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Species-Specific Sequences at the *omp2* Locus of *Brucella* Type Strains

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A DNA sequence analysis of the *omp2* locus of *Brucella* type strains revealed nucleotide differences that can be used for species identification. We developed specific probes which were used to verify the observed differences among the type strains following PCR amplification of portions of the *omp2* locus.

Organisms belonging to the genus *Brucella* are typically identified by using phenotypic properties which have been shown to vary among isolates (3, 5, 12). Previously, workers in several laboratories have shown that there are species-specific restriction fragment length polymorphisms which can be used to identify members of the genus *Brucella* (2, 4, 6, 8, 13). The results of genetic analyses have suggested that the *Brucella* species (*Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis*, *Brucella canis*, and *Brucella neotomae*) represent a monospecific genus or a genomic species (15). In this study we extended our previous work in order to characterize oligonucleotide probes which can be used to identify individual *Brucella* species on the basis of DNA sequence variations at the *omp2* locus.

Five overlapping portions of the *omp2* locus of *Brucella* type strains were amplified by using the primers shown in Table 1 and selected by using Oligo 4.0 (National Biosciences, Inc.) on the basis of a previously published sequence available from the GenBank database (accession number M26034). Amplified segments of the *omp2* locus from three separate amplification reactions were cloned into plasmid pCR or pBluescript, and the sequence of at least one recombinant from each reaction mixture was determined as described previously by using internal primers and vector sequencing primers supplied by manufacturers (7). The sequences shown in Table 2 have been deposited in the GenBank database.

The results of an analysis of the sequences obtained for the *omp2* locus for the type strains confirmed that the gene duplication in a 3,400-bp region was conserved in all six species. Variations in nucleotides were observed at 183 positions in a 3,400-bp region representing 6% of the locus; this number does not include variations due to insertions or deletions of blocks of nucleotides (the total number of changes was approximately 185 bp). One of the deletions was a block of 138 nucleotides from the central region of the *omp2a* gene in *B. abortus* biovars 1, 2, and 4 (7). The mechanism for removing this sequence may involve imperfect direct repeats of 26 nucleotides 5′ to both deletion endpoints in the *omp2a* gene in all *Brucella* taxa except *B. abortus* biovars 1, 2, and 4. This sequence can form cruciform structures up to 9 bp long which may represent recognition sites for DNA cleavage (1). Although found in these three organisms, the deletion may have occurred independently in them and should not be used by itself to infer a close relationship. This deletion preserves the original open reading frame, expression from this locus in porin-deficient *Escherichia coli* results in altered membrane permeability (10), and the purified protein forms pores in black lipid bilayers (9).

When we compared the sequences of the *omp2a* and *omp2b* genes obtained from all of the species, we also found a deletion in each *omp2b* gene that corresponded to the 5′ copy of the direct repeat. It seems reasonable to assume that if this sequence represents a site for homologous or site-specific recombination events, this feature could result in deletion within the active porin gene and is not compatible with survival.

A phylogenetic analysis of the *omp2* nucleotide sequences of the type strains of *Brucella* species was performed by using the computer program PAUP (14). The resulting cladogram is the single most parsimonious representation of the relationships among these species (Fig. 1). This tree is similar to the traditional trees obtained for these species when phenotypic analysis is used. For example, *B. abortus* and *B. melitensis* have long been considered closely related on the basis of phenotypic characteristics, and they formed a distinct monophyletic group when their nucleotide sequences at the *omp2* locus were studied. In addition, also because of phenotypic similarities, it has been postulated that *B. canis* and *B. suis* are recently diverged and closely related species (11). The results of our analysis of the *omp2* gene region strongly suggest that these two species are sister taxa that share a common ancestor that is not shared with the other *Brucella* species. Phenotypically, *B. neotomae* and *B. ovis* are very different from the other *Brucella* species, and these two species were the most divergent taxa in our study on the basis of their *omp2* nucleotide sequences. The extreme divergence of *B. ovis* from the other *Brucella* species is the result of a lack of divergence between its *omp2a* and *omp2b* gene sequences. When the *omp2a* and *omp2b* genes of most species are aligned, similar numbers of nucleotide differences (average, 130 differences) are observed in the two gene copies. The exception is *B. ovis*, in which the genes differ by only 30 nucleotides at the 3′ ends of the genes. The sequence differences are similar to the differences observed in other species. The 5′ ends of the two genes are identical, and the sequences in these regions correspond to the sequences found in the *omp2a* genes of other species. This suggests that either the progenitor gene closely resembled *omp2a* or gene conversion corrected the 5′ end of the *omp2b* gene in *B. ovis* by using *omp2a* as the template. Similarly, the genes in *B. neotomae* differ at only 112 positions. At the other 28 positions (all at the 3′ end) the sequences are identical and correspond to the profile obtained for *omp2a*, once again suggesting that the *omp2a* gene was used to correct sequence variation within

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TABLE 1. Sources of bacterial strains used in this study

Species	Biovar	Strain	Source[a]	GenBank nucleotide sequence accession no.
B. abortus	1	2308	NADC[a]	M26034
	5	B3196^{Tb}	ATCC 23452^{T}	BAU26438
B. canis		NCTC 10854^{T}	ATCC 23365^{T}	BCU26439
B. melitensis	1	5K33^{T}	ATCC 23459^{T}	BNU26441
B. neotomae		16M^{T}	ATCC 23456^{T}	BMU26440
B. suis	1	1330^{T}	ATCC 23444^{T}	BSU26443
B. ovis		63/290^{T}	ATCC 25840^{T}	BOU26442

[a] NADC, National Animal Disease Center, Ames, Iowa; ATCC, American Type Culture Collection, Rockville, Md.
[b] T = type strain.

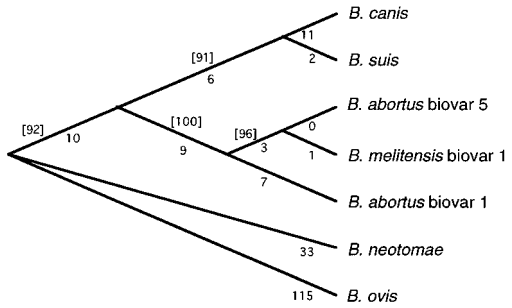

FIG. 1. Maximum parsimony analysis of the *Brucella omp2* gene locus. The tree was derived from the results of an analysis of nucleotide sequence variations in the *omp2* genes of six *Brucella* species by using the PAUP package. The tree was rooted by using *B. ovis* as the outgroup with equal character weighting and accelerated transformation character state optimization. An exhaustive search of all tree topographies resulted in the construction of this single most parsimonious tree. The tree length was 197 steps, and the consistency index (excluding uninformative characters) was 0.78. The numbers not in brackets are branch lengths from the nodes. No other resolutions were found within two steps of this single most parsimonious tree. A bootstrap analysis (1,000 replications) was also performed, and the bootstrap values are given in brackets at the branch points.

omp2b. Gene conversion may represent an enzymatically active system designed to rectify changes that occur in *omp* genes. Repair does not appear to be a frequent occurrence, since most organisms have two *omp2* genes which differ by as much as 15%.

There may be a mechanism in the genus *Brucella* to invert the promoter-containing fragment and shift expression from *omp2b* to *omp2a* in response to changes in environmental conditions. However, expression of the resulting protein has not been detected in *Brucella* strains under a number of in vitro and in vivo conditions (data not shown). A $tRNA^{Ser}_{AGC}$ gene was found 5′ to the silent *omp2a* locus in all of the *Brucella* type strains. This insertion may have interrupted upstream transcriptional control elements, which would explain the apparent inability of the organisms to express the *omp2a* gene. However, a consensus promoter upstream from this region could not be identified. A similar situation exists for *omp2b*, and this finding is consistent with positive regulation of gene expression. The frequency of occurrence of the codon recognized by $tRNA^{Ser}_{AGC}$ is not significantly higher in *omp2a* than in *omp2b*, which rules out a potential role for this tRNA in expression from *omp2a*.

When we compared the DNA sequences of the different *Brucella* species, it became evident that a relatively small number of oligonucleotide probes could be used to distinguish the species when the 3′ end of the *omp2a* gene was amplified by PCR. Following amplification in which either extracted genomic DNA or heat-killed cells were used, the amplified

TABLE 2. *Brucella omp2* primers and probes

Primer or probe	Sequence	Organism(s)
Sequencing primers		
2.20	TCGTGATGTC GCTGATGG	
2.21	TGGCTCAATC CTTTACAA	
2.33	GGGGATGGGG ACAGGTTGTC C	
2.36	CAGGCGATCT TCCGCGACCC C	
2.43	GCAGCTTCCG GCGCTCAGGC TGCC	
2.45	CGAGCGCTGG AAGCGAACGA TACC	
2.46	CGTTGTCAAC GTCTTCGCCA CCC	
2.47	CGCGAACTCC ATGACGGTGC CGC	
2.50	GAATTCTCGC CAGAATTTTG AATAGCCATT AC	
2.51	GAATTCTGGG TCTGGGCATT CTGATTTGGC TG	
2.54	GAATTCACGC GGACATATTC AACGGCTTCG	
2.55	ACTCCCTTAG CAGGGGAGCG CCTT	
2.56	TTCGTTTCAA CATCGAATAT GTTC	
2.57	GTGAAGCCAG GAACCAGT	
2.58	AATTCGGGCG TAGATGGT	
2.59	ATCTCGGCTG GCTCCTAC	
Diagnostic probes		
1	AtGTcgTCgC tGcTGGCTCC	*B. suis*, *B. canis*, *B. abortus*, *B. melitensis*
	....a.......	*B. neotomae*
	.c..ga..t. g.........	*B. ovis*
2	TGtTGTTGCC TATGACtCGG	*B. abortus*, *B. melitensis*, *B. neotomae*, *B. ovis*
	..c....... ......c...	*B. suis*, *B. canis*
3	ATTGCCcCCG AAAAGGcAAC	*B. abortus*, biovars 1 and 5, *B. melitensis*
	......a...	*B. canis*, *B. neotomae*, *B. ovis*
	......a... ......t...	*B. suis*
5	GTcGCTTAtc AgCTCGTTCC	*B. suis*, *B. canis*, *B. abortus*, *B. melitensis*, *B. ovis*
	..t.....cg .a........	*B. neotomae*

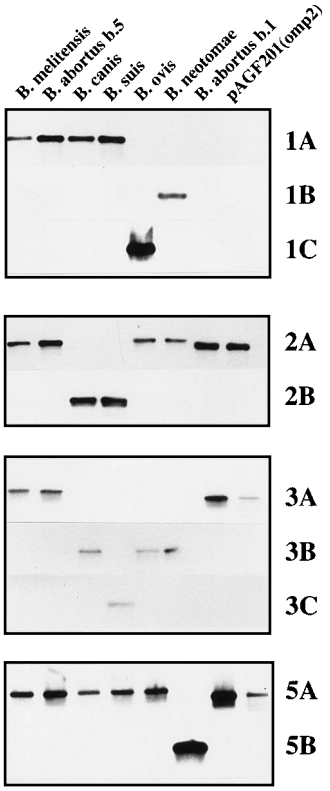

FIG. 2. Hybridization with PCR-amplified portions of the *omp2* locus. Portions of the *omp2* locus were amplified by PCR as described in the text, and the products were hybridized with a battery of oligonucleotide probes to identify the *Brucella* species. The numbers to the right refer to probes used in hybridization (Table 2).

625- to 765-bp products were electrophoresed on agarose gels, and the amplified fragments were characterized by hybridization with diagnostic probes (Fig. 2). Hybridization under low-stringency conditions (37°C) and washing under high-stringency conditions (i.e., temperatures just below the predicted melting temperature) were performed as described previously (7), and the results allowed us to identify the type strains on the basis of retention of the signal under high-stringency conditions. Amplification products from all species were detected under low-stringency conditions, and no signal was detected under any conditions when other procaryotic DNAs were amplified (data not shown). Sequence variations among field strains are currently being examined, and the results may provide stable genetic markers that are useful for tracking individual strains epidemiologically.

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