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Phenotypic and molecular characterization of a *Brucella* strain isolated from a minke whale (*Balaenoptera acutorostrata*)

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Isolation of *Brucella* spp. in marine mammals has been reported during the past several years. A *Brucella* strain from the spleen and liver of a minke whale (*Balaenoptera acutorostrata*) was isolated. Conventional typing methods indicated that this isolate was related to the genus *Brucella* but did not match the profiles of any known *Brucella* species or biovar. Successful PCR amplification of the *Brucella rrs-rrl* spacer sequence and of the insertion sequence IS*6501* also indicated that the minke whale strain was related to the genus *Brucella*. In addition, the *rrs* gene of this strain shared a very high degree of nucleotide identity (>98%) with published *Brucella* spp. *rrs* sequences. However, RFLP studies using an IS*6501*-specific probe showed a unique profile for this strain in comparison with the profiles of the six known *Brucella* species. Moreover, analysis of the *omp2* locus by PCR-RFLP, by Southern hybridization using *omp2a*- and *omp2b*-specific probes, and by DNA sequencing showed that the minke whale isolate possesses two copies of the *omp2b* gene instead of one *omp2a* and one *omp2b* gene copy or two copies of the *omp2a* gene described in the six known *Brucella* species. Thus, molecular typing methods showed that this isolate is clearly distinct from all other known *Brucella* species and strains. The specific molecular features of this minke whale *Brucella* isolate raise questions about the lineage between the *Brucella* strains isolated from marine mammals and the *Brucella* species isolated from terrestrial mammals.

Keywords: *Brucella* sp., brucellosis, minke whale (*Balaenoptera acutorostrata*)

INTRODUCTION

Brucellae are Gram-negative, facultative intracellular bacteria that can infect many species of animals and man (for review, see Young, 1995). Six species have been described within the genus *Brucella*: *Brucella abortus*, *Brucella melitensis*, *Brucella suis*, *Brucella ovis*, *Brucella canis* and *Brucella neotomae* (Corbel & Brinley-Morgan, 1984). This classification is based mainly on differences in pathogenicity and host preference. The main pathogenic species worldwide are *B. abortus*, responsible for bovine brucellosis, *B. melitensis*, the main aetiologic agent of ovine and caprine brucellosis, and *B. suis*, responsible for swine brucellosis. These three *Brucella* species may cause abortion in their hosts resulting in huge economic losses. *B. ovis* and *B. canis* cause ovine epididymitis and canine brucellosis, respectively. As for *B. neotomae*, only strains isolated from desert rats have been reported. Moreover, *Brucella* strains are often isolated from a great variety of wildlife species such as bison (Lawler, 1997), elk (Jensen *et al.*, 1996), feral swine (Drew *et al.*, 1992), wild boar (Godfroid *et al.*, 1994), hare (Kautzsch *et al.*, 1995), African buffalo (Waghela & Karstad, 1986) and caribou (Ferguson, 1997).

The broad spectrum of *Brucella* hosts has recently been enlarged to include sea mammals. A number of recent

Abbreviation: OMP, outer-membrane protein.

The GenBank accession numbers for the sequences reported in this paper are AF027600 (*rrs*) and AF027601 (*omp2*).

reports have described the isolation and characterization of *Brucella* strains from a wide variety of sea mammals, including the bottlenose dolphin (*Tursiops truncatus*) (Ewalt *et al.*, 1994), common seal (*Phoca vitulina*), harbour porpoise (*Phocoena phocoena*), common dolphin (*Delphinus delphis*) (Ross *et al.*, 1994, 1996), Atlantic white-sided dolphin (*Lagenorhynchus acutus*), striped dolphins (*Stenella coeruleoalba*), hooded seal (*Cystophora cristata*), grey seal (*Halichoerus grypus*) and European otter (*Lutra lutra*) (Foster *et al.*, 1996). Although the general characteristics of these isolates have been analysed by conventional typing methods, their profiles could not be assigned to any of the six *Brucella* species listed above. *Brucella* species and their different biovars are currently distinguished by differential tests based on serotyping, phage typing, dye sensitivity, CO_2 requirement, H_2S production and metabolic properties (Alton *et al.*, 1988; Verger & Grayon, 1977).

Because the brucellae share a high degree of DNA homology (>90% for all species), *Brucella* has been proposed as a monospecific genus (Verger *et al.*, 1985, 1987). Several techniques have been employed to find DNA polymorphisms which would enable the molecular typing of *Brucella* species and their different biovars. The genes encoding the major outer-membrane proteins (OMPs), *omp25*, *omp31*, *omp2a* and *omp2b*, have been found to be particularly useful for this purpose because they exhibit sufficient polymorphism to allow differentiation between *Brucella* species and some of their biovars (Cloeckaert *et al.*, 1995, 1996a; Ficht *et al.*, 1990, 1996; Vizcaino *et al.*, 1997). An insertion sequence named IS*6501* (Ouahrani *et al.*, 1993) or IS*711* (Halling *et al.*, 1993) has also been found to be useful for discriminating *Brucella* strains at the species or strain level (Ouahrani *et al.*, 1993). The number of IS*6501* copies in chromosomal DNA varies from 5 to 35, depending on the *Brucella* species, and can be visualized by hybridization of an IS*6501* probe to *Eco*RI-digested total DNA (Ouahrani *et al.*, 1993). Other molecular techniques have been designed for the specific detection of *Brucella* species which are based on the PCR amplification of the *rrs-rrl* ribosomal spacer DNA (Rijpens *et al.*, 1996) and of IS*6501* DNA using specific primers (S. Henault & B. Garin-Bastuji, personal communication).

In November 1996, we isolated a *Brucella* strain for the first time from the spleen and the liver of a minke whale (*Balaenoptera acutorostrata*) that had been caught during commercial whaling off the Norwegian coast of Finnmark in May 1995. The purpose of the present study was to characterize this new isolate by conventional typing methods and by the molecular methods cited above.

METHODS

Bacterial strains and growth conditions. The minke whale (B202R) isolate was compared with FAO/WHO *Brucella* reference strains (Alton *et al.*, 1988). The *Brucella* strains were grown on *Brucella* agar (Difco) supplemented with 5% horse serum (Gibco) at 37 °C for 96 h.

Conventional typing methods. The tests were performed according to the techniques described by Alton *et al.* (1988): culture on *Brucella* agar supplemented with 5% horse serum (Gibco) in the presence of basic fuchsin (20 μg ml^{-1}), thionin (10, 20 or 40 μg ml^{-1}) and safranin O (100 μg ml^{-1}). Urease and catalase activity, H_2S production, CO_2 and serum requirement were evaluated. Serotyping was determined by agglutination tests using A- and M-monospecific antisera (Wellcome). The strain was also tested for its susceptibility to Tb, Wb, Iz and R/C brucellaphages. Oxidative metabolic tests were performed as described by Alton *et al.* (1988) and Verger & Grayon (1977).

DNA preparation, PCR primers, probes and amplification conditions. DNA was prepared as described by Ausubel *et al.* (1990). PCR amplification of chromosomal DNA from the B202R isolate was performed with several sets of primers specific for *Brucella* spp.: one was specific for the *rrs-rrl* spacer rDNA (Rijpens *et al.*, 1996) and another for IS*6501* (S. Henault & B. Garin-Bastuji, personal communication) (Table 1). Amplification reactions were prepared in 100 μl volumes containing 10 mM Tris/HCl (pH 7·4), 1·5 mM $MgCl_2$, 50 mM KCl, 0·1% Triton X-100, 200 μM dNTPs, 1 μM primer, 100 ng genomic DNA and 2 U Dynazyme II DNA polymerase (Finnzymes). PCR amplifications were performed in a Techne PHC-3 thermocycler (New Brunswick). The amplification conditions were the same as those described in the references in Table 1.

RFLP and IS*6501* hybridization. The IS*6501* biotinylated probe was generated by random octamer priming of the PCR product with the NEBlot Phototope Kit (New England Biolabs). Genomic DNA was treated for 3 h with 40 U *Eco*RI (Boehringer Mannheim) at 37 °C before electrophoresis through a 0·8% agarose gel for 20 h at 25 V. The DNA was then transferred to a nylon membrane (Biodyne A, Pall). The membrane-bound DNA was hybridized at 68 °C with the biotinylated probe (IS*6501*) and then washed as described in the Phototope*-Star Detection Kit manual (New England Biolabs). Hybridized IS*6501* probe was detected using the Phototope*-Star Detection Kit and by exposure of the hybridized membrane to an X-ray film (Kodak Scientific Imaging Film).

***omp2a* and *omp2b* hybridization.** One microlitre of PCR product was blotted onto a nylon membrane (Biodyne A, Pall). After denaturation and neutralization, the DNA was fixed by UV exposure. Membrane-bound DNA was hybridized at 55 °C with the biotinylated probe (Table 1). The membrane was washed twice with 6 × SSC/0·1% SDS at 55 °C for 10 min, once with 4 × SSC/0·1% SDS at 55 °C for 5 min and then once with 4 × SSC/0·1% SDS at 60 °C for 5 min (1 × SSC is 0·15 M sodium chloride/0·015 M sodium citrate). Biotinylated DNA was detected by using the Phototope*-Star Detection Kit.

PCR-RFLP of the major OMP genes. The major OMP genes, *omp25*, *omp31*, *omp2a* and *omp2b*, were characterized for polymorphism by PCR-RFLP as described previously (Cloeckaert *et al.*, 1995; Vizcaino *et al.*, 1997). Primers used are shown in Table 1.

***rrs* rDNA and *omp2* locus DNA sequencing and *omp2* phylogenetic analysis.** DNA sequences were obtained by the dideoxy-chain termination method of Sanger *et al.* (1977). The DNAs used as sequencing templates were PCR products purified with Centricon-100 columns (Amicon). Sequencing

Table 1. Nucleotide sequences of primers and probes used

The primers and probes were obtained from Biosource Europe (Belgium).

Primer and probe	Sequence (5′–3′)	Target DNA
rP2	ACGGCTACCTTGTTACGACTT	16S DNA (Weisburg *et al.*, 1991)
fD1	AGAGTTTGATCCTGGCTCAG	16S DNA (Weisburg *et al.*, 1991)
16S-677	GCGTCAGTAATGGTCCAGTG	16S DNA
16S-1071	AGAGTGCCCAACTGAATGCTG	16S DNA
Ba148-167F	TGCTAATACCGTATGTGCTT	16S DNA (Herman & De Ridder, 1992)
Ba928-948	TAACCGCGACCGGGATGTCAA	16S DNA (Herman & De Ridder, 1992)
P5	TCGAGAATTGGAAAGAGGTC	16S–23S rRNA spacer (Rijpens *et al.*, 1996)
P8	GCATAATGCGGCTTTAAGA	16S–23S rRNA spacer (Rijpens *et al.*, 1996)
2bC	AATATGCGCCCTGCAGCCATAC	*omp2* locus
2bG	CCGAATTGTTCGCAGCATAG	*omp2* locus
2aE	GTTTCCACCGGTTCGGAAAC	*omp2* locus
2bH	TAAGCGTCGCAAACGCGGAC	*omp2* locus
2IG1	CCGAAGCGCTCCTTCTTCTG	*omp2* locus
2IG2	TCGCCTGCCGAATAAAGCCG	*omp2* locus
2IG3	AGCCAAGGAGAAGGCTCTTG	*omp2* locus
2aF	TGACCGTAGTTCTGGTTCGG	*omp2* locus
2aA	GGCTATTCAAAATTCTGGCG	*omp2* locus (Cloeckaert *et al.*, 1995)
2aB	ATCGATTCTCACGCTTTCGT	*omp2* locus (Cloeckaert *et al.*, 1995)
2bA	CCTTCAGCCAAATCAGAATG	*omp2* locus (Cloeckaert *et al.*, 1995)
2bB	GGTCAGCATAAAAAGCAAGC	*omp2* locus (Cloeckaert *et al.*, 1995)
promomp2b	GCCACCTTGCCGACATATTCCGTT	*omp2* locus
IS*6501A*	ACGCCGGTGTATGGGAAAGGCTTTT	IS*6501*
IS*6501B*	GATAGAAGGCTTGAAGCTTGCGGAC	IS*6501*
omp2a probe	GGGTGGCGAAGACGTTGACAACGA	*omp2a* (Ficht *et al.*, 1990)
omp2b probe	GCGGCACCGTCATGGAGTTCGCG	*omp2b* (Ficht *et al.*, 1990)

reactions were performed according to the manufacturer's instructions using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). A phylogenetic tree was derived from CLUSTAL W (http://www2.ebi.ac.uk/clustalw/) (Higgins *et al.*, 1994) alignment of *omp2a* and *omp2b* sequences available in GenBank using the NJplot program (http://pbil.univ-lyon1.fr/software/njplot.html) (Perrière & Gouy, 1996).

RESULTS

The isolate from minke whale is related to the genus *Brucella* as suggested by conventional typing

Gram staining of the B202R strain isolated from the spleen of the minke whale revealed small, Gram-negative coccobacilli (not shown). The strain grew on *Brucella* agar supplemented with 5 % horse serum in the presence of basic fuchsin or thionin but did not grow in the presence of safranin O. CO_2 was not required for growth and H_2S was not produced. The isolate showed catalase, oxidase and urease activity. At the routine test dilution (RTD), the strain was lysed by the brucella-phages Wb and Iz, but not by Tb and R/C. The strain was lysed by Tb at an RTD of 10^4. Serotyping showed that the strain was A-dominant. The oxidative metabolic profile of strain B202R did not resemble the profiles of any of the known *Brucella* species and biovars (data not shown) (Alton *et al.*, 1988; Verger & Grayon, 1977). Thus, conventional typing methods suggested that the isolate was related to the genus *Brucella* but did not match the profiles of any established species or biovar.

The isolate from minke whale belongs to the genus *Brucella* according to molecular detection methods

For each set of primers specific for the *rrs-rrl* spacer rDNA (Rijpens *et al.*, 1996) and for IS*6501* (S. Henault & B. Garin-Bastuji, personal communication), an amplification product was obtained of the expected size, suggesting that the isolate belongs to the genus *Brucella*.

The nucleotide sequence of part of the *rrs* rDNA of strain B202R was determined. A homology search using BLAST (Altschul *et al.*, 1990) showed the highest identity (>99·5 %) with *rrs* rDNA of the six *Brucella* species, thus indicating that this strain probably belongs to the genus *Brucella*.

The isolate from minke whale cannot be classified within the known species of *Brucella* according to molecular typing methods

(i) RFLP and IS*6501* hybridization Fig. 1. shows IS*6501* hybridized to *Eco*RI-digested total DNA RFLP patterns of strain B202R compared with *Brucella* reference

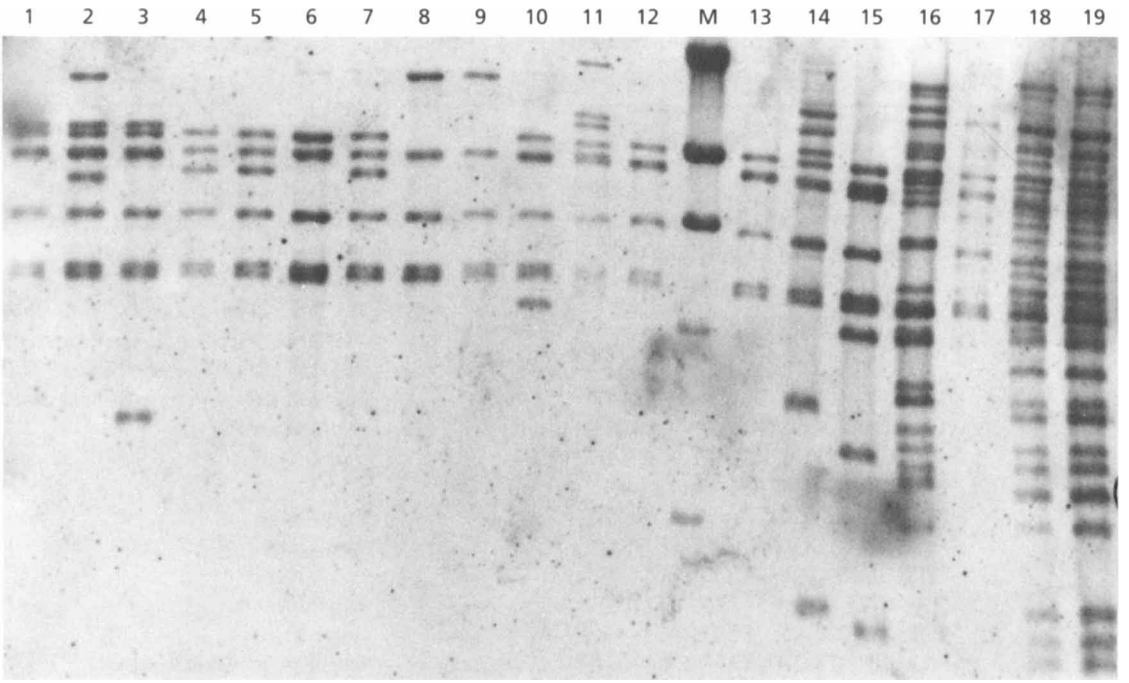

Fig. 1. IS*6501* RFLP. Southern blot of *Eco*RI-digested DNA from *Brucella* reference strains and B202R strain probed with IS*6501*. Lanes: 1, *B. melitensis* bv1; 2, *B. melitensis* bv2; 3, *B. melitensis* bv3; 4, *B. abortus* bv1; 5, *B. abortus* bv2; 6, *B. abortus* bv3; 7, *B. abortus* bv4; 8, *B. abortus* bv6; 9, *B. abortus* bv9; 10, *B. suis* bv1; 11, *B. suis* bv2; 12, *B. suis* bv3; 13, *B. suis* bv4; 14, *B. suis* bv5; 15, *B. neotomae*; 16, *B. ovis*; 17, *B. canis*; 18–19, B202R; M, molecular mass marker (λ *Hin*dIII).

strains. More than 25 IS*6501* bands appeared in the restricted DNA of strain B202R, which is close in number to *B. ovis* and much higher than the number of bands occurring in the other reference strains. Nevertheless, the pattern of strain B202R was very different from the *Brucella* type strains, including *B. ovis*.

(ii) *omp25* and *omp31* PCR-RFLP Species-specific markers on *omp25* and *omp31* have been identified previously for *B. melitensis* (absence of the *Eco*RV site in *omp25*), *B. ovis* (short deletion in *omp25*), *B. abortus* (absence of the *omp31* gene) and *B. canis* (specific *Ava*II restriction pattern of *omp31*) (Cloeckaert *et al.*, 1995, 1996a, b; Vizcaino *et al.*, 1997). The *omp25* and *omp31* genes were successfully amplified from the DNA of strain B202R, indicating again that it is probably a member of the *Brucella* genus (data not shown). None of the species-specific markers cited above were detected for strain B202R (data not shown) and no additional polymorphism was detected with the restriction enzymes used for *omp25* and *omp31* PCR-RFLP. Thus, it appeared that strain B202R could not be classified within the *B. abortus*, *B. melitensis*, *B. ovis* or *B. canis* species.

(iii) The isolate from minke whale possesses two copies of *omp2b* Two closely related genes, *omp2a* and *omp2b*, located in the *Brucella omp2* locus, encode and potentially express the 36 kDa porin OMP (Ficht *et al.*, 1989). The genes share about 85% DNA homology. They are separated by 900 bp and are oriented in opposite directions. The gene arrangement at the *omp2* locus appears to be conserved in all *Brucella* species (Ficht *et al.*, 1990). However, *B. ovis* has two genes closely related to *omp2a* instead of the one copy of *omp2a* and one copy of *omp2b* detected in the other *Brucella* species (Ficht *et al.*, 1990). These genes are of particular interest because biovar-specific markers for *B. suis* biovars and species-specific markers for *B. neotomae* have been identified (Cloeckaert *et al.*, 1995).

PCR-RFLP of the *omp2a* and *omp2b* genes successfully amplified from the DNA of strain B202R showed some unexpected results. In particular, restrictions performed with most enzymes on its amplified *omp2a* gene showed restriction patterns that were different from previously characterized *omp2a* genes. In fact, restriction patterns with most of the restriction enzymes of the B202R strain *omp2a* gene appeared to be almost identical to those of its *omp2b* gene (Fig. 2). This observation suggests that strain B202R carries two *omp2b* gene copies which is a characteristic that has not yet been reported in strains of the six *Brucella* species.

Further hybridization of an *omp2b*-specific probe to both the amplified *omp2a* and *omp2b* genes of strain B202R also suggested that this strain possesses two copies of *omp2b* (not shown).

The nucleotide sequence of the *omp2* locus of strain B202R was determined from three overlapping PCR products and sequenced as described in Methods. The complete nucleotide sequence of this locus (3146 bp)

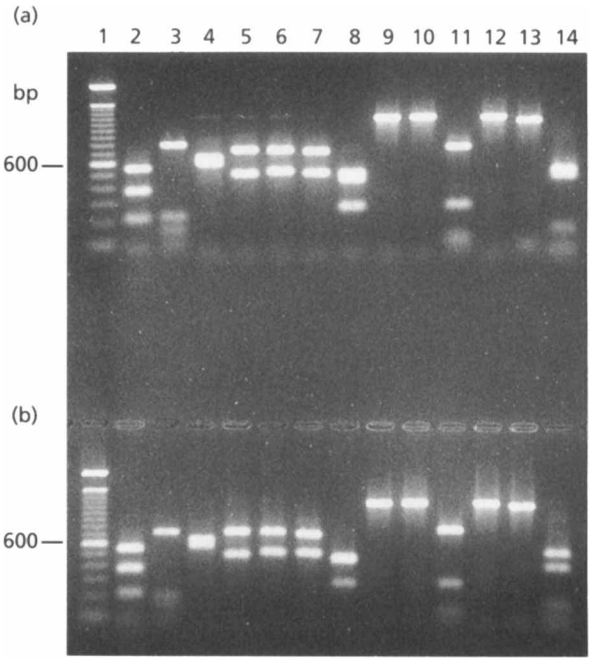

Fig. 2. Restriction patterns of PCR-amplified *omp2b* (a) and *omp2a* (b) genes of the minke whale isolate (B202R) cut by *Alu*I (lanes 2), *Ban*I (3), *Bgl*II (4), *Cla*I (5), *Eco*RI (6), *Hae*III (7), *Hin*fI (8), *Kpn*I (9), *Nco*I (10), *Pst*I (11), *Pvu*II (12), *Sty*I (13) and *Taq*I (14). Lanes 1: molecular mass marker (100 bp DNA ladder; Gibco).

was compared with those from the six known *Brucella* species. As seen in these species, the two *omp2* genes of strain B202R were separated by 900 bp and oriented in opposite directions. However, alignment of the two homologous regions (*omp2a* and *omp2b*) of strain B202R revealed that the two genes share about 98·9% identity (nucleotide differences at only five positions) without any insertion or deletion as seen in those from the other *Brucella* species. In addition, alignment of both genes with those of the other *Brucella* species showed that they had the highest identity levels with the *omp2b* gene of *B. neotomae* reference strain 5K33 (96·9% identity). This is also illustrated in the phylogenetic tree in Fig. 3 derived from CLUSTAL W-aligned *omp2a* and *omp2b* nucleotide sequences. Thus, the nucleotide sequence determined from the *omp2* locus of strain B202R confirmed the presence of two *omp2b* gene copies which were nearly identical to the *omp2b* gene of *B. neotomae*.

DISCUSSION

In this paper we describe a *Brucella* strain (B202R) isolated for the first time from a minke whale. Colony morphology, Gram staining, biochemical tests, phage-typing and serotyping indicated that strain B202R belongs to the *Brucella* genus. However, biotyping and the oxidative profile showed that this strain has unique characteristics in comparison with those of the six known species.

The specific PCR amplification of a portion of IS*6501* as well as the *rrs-rrl* rDNA spacer and the *rrs* rDNA nucleotide sequence determination confirm the results of the conventional typing methods. In addition, the *omp25* and *omp31* genes were successfully PCR-amplified but did not reveal any species-specific markers identified for four *Brucella* species and did not show additional polymorphism allowing classification of strain B202R. The *omp2* locus of strain B202R had the same general organization as the other *Brucella* species, i.e. the presence of two *omp2* genes separated by 900 bp and oriented in opposite directions. However, in contrast to the six known *Brucella* species (Ficht *et al.*, 1990), strain B202R appeared to have two copies of the *omp2b* gene as shown by PCR-RFLP, hybridization with an *omp2b*-specific probe and by determining the nucleotide sequence of the *omp2* locus. The identity of both *omp2b* genes of strain B202R reached 96·9% with the *omp2b* gene of *B. neotomae* 5K33.

On the basis of polymorphism at the *omp2* locus, *B. neotomae* and *B. ovis* are the most divergent taxa of the *Brucella* species (Ficht *et al.*, 1996). The extreme divergence of *B. ovis* (presence of two *omp2a* gene copies) from the other *Brucella* species has been shown to be due to a lack of divergence between its *omp2a* and *omp2b* gene sequences. Indeed, the genes in *B. ovis* differ by only 30 nt at their 3′ ends. In *B. abortus*, *B. melitensis*, *B. suis* and *B. canis* a mean of 130 nt differences are observed in the two gene copies (at the 3′ ends). 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*. It was therefore suggested 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 (Ficht *et al.*, 1996). As shown in the present study, the occurrence of *Brucella* strains such as the one isolated from the minke whale carrying two *omp2b* gene copies contrasts with the hypothesis of a progenitor species with two *omp2a* genes. In addition, the very high identity between the two *omp2b* gene copies in strain B202R with no deletion or addition would suggest a recent duplication of the gene and/or perhaps a much slower evolution of marine brucellae.

RFLP and IS*6501* hybridization also demonstrated a profile for strain B202R distinct from the profiles of the *Brucella* type strains. Thus, IS*6501* RFLP up to now has shown two groups of patterns: one has a low number of bands (<10; *B. melitensis*, *B. suis*, *B. neotomae*, *B. abortus*, *B. canis*) and the other has a high number of bands (>25; *B. ovis* and strain B202R). However, within the second group, the two profiles were clearly different, suggesting that strain B202R could be phylogenetically more distant from the other *Brucella* species.

Thus, the most significant observations in this present study were (i) isolation of a *Brucella* strain from a minke whale which has some phenotypic characteristics that differ from type strains of the six known *Brucella* species and their biovars and (ii) the presence in this strain of

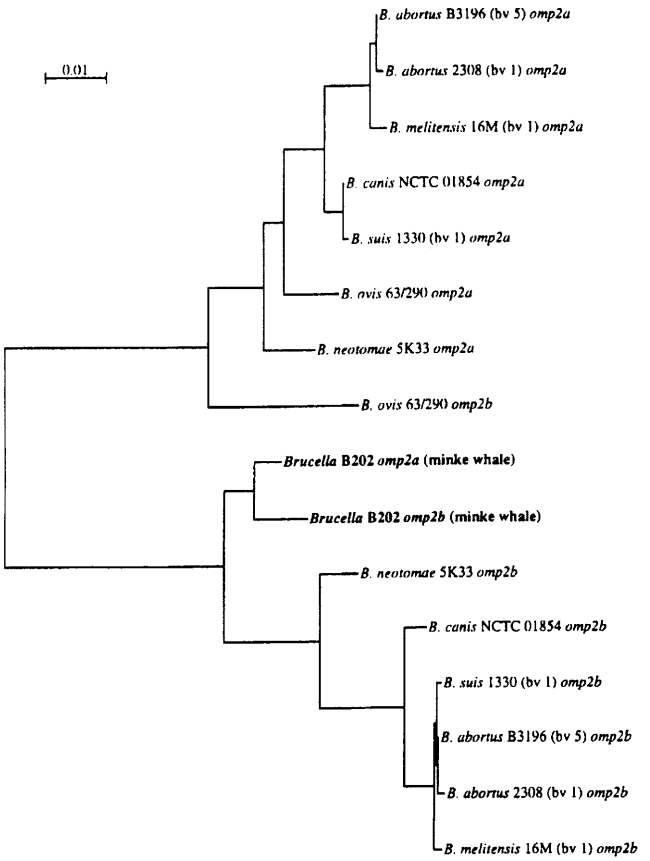

Fig. 3. Phylogenetic tree derived from CLUSTAL W-aligned *omp2a* and *omp2b* nucleotide sequences. The figure clearly shows that the *omp2a* and *omp2b* nucleotide sequences from the minke whale isolate are closely related and belong to the *omp2b* group of genes.

two *omp2b* gene copies and an IS*6501* RFLP pattern which is clearly distinct from other *Brucella* strains, making the isolate genetically more distant from the six known *Brucella* species. Molecular characterization of other isolates from marine mammals is now required to determine if these features are specific to marine brucellae.

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