa

was found with the hypothetical protein 1 of *A. tumefaciens*, a protein encoded by IS869 (Paulus *et al.*, 1991). Similarly, 34.2% identity was found over 76 aa of a *M. tuberculosis* transposase sequence available in the database but unpublished (accession no. S21394). Fig. 4 shows an alignment of the deduced amino acid sequence of the three proteins. There is a very strong conservation of residues in the middle of the putative transposase. Inspection of the alignment showed strict conservation of 14 contiguous residues and also a large number of identical residues and conservative replacements at other locations.

The putative protein contains a high number of basic residues. The calculated isoelectric point (pI) of 11.35 suggests a possible interaction with nucleic acids. The propensity of the sequence to adopt definite secondary structures was analysed using the method of Garnier *et al.* (1978). The results clearly show a succession of α -helices and β -sheets, suggesting an α/β structure. At the carboxy terminus of the molecule, a typical helix-turn-helix structure, thought to be the DNA binding site of the transposase (as discussed by Galas & Chandler, 1989) was found (data not shown).

Polymorphism revealed by IS6501 in the *Brucella* genome

The presence of IS6501 in the nomen species of *Brucella* (*B. abortus*, *B. canis*, *B. melitensis*, *B. ovis* and *B. suis*) was investigated. Oligonucleotides were calculated in the IS fragment to design a PCR method that specifically detects IS6501. All the tested isolates exhibited an amplified fragment of the same expected size (data not shown).

-35

CGGTCGTGCATTTCATAGGCTAGGGCTTGTCTGCATTCAAGGATTCCTTTTGTACGAA 60
GATCCCGAACACAGCTAAGTTCCCTAAGGGAACATGCTT
 -----*----->

-10 SD

ATTCTGATTCAAGGTTGTTAAAGGAGAACACCGGTGAGCAGACGAAGCCTTACAGATGAG 120
 TAAGACTAAGTTCCAACAATTTCTCTGTGCGCAGTCTCTGCTTCGGAATGTCTACTC

M E P D R S I S S G A S W Y A R P Q W
 CAATGGAACCGATCGAAGCATATCTTCGGGGCGAGTTGGTACGCCCGGCCGAGTGGG 180
 GTTACCTTGGCCTAGCTTCGTATAGAAAGGCCCTCAACCATGCGGGCCGCGTCAACC

V D N R L F V D A I L W M A A N A G T G
 TTGATAACCGAATTAATTTGTGACGCGCATCTTGTGGATGGCTGCAATGCAGGCACTGGCG 240
 AACTATTGGCTAATAAACAGCTGCGGTAGAACACCTACCGACGGTTACGTCCTGACCGC

D L P A T F G K W T A V H A R F R R W S
 ATCTGCTCGGACCTTCGGCAATGACAGCGGTTTCATGCCCGCTTTCGGCGCTGGTGGC 300
 TAGACGGACGCTGGAAGCCGTTTACCTGTGCGCAAGTACGGGCGAAAGCCGCGACGCG

H A G V W E R L F H A L A D T P D F E Y
 ACGCCGTGTATGGGAAGGCTTTTCCATGCCCTGGCTGATACGCCGACTTTGAATATG 360
 TGGCGCCACATACCCCTTTCGAAAAGGTACGGGACCGACTATGCGGCTGAAACTATATC

V L I D S T I S K V H A D A A G A K G G
 TCCTCAATTGATAGCACCATATCGAAAGTCCACGAGATGCGCGGGCGCAAAAGGGGGCG 420
 AGGAGTAACATCGTGGTATAGCTTTTCAGGTGCGTCTACGCCCGCCGCTTTTCCCCCG

L K L P A S V S R G G L T T K L H A V V
 TGAAGCTGCTGCATCGGTCTCGCGCGGTGGATTGACGACCAAGCTGCGTCTGTTGTGCG 480
 ACGTTCGAGGACGTAGCGAGCGCGCCACCTAAGTCTGCTGCTGACGTACGACAAACAGC

D A I G L P L R I K P T P G H Y G D C P
 ATGCTATCGGCTTACCGCTGCGAATAAAGCAACACCCGCGCAATTATGGTGAATCTCCGC 540
 TACGATAGCCGATGGCGACGCTTATTTTCGGTTGTGGCGCGTAATACCACTGACAGGCG

Q A S S L L S G L K G V G H V L A D A A
 AAGCTTCAAGCCTTCTATCCGGCTTGAAGGGTGTGGGCGATGCTCTGCTGATGACGAGCT 600
 TTGGAAGTTTCGGAAGTAGGCGCAACTTCCCAACCCGCTACAGGAACGACTACGTGGA

HindIII
 Y D A D H L R A F I A S N L K A T A Q I
 ATGATGCCGATCACTTAAGGGCTTCAATGCGCAGCAATCTCAAGGCAACGGCTCAGATCA 660
 TACTACGCGTAGTGAATCCCGAAGTAACGGTCTGTAGAGTTCGGTTCGCGAGTCTAGT

K V N P T R S S V P T I D W R L Y K E R
 AGGTCAATCCACACGTTCCAGTGTCACCAATCGACTGAGGCTGTACAGGAAGACGCC 720
 TCCAGTTAGGTTGTGCAAGGTCAAGGTTGTAGCTGACCTCCGACATGTTCTTCGCGG

H Q I E C F F N K L K R Y R R I A L R C
 ATCAGATTGAATGCTTTTAAACAAGTTGAAACGCTATCGTCTGATTTGCGCTGCGATGCG 780
 TAGCTCAACTTACGAAAAAATTTGTTCAACTTTGCGATAGCAGCATAACGCGACGCTACGC

E K T L T A F M G L R S I S H A L
 AGAAACATTGACCGCATTCATGGGGCTTCGGTCCATCTCGCATGCGCTATGATCTGGTT 840
 TCTTTGTAACTGCGCTAAGTACCCGAAGCCAGGTAGAGCTACGCGATACAGACCA

ACGTGTAATGCAGACACGCCCTAGAGCAITTTCCAGCAAAAGCGT
 TGCAACTTACGCTCTGTGCGGATC
 <-----*----->

Fig. 2. Nucleotide sequence of IS6501. Dashed arrows indicate the 17 base inverted repeats of the IS, with one mismatch (*), and the 4 bp target duplications are double-underlined. The *HindIII* site is also indicated. The site of insertion is at position 1750 of the BCSP31 gene. The deduced amino acid sequence of the putative transposase, indicated over the sequence in single-letter code, is obtained from the complementary strand with regard to the 5' → 3' orientation of the BCSP31 gene. The -10, -35 and the Shine-Dalgarno (SD) motifs are overlined. Five consensus sequences for Dam sites (GATC) and three consensus sequences for IHF are underlined (AANNNTTGATA).

The possibilities of discriminating the different strains by RFLP, using IS6501 as a probe, were investigated. Southern blots of *EcoRI*-digested DNA of *Brucella* strains were probed with radiolabelled IS6501. Multiple banding patterns differentiated the strains into different RFLP types (Fig. 5). The differences in intensity of some

5' element insertion site junction

PCR product	CGGTCGTGCATTTCATAGGCTAG----	IS6501 -
pBOH20/36	CGGTCGTGCATTTCATAGGCTAG----	IS6501 -
pBOH13/36	GATTGAATCAGATCGGCGCTCTAG----	IS6501 -
pBOH12/36	ATTGTGGGCATAGCCCCCTCCCTAG----	IS6501 -

3' element insertion site junction

PCR product	- IS6501 ----CTAGAGCATTTCCAGCAAAAGCGT
pBOH10/37	- IS6501 ----CTAGTGGATTGAATTTGACGTTTG
pBOH08/37	- IS6501 ----CTAGAGCGCGTTTCGATCTGGTTG
pBOH11/37	- IS6501 ----CTAGAGCGCGGATCTGATTCAATC

Fig. 3. Flanking sequences of isolated copies of IS6501. Designations of plasmids containing the different IS6501 copies are indicated. Direct repeats are in bold type. pBOH20/36 has the same sequence as the PCR-amplified fragment because it contains the BCSP31 gene.

	MAKGGC---LG---S---CSGRDFQ	<i>At</i>
	MEPDRSISGASWYARQPQWDNRLFVDI---LWMAANAG---TG---DLPATFGKWT	<i>Bo</i>
	MTRVGVISDEFWAVVEPLMPSEHGKPGRFSDHRLILEGIARFRFTGSPWRDLPAEFGPWQ	<i>Mt</i>
	GFRRRYRDRQLL-----CPCSSTC-----	<i>At</i>
	AVHARFRRWSHAGVWERLF-HALA---DTP---DPE-YVLIDSTISKVH---ADAA---	<i>Bo</i>
	TVWKRHRHRLSDGTCDEVFAHVAAVFGVDAEVAEDIEKLLSVSDTNVRAHQHSAGAARTR	<i>Mt</i>
	---GHKKGDKHDGC-----MGRSRGGLTKIHAVVDADGRPIRLALTAGQAHDGRMAEP	<i>At</i>
	---GA-K-G---GLKLPASV---SRGGLTKLHAVVDAIGLPLRIKPTPGHYGDCPQASS	<i>Bo</i>
	SPQAGLSDYKKSADPEDDHAIGRSRGLTTKIHALTDQREAPVRIRLTAGQAGDNPLL	<i>Mt</i>
	LLQTSKGAILLADKAYDTNIRAFAKQRQ-AWANIP-----AKS	<i>At</i>
	LLSGLKGVGHVLADAAYDADHLRAFIASNLKATAQIK-----VNP	<i>Bo</i>
	LLDDYRHASTEVALGSTDFRLADKAYSHPSRAALRSKKIKHTIPERHQIDRRKAKGS	<i>Mt</i>
	NRKGSFPFSQWVYRQRNLVERFFSKLKQFRGIATRYDKDPLNFLAAVLAARVIRSL	<i>At</i>
	TRSSVPTIDWRLYKERHQIECFNKLKRYRRIALRCEKTLTAFMGLRSISHAL	<i>Bo</i>
	AGGRFPADFDAALYGLRNTVERGFHRLKQWRGIATRYDKYALTYLGGVLLACAVIHARVG	<i>Mt</i>
	TPKLGDTPT	<i>Mt</i>

Fig. 4. Homology between proteins encoded by IS869 of *A. tumefaciens* (*At*), IS6501 of *B. ovis* (*Bo*) and the transposase of *M. tuberculosis* (*Mt*) (NBRF accession no. S21394). Dashes indicate gaps introduced to increase the number of matches. Identical and similar amino acids are represented by double and single dots, respectively.

of the bands may be due to a double band or to the presence of two or more copies of IS6501 in the fragment. Alternatively, the possibility of sequence heterogeneity within a strain cannot be excluded. These possibilities are under investigation. However, as expected, the results clearly show that an IS6501-based RFLP is able to discriminate between *Brucella* strains.

Fig. 5. Southern blot of *Eco*RI-digested DNA from several *Brucella* spp. probed with IS6501. Lanes: 1, *B. ovis*; 2, *B. canis*; 3, *B. suis* S4; 4, *B. suis* S3; 5, *B. suis* S2; 6, *B. suis* S1; 7, *B. abortus* A9; 8, *B. abortus* A6; 9, *B. abortus* A5; 10, *B. abortus* A4; 11, *B. abortus* A3; 12, *B. abortus* A2; 13, *B. abortus* A1; 14, *B. melitensis* M3; 15, *B. melitensis* M2; 16, *B. melitensis* M1.

Comparison of *B. melitensis* and *B. ovis* strains by PFGE

Because of the polymorphism revealed by RFLP using IS6501 as a probe, it was interesting to compare this finding with the polymorphism revealed by PFGE. For this purpose, genomic DNA isolated from the reference strain and seven field strains belonging to biovar 3 of *B. melitensis* were digested by *Xba*I and the fragments were separated by PFGE. The reference strain and nine field isolates of *B. ovis* were also analysed in the same manner. As in our previous studies (Allardet-Servent *et al.*, 1988), no difference could be demonstrated between the field strains of *B. melitensis* biovar 3 (data not shown). The electrophoretic pattern of the *B. ovis* isolates appeared to be very similar (data not shown). Nevertheless, four patterns could be distinguished, differing by one or two bands: pattern I contains three strains (208, 212 and 217, all isolated near Bilbao, Spain); pattern II contains one strain (247, isolated near Toulon, France); pattern III

Fig. 6. Southern blot of *Eco*RI- (a) and *Xba*I- (b) digested DNA from reference and field strains of *Brucella ovis* probed with IS6501. Lanes: 1, 91/268; 2, 91/266; 3, 91/264; 4, 91/217; 5, 91/212; 6, 91/208; 7, 91/170; 8, 91/66; 9, 76/247; 10, 76/250; 11, RM6/66^T (ATCC 25840).

contains one strain (268, isolated in Switzerland); pattern IV contains all the other strains (isolated in Argentina, Spain or France).

Comparison of B. melitensis and B. ovis strains by hybridization with IS6501

Genomic DNA from the same strains were digested with *EcoRI* and *XbaI*. The fragments were separated either by conventional methods (*EcoRI*) or by PFGE (*XbaI*) and blotted onto nylon membranes which were then hybridized with the IS6501 probe. No differences could be demonstrated within the *B. melitensis* strains (data not shown). Among the *B. ovis* isolates, five hybridization patterns were observed, differing by one or two bands in the digests with *EcoRI* and seven bands in the digests with *XbaI* (Fig. 6). However, the grouping of the strains was different when DNA was digested by *EcoRI* or by *XbaI*: the Bilbao group (strains 208, 212, 217) and strain 247, both individualized by PFGE, always exhibited a unique pattern; strains 66 and 70 (isolated in Aragon, Spain) were individualized by the two methods, but strain 250 (isolated near Biarritz, France), 264 and 266 (isolated in Argentina), and 268 (isolated in Switzerland) were distinguishable by IS6501 hybridization on *EcoRI* and *XbaI* fragments.

Discussion

This study reports the identification, cloning and characterization of an IS from *B. ovis* called IS6501. It was first identified downstream of the coding sequence of BCSP31 described by Mayfield *et al.* (1988) at position 1750.

To determine precisely the sequence of this element, the sequence of other copies of IS6501, identified from a genomic bank, was also determined. The sequence is conserved in all the sequences determined of the various copies of the IS6501 of *B. ovis*. Analysis and comparison of the nucleotide sequence of different clones identified in the library showed that IS6501 has 4 bp duplicated at the insertion site and the presence of inverted repeats has been assessed. Concerning the flanking regions of this IS, no specific consensus sequence for its integration in the genome was found. These data confirmed that the postulated IS contains all the structural and biological transposition characteristics of a prokaryotic IS element.

Analysis of the sequence of IS6501 revealed five Dam-methylase sites and three IHF sites (Fig. 2). The search for a specific Dam-methylase in *Brucella* was negative, thus it is difficult to know if this sequence is meaningful. In contrast, the three IHF sites are localized near the end (but not at the end) of IS6501. IHF sequences have been reported at the end of *E. coli* IS1 (Gamas *et al.*, 1987),

near the end of IS10 and within the sequence of numerous ISs (for a review, see Galas & Chandler, 1989). A role in transposition has been suggested, however in spite of suggestive observations, no convincing effect *in vivo* has been obtained with IS1 (Galas & Chandler, 1989). Similarly, an IHF site present near one of the ends of Tn1000 binds IHF cooperatively with the transposase at an adjacent site and, *in vitro*, IHF binding enhances transposase binding (Grindley & Wiater, 1988). However, IHF-negative mutant strains support apparently normal Tn1000 transposition. The presence of IHF sites is common in many ISs, but their exact role remains to be established.

The larger ORF encodes a protein rich in basic amino acids, with a calculated pI of 11.35, and a predicted secondary structure compatible with a helix-turn-helix motif, suggesting a possible interaction with DNA (for a review, see Galas & Chandler, 1989). Furthermore, homologies with a transposase from *Mycobacterium* confirm that in *Brucella* this ORF certainly encodes a transposase.

IS6501 is present in all species and strains of *Brucella* tested. The existence of a genus-specific IS might have important theoretical and practical consequences. The homology with *A. tumefaciens* is highly significant and argues in favour of the phylogenetic proximity between these bacteria. This observation is in agreement with evolutionary analysis based on the chemical analysis of the lipid A fraction (Moreno *et al.*, 1990) and 16S rRNA (Weisburg *et al.*, 1991). Both belong to the Alpha-2 subdivision of the class Proteobacteria (Woese *et al.*, 1984), containing many bacteria that form intimate pericellular or intracellular associations with eukaryotic cells. The finding of a related IS in both organisms confirms the close relationship between them. However, there are two ways of explaining the homology of the IS observed in the two genera: the IS may have existed either before the separation of the two genera or a horizontal transfer may have occurred later after the separation. If the first hypothesis is true, then this IS should constitute a molecular marker to construct a phylogenetic tree among these bacteria. The second hypothesis is more problematic and cannot be proved in the light of our present knowledge.

ISs are mobile elements. Transposition of IS leads to several genetic events such as deletion or inversion (Galas & Chandler, 1989), introducing great variability in the genome. On this basis, IS fingerprinting was proposed as a typing method for an epidemiological survey of bacterial infections (Glare *et al.*, 1990; Hermans *et al.*, 1990). The copy number of ISs varies widely from strain to strain within the same species. For instance, in a sample of 71 independent isolates of *E. coli* (the ECOR collection), 11 did not carry genomic copies

of IS1, whilst 16 carried between 10 and 40 copies (Galas & Chandler, 1989). However, the copy number of ISs is usually lower, since the average copy number in these 71 *E. coli* strains was determined to be 6.4 for IS1, 2.7 for IS3, 2 for IS4, 1.3 for IS5, and 0.9 for IS30. The genomic location could be associated with specific pathogenicity for a preferential host (Mills *et al.*, 1992). The copy number of the IS6501 sequence is moderately high in *Brucella* and varies in the different species. It was determined to be 7–10 in the three biovars of *B. melitensis*, 6–8 in the seven *B. abortus* biovars, as in the four *B. suis* biovars and in *B. canis*, and more than 28 in *B. ovis*. Moreover, IS fingerprinting showed that the genomic localization of IS6501 is species-specific, confirming at the genomic level the species and the biovars described in the genus by phenotypic methods, as it was found previously with PFGE. However, RFLP revealed by PFGE or by IS hybridization is not sufficient to identify a given strain at the species or subspecies level. More probably, differences in restriction or hybridization patterns are likely to be related to evolving lineages within the genus. In our study, IS6501 localization on the genome was compared in *B. melitensis* and *B. ovis* isolates, including reference and field strains. Two main differences were observed between the two species. Firstly, the copy number of the IS is very different in the genomes of the two species since *B. ovis* contains three times more copies than *B. melitensis*. Secondly, in contrast to the polymorphism demonstrated by PFGE or IS6501 hybridization in *B. ovis* (our results and Halling & Zehr, 1990), surprisingly no differences were seen between the *B. melitensis* isolates tested by either method.

The polymorphism observed by PFGE of DNA fragments generated by rare cutting restriction endonucleases can be explained either by mutations affecting the restriction sites or by chromosomal rearrangements. The correlation between high copy number of the IS and the polymorphism demonstrated by digestion of the genomic DNA with rare cutting enzymes suggests that the differences observed by PFGE may be more related to chromosomal rearrangements than mutations. This may be mediated at least partly by IS transposition. Several authors have proposed such a mechanism for PFGE polymorphism observed in *Bacillus cereus* or *Clostridium perfringens* strains by other arguments, such as physical mapping of chromosomes of different strains belonging to the same species (Carlson *et al.*, 1992; Canard *et al.*, 1992). This proposal is strengthened by the results of IS6501 hybridization on *EcoRI* or *XbaI* fragments from *B. ovis* DNA, which showed only two polymorphic bands with the first endonuclease and at least seven with the second.

The simplest explanation of the differences in copy number observed in the two *Brucella* species is that

IS6501 is always active in *B. ovis* leading to the polymorphism observed in the species, and inactive in *B. melitensis*, in which the IS would be unable to transpose. This implies that the positions of insertions in the *B. melitensis* genome are conserved in the other species. This seems to be the case when one examines the gels (not shown). Indeed, most of the bands present in *B. melitensis* also appear in other species.

ISs are responsible for a number of genetic rearrangements, including gene inactivation by insertional mutagenesis or deletion, gene activation by insertion or formation of upstream promoters, or plasmid integration (Iida *et al.*, 1983; Galas & Chandler, 1989). The presence of IS6501 may confer a possible selective advantage to the organism such as altered pathogenicity or modified host specificity. It is noteworthy that *B. ovis* is an avirulent species for humans and it contains the highest IS copy number. The existence of altered virulence due to an IS has been reported in other pathogenic bacteria (Mills *et al.*, 1992).

For practical purposes, genus-specific elements such as IS6501 might be useful as molecular probes for the identification of clinical isolates. We are currently developing PCR methodology for specific and sensitive detection of *Brucella* to complement the method described by Fekete *et al.* (1990a, b). As IS6501 is present as multicopies in the *Brucella* genome, this will increase the sensitivity of detection. Such PCR assays will be useful for the detection of infectious *Brucella* in the veterinary field and in dairy products. When used as a probe, IS6501-based RFLP is able to distinguish between *Brucella* species and strains.

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