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Intraspecies Biodiversity of the Genetically Homologous Species *Brucella microti*

Sascha Al Dahouk,[a,b] Erwin Hofer,[c] Herbert Tomaso,[d] Gilles Vergnaud,[e,f,g] Philippe Le Flèche,[e,f,h] Axel Cloeckaert,[i] Mark S. Koylass,[j] Adrian M. Whatmore,[j] Karsten Nöckler,[a] and Holger C. Scholz[k]

Federal Institute for Risk Assessment, Berlin, Germany[a]; RWTH Aachen University, Department of Internal Medicine III, Aachen, Germany[b]; Austrian Agency for Health and Food Safety, Mödling, Austria[c]; Friedrich Loeffler Institute, Institute of Bacterial Infections and Zoonoses, Jena, Germany[d]; Université Paris Sud, Institut de Génétique et Microbiologie, UMR 8621, Orsay, France[e]; Centre National de la Recherche Scientifique (CNRS), Orsay, France[f]; Mission pour la Recherche et l'Innovation Scientifique (DGA/MRIS), Bagneux, France[g]; Centre d'Etudes du Bouchet, Division of Analytical Microbiology, Vert le Petit, France[h]; Institut National de la Recherche Agronomique (INRA), UR1282, Infectiologie Animale et Santé Publique (IASP), Nouzilly, France[i]; Animal Health and Veterinary Laboratories Agency (AHVLA), Department of Bacteriology, New Haw, United Kingdom[j]; and Bundeswehr Institute of Microbiology, Department of Bacteriology, Munich, Germany[k]

Brucellosis is one of the major bacterial zoonoses worldwide. In the past decade, an increasing number of atypical *Brucella* strains and species have been described. *Brucella microti* in particular has attracted attention, because this species not only infects mammalian hosts but also persists in soil. An environmental reservoir may pose a new public health risk, leading to the reemergence of brucellosis. In a polyphasic approach, comprising conventional microbiological techniques and extensive biochemical and molecular techniques, all currently available *Brucella microti* strains were characterized. While differing in their natural habitats and host preferences, *B. microti* isolates were found to possess identical 16S rRNA, *recA*, *omp2a*, and *omp2b* gene sequences and identical multilocus sequence analysis (MLSA) profiles at 21 different genomic loci. Only highly variable microsatellite markers of multiple-locus variable-number tandem repeat (VNTR) analysis comprising 16 loci (MLVA-16) showed intraspecies discriminatory power. In contrast, biotyping demonstrated striking differences within the genetically homologous species. The majority of the mammalian isolates agglutinated only with monospecific anti-M serum, whereas soil isolates agglutinated with anti-A, anti-M, and anti-R sera. Bacteria isolated from animal sources were lysed by phages F1, F25, Tb, BK2, Iz, and Wb, whereas soil isolates usually were not. Rough strains of environmental origin were lysed only by phage R/C. *B. microti* exhibited high metabolic activities similar to those of closely related soil organisms, such as *Ochrobactrum* spp. Each strain was tested with 93 different substrates and showed an individual metabolic profile. In summary, the adaptation of *Brucella microti* to a specific habitat or host seems to be a matter of gene regulation rather than a matter of gene configuration.

Brucella species are facultatively intracellular pathogens responsible for one of the world's most widespread zoonotic diseases. The bacteria may cause reproductive failure and abortion in domestic animals and a potentially debilitating multiorgan infection in humans. Like *Agrobacterium* and *Rhizobium* spp., brucellae belong to the order of *Rhizobiales* of the α-2 subgroup of *Proteobacteria*. Members of the class *Alphaproteobacteria* include organisms that are either mammalian or plant pathogens or symbionts (12). Within the family *Brucellaceae*, *Ochrobactrum*, a genus comprising soil-associated facultative human pathogens, contains the closest phylogenetic neighbors to *Brucella*. *Ochrobactrum intermedium* and *Brucella* spp. are 98.8% identical in their 16S rRNA gene sequences (14). Furthermore, *Brucella* species are closely related to each other (monophyletic genus), showing 98 to 99% similarity in most of the coding sequences. Despite this high genetic homology, brucellae differ widely in host tropism, phenotypic characteristics, and pathogenicity (38).

The phylogeny of *Brucella* species does not always match that of their nominal mammalian hosts (36). Currently, the genus *Brucella* consists of 10 species. With the exception of *Brucella inopinata* (29), at least one animal host has been described for each species. Although the host range of *Brucella* spp. can be variable, most species display strong host preferences. The classical *Brucella* spp. of terrestrial origin, i.e., *Brucella melitensis*, *B. abortus*, *B. ovis*, *B. canis*, *B. suis*, and *B. neotomae*, are best characterized as facultatively intracellular pathogens. An intracellular lifestyle has also been found for *Brucella* species that infect marine mammals (*B. ceti* and *B. pinnipedialis*) (20). Hence, *Brucella* spp. are commonly regarded as intracellular pathogens with an animal reservoir, although they share close relationships with soil organisms such as *Ochrobactrum* spp., with plant symbionts such as *Rhizobium* spp., and with phytopathogens such as *Agrobacterium* spp. (38). No natural reservoir outside infected mammalian hosts has been identified yet. *Brucella* spp. generally appear as fastidious bacteria, because their survival in the nutrient-poor phagosome requires a low overall metabolic activity.

In contrast to the classical *Brucella* spp., the recently described species *B. microti* is characterized by fast growth on standard media and exhibits remarkable metabolic capabilities (28). Indeed, the phenotype of *B. microti* resembles that of *Ochrobactrum* rather than that of *Brucella*. Hence, *B. microti* has been misidentified as *Ochrobactrum intermedium* by use of commercially available biochemical tests, such as the API 20 NE test (bioMérieux, Nürtingen, Germany) (17).

Initially, two strains of this novel *Brucella* species that had been isolated from systemically diseased common voles (*Microtus ar-*

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Address correspondence to Sascha Al Dahouk, Sascha.Al-Dahouk@gmx.de.

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TABLE 1 *Brucella microti* strains ($n = 11$) isolated from different sources and of different geographical origins

Strain	Geographical origin	Source	Yr of isolation	Reference(s)
CCM 4915^T	South Moravia, Czech Republic	Common vole	2000	17, 28
CCM 4916	South Moravia, Czech Republic	Common vole	2000	17, 28
IBM 284	Gmünd, Lower Austria	Red fox	2007	26
IBM 257	Gmünd, Lower Austria	Red fox	2007	26
FH 2208	Horn, Lower Austria	Red fox	2008	This study
FK 21908	Korneuburg, Lower Austria	Red fox	2008	This study
FW 70608	Waidhofen/Thaya, Lower Austria	Red fox	2008	This study
F 303 Mi	Mistelbach, Lower Austria	Red fox	2009	This study
BMS 10	South Moravia, Czech Republic	Soil	2008	This study
BMS 17	South Moravia, Czech Republic	Soil	2008	27
BMS 20	South Moravia, Czech Republic	Soil	2008	27

valis) in the Czech Republic (17) were analyzed in detail (28). Subsequently, *B. microti* was also isolated from the mandibular lymph nodes of red foxes in a district of Lower Austria (26) and even directly from soil (27). These findings indicated that *B. microti* may persist in a geographical area comprising most parts of Moravia (Czech Republic) and Lower Austria. Long-term environmental persistence outside mammalian hosts and the diversity of reservoir species may play a key role in the epizootic spread of this *Brucella* species.

The aim of this study was to characterize all currently available *B. microti* isolates originating from different animal species and various geographical regions by using a selection of widely recognized classical techniques, comprehensive biotyping, and molecular analyses in direct comparison. The data generated would reveal intraspecies diversity among *B. microti* strains, which might help to explain their ability to survive in multiple hosts and environments.

MATERIALS AND METHODS

Bacterial strains. A total of 11 *B. microti* strains, including the type strain, CCM 4915, isolated from both environmental and animal sources and originating from different geographical regions, were analyzed (Table 1). The bacteria were grown on *Brucella* agar for 48 h at 37°C both with and without 10% CO_2.

Molecular analyses (16S rRNA [*rrs*], *omp2a*, *omp2b*, and *recA* gene sequencing, multilocus sequence typing [MLST], and multiple-locus variable-number tandem repeat analysis [MLVA]) and phenotypic characterization (biochemical profiling, agglutination, phage lysis) were carried out essentially as described previously for the *B. microti* type strain, CCM 4915 (28).

Molecular analysis. (i) DNA preparations. Crude DNA was prepared by transferring a single colony of each strain from the agar plate to 200 μl 5× lysis buffer D (PCR Optimizer kit; Invitrogen, De Schelp, The Netherlands) diluted 1:5 in distilled water, supplemented with 0.5% Tween 20 (ICI America Inc., Merck, Hohenbrunn, Germany) and 2 mg/ml proteinase K (Roche Diagnostics, Mannheim, Germany). After incubation at 56°C for 1 h and inactivation at 95°C for 10 min, DNA samples were purified using the QIAamp DNA Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). A total of 2 μl of the cleared lysates was used as a template in the PCR assays.

(ii) PCR analysis. *(a) bcsp31 PCR and AMOS-PCR.* In a genus-specific PCR, the published B4 and B5 primers were used to amplify a 223-bp target within the *bcsp31* gene encoding a 31-kDa immunogenic protein conserved among *Brucella* spp. (6). AMOS-PCR for the detection of *B. abortus* bv. 1, 2, and 4, *B. melitensis*, *B. ovis*, and *B. suis* bv. 1 was carried out basically as described by Bricker and Halling (7).

(b) Bruce-ladder multiplex PCR. The Bruce-ladder multiplex PCR first established by García-Yoldi and colleagues (13) was used in its modified version as described previously (21). Briefly, primer pair Bmispec_f (5′-AGATACTGGAACATAGCCCG-3′) and Bmispec_r (5′-ATACTCAGG CAGGATACCGC-3′), targeting a 12-kb genomic island specific for *B. microti*, was added (5, 27). The 25-μl reaction mixture contained 2.5 μl primer mix (with each primer at 2 pmol/μl), 1 μl template DNA, and 12.5 μl 2× Qiagen Multiplex PCR master mix. Thermal cycling was carried out with a model 2720 thermal cycler (Applied Biosystems, Foster City, CA). The initial denaturation step at 95°C for 15 min was followed by template denaturation at 94°C for 30 s, primer annealing at 58°C for 90 s, and a 3-min primer extension at 72°C. After a total of 25 cycles, a final extension phase of 10 min at 72°C completed the reaction, and the PCR products were analyzed using a 1.5% agarose gel.

(iii) Analysis of the 16S rRNA (*rrs*), *recA*, *omp2a*, and *omp2b* genes. *(a) 16S rRNA (rrs) and recA.* The *rrs* (16S rRNA) and *recA* (recombinase A) genes were amplified and sequenced as described previously (30). Briefly, almost the complete *rrs* sequence was amplified using the universal primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492r (5′-A AGTCGTAACAAGGTARCCG-3′). The primer pair *recA*-BrucOchro-f (5′-ATGTCTCAAAATTCATTGCGAC-3′) and *recA*-BrucOchro-r (5′-A GCATCTTCTTCCGGTCCGC-3′) generated a fragment comprising the entire *recA* gene (1,086 bp). The PCRs were performed in 50 μl Ready-To-Go master mix (Eppendorf GmbH, Hamburg, Germany) with the addition of 15 pmol of each primer. Amplification was carried out in a Perkin-Elmer GeneAmp 2400 thermal cycler (Perkin-Elmer, Applied Biosystems, Foster City, CA). A total of 30 cycles were conducted, each consisting of 30 s of denaturation at 94°C, 30 s of annealing at 58°C (*rrs*) or 65°C (*recA*), and elongation at 72°C for 90 s (*rrs*) or 60 s (*recA*). A final elongation step of 7 min at 72°C completed the run. Finally, PCR products were analyzed for the presence of the respective amplicons by agarose gel electrophoresis (1% [wt/vol] in Tris-acetate-EDTA [TAE] buffer).

The purified fragments of the *recA* and *rrs* sequences were sequenced with an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA) using the *recA*-BrucOchro-f/*recA*-BrucOchro-r primers and the internal primer set consisting of 341fw (5′-CCTACGGGAGGCAGCAG-3′), 518r (5′-ATTACCGCGGCTGCTGG-3′), and 926f (5′-AACTYAAAKGAATTGACGG-3′), respectively. Multiple sequence alignments were performed with ClustalW, version 1.8 (http://clustalw.genome.jp/).

(b) omp2a and omp2b. The *omp2a* and *omp2b* genes were amplified and sequenced as described previously (8, 9). Primers 2aA (5′-GGCTAT TCAAAATTCTGGCG-3′) and 2aB (5′-ATCGATTCTCACGCTTTCGT-3′) were used to amplify the *omp2a* gene, and the primer set 2bA (5′-CC TTCAGCCAAATCAGAATG-3′) and 2bB (5′-GGTCAGCATAAAAAGC AAGC-3′) was used to amplify *omp2b*. The purified PCR products were sequenced by the dideoxy-chain termination method (25).

MLSA and MLVA comprising 16 loci (MLVA-16). (i) MLST. Extended multilocus sequence analysis (MLSA) was carried out, examining 21 distinct genomic fragments equating to >10.2 kb of sequence. Most of these genetic loci were housekeeping genes. Nine loci, *gap*, *aroA*, *glk*,

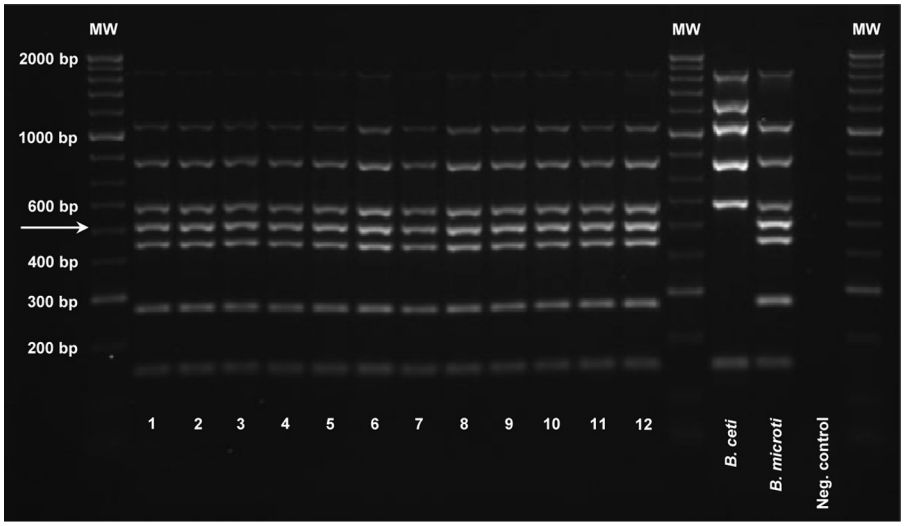

FIG 1 Identification of *Brucella microti* using the Bruce-ladder multiplex PCR. The *B. microti* strains showed a consistent amplicon pattern comprising seven fragments also found in *B. suis* (1,682 bp, 1,071 bp, 794 bp, 587 bp, 450 bp, 272 bp, and 152 bp) and an additional, *B. microti*-specific 510-bp amplicon (indicated by the arrow). Lane 1, *B. microti* type strain CCM 4915; lane 2, strain CCM 4916; lane 3, field isolate IBM 257; lane 4, field isolate IBM 284; lanes 5 and 6, field isolate BMS 10, presented with a rough and a smooth phenotype, respectively; lane 7, field isolate F 303 Mi; lane 8, field isolate FK 210908; lane 9, field isolate FH 2208; lane 10, field isolate FW 70608; lane 11, field isolate BMS 17; lane 12, field isolate BMS 20. A 1-kb Plus DNA ladder (Invitrogen Ltd.) was used as molecular weight (MW) markers. The *B. ceti* NCTC 12891 and *B. microti* CCM 4915^T reference strains were used as positive controls for all possible amplicons in the Bruce-ladder PCR, and distilled water was used as a negative control.

dnaK, *gyrB*, *trpE*, *cobQ*, *omp25*, and *int-hyp*, had already been demonstrated to be useful in describing genetic relatedness among *Brucella* spp. (37). An additional 12 loci (*ddlA*, *csdB*, *putA*, *fbaA*, *mutL*, *fumC*, *prpE*, *leuA*, *acnA*, *soxA*, a gene encoding an acyl coenzyme A [acyl-CoA] dehydrogenase, and a gene encoding a glucose-fructose oxidoreductase precursor), part of an extended MLSA scheme designed to increase resolution (A. M. Whatmore, unpublished data), were examined in order to characterize *B. microti* strains. MLST was conducted essentially as described previously (37). Each allele at each locus was given a distinct arbitrary numerical designation.

(ii) MLVA-16. MLVA was carried out as described by Le Flèche and colleagues (19) and modified by Al Dahouk and colleagues (1) using eight minisatellite markers (panel 1: bruce06, bruce08, bruce11, bruce12, bruce42, bruce43, bruce45, and bruce55) and eight microsatellite markers (panel 2). The panel 2 markers were split into two groups, panel 2A and 2B, comprising three (bruce18, bruce19, and bruce21) and five (bruce04, bruce07, bruce09, bruce16, and bruce30) markers, respectively. The most highly variable markers were included in panel 2B.

The clustering analysis was based on the categorical coefficient and the unweighted-pair group method using arithmetic averages (UPGMA). The same weight was given to a large and a small number of differences in the repeats at each locus. Three different character data sets were defined and were combined using the composite data set tool provided by Bionumerics. Different weights were assigned to the markers depending on the panel to which they belonged: individual weights of 2 for panel 1 markers, 1 for panel 2A markers, and 0.2 for panel 2B markers (1).

Analysis of phenotypic characteristics. (i) Classical microbiological methods. All isolates were characterized using the classical microbiological methods described by Alton and colleagues, i.e., CO_2 requirement, H_2S production, urea hydrolysis, agglutination with monospecific sera (anti-A, anti-M, and anti-R), dye sensitivity (basic fuchsin and thionine), and phage typing (F1, F25, Tb, BK2, Iz, Wb, R/C) (4).

(ii) Biotyping by metabolic activity testing. Metabolic activity was assessed by using a commercial biotyping system (Micronaut; Merlin Diagnostika, Bornheim-Hersel, Germany) as described previously (3) to determine if a set of phenotypic features may reveal intraspecies variability. The recently developed 96-well "*Brucella* identification and typing" plate tested for 29 aminopeptidases, 2 phosphatases, 4 glucosidases, 1 esterase, and the metabolism of 11 monosaccharides, 3 disaccharides, 7 sugar derivates, 15 amino acids, 11 organic acids, 1 salt, 1 amino acid derivate, 1 peptide, 1 base, and 6 classical reactions (nitrite and nitrate reduction, pyrazinamidase, Voges-Proskauer medium, urease, and H_2S production). Each enzyme-substrate reaction was carried out 3 to 5 times.

Hierarchical cluster analysis was performed by Ward's linkage algorithm using binary coded data based on empirically set cutoffs, and a dendrogram was generated. Each character was considered equal to every other character within the data set.

RESULTS

Molecular analysis. (i) PCR analysis. The genus-specific *bcsp31* PCR correctly identified all *B. microti* strains as members of the genus *Brucella*. In the IS*711*-based AMOS-PCR, a 1,900-bp fragment was generated in all *B. microti* strains with the *Brucella ovis*-specific primers, as previously shown for the *B. microti* type strain, CCM 4915 (data not shown).

By use of the modified Bruce-ladder multiplex PCR (13, 21), the *B. microti* field isolates gave a consistent amplicon pattern comprising the seven fragments found in *B. suis* (1,682 bp, 1,071 bp, 794 bp, 587 bp, 450 bp, 272 bp, and 152 bp) and the distinguishing 510-bp amplicon of the *B. microti*-specific 12-kb genomic insertion (Fig. 1).

(ii) Analysis of 16S rRNA, *recA*, *omp2a*, and *omp2b* genes. The 16S rRNA gene sequences of the 11 *B. microti* strains were identical to the consensus sequence of the classical *Brucella* species. Thus, they did not reveal even the minor differences that have been observed in other atypical strains, such as BO1 (*B. inopinata*), BO2, and the newly described rodent strains originating from Australia (10, 33, 34).

The *recA* gene sequences of the *B. microti* strains investigated were successfully amplified by PCR with the primer set *recA*-BrucOchro-r/*recA*-BrucOchro-f, initially constructed from se-

Brucella species and strain	Host	GenBank accession n°	*omp2* gene	90	180	270	360	450	540	630	720	810	900	990	1080	1140
B. microti	common vole	AM698039	*omp2b*													
str. CCM 4915^T		AM698038	*omp2a*	1	4 1 9 1	10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1 2	2
***B. melitensis* bv 1**	goat	U26440	*omp2b*	1		1										
str. 16M		U26440	*omp2a*	1		1 10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1 2	2
***B. abortus* bv 1**	cattle	M26034	*omp2b*	1												
str. 2308		M26034	*omp2a*	1		1 10 9 12	5(12) 6(18)	-	- - -	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1 2	2
***B. abortus* bv 5**	cattle	U26438	*omp2b*	1												
str. B3196		U26438	*omp2a*	1		1 10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1 2	2
***B. suis* bv 1**	pig	U26443	*omp2b*	1												
str. 1330		U26443	*omp2a*	1		9 1 10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1 2	2
***B. suis* bv 4**	reindeer	AM712086	*omp2b*	1		9										
NCTC 11364		AM712085	*omp2a*	1		9 1 10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1 2	2
***B. suis* bv 5**	wild rodent	AM712078	*omp2b*	1												
str. 513		AM712077	*omp2a*	1	4 1 9 1	10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	6 1 2	2
B. canis	dog	U26439	*omp2b*	1		9										
NCTC 01854		U26439	*omp2a*	1		9 1 10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	6 1 2	2
B. neotomae	desert rat	U26441	*omp2b*	1									5	14 1		
str. 5K33		U26441	*omp2a*	1	4 1 9 1	10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1	1 2	2
B. ovis	sheep	NC_009505	*omp2b*	1	4 1 9 1	10 9 12	5(12) 6(17,**1**)	8	7 1 1	2 1 1	8(8) 3(7) 1	2	2 5			
str. 63/290		NC_009505	*omp2a*	1	4 1 9 1	10 9 12	5(12) 6(17,**1**)	8	7 1 1	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1	***2** 2
B. pinnipedialis	common seal	AF300818	*omp2b*	1	4 1 9								2 5	14 1 4	5	
B2/94		AF300819	*omp2a*	1	4 1 9 1	10 9 12	5(12) 6(18)	8	7 1 6	2 1 1	8(8) 3(7) 1	2	2 5	14 1 4	7 1 2	2
B. ceti	porpoise	AF300816	*omp2b*	1	4 1 9							2	2 5	14 1 4	4	
B1/94		AF300817	*omp2a*	1	4 1 9							2	2 5	14 1 4	4	2 2

FIG 2 Schematic multiple nucleotide sequence alignment of the *omp2a* and *omp2b* genes of *Brucella* strains. Nucleotide sequences are represented by rectangles divided into boxes of 30 nucleotides. The *B. microti omp2b* gene sequence was used as a reference. *B. microti omp2a*-specific nucleotides are shaded. The numbers in the corresponding boxes indicate the number of *omp2a*-specific nucleotides present in the sequence considered. Numbers in parentheses represent insertions and deletions. Numbers in boldface indicate nucleotide differences that are not due to gene conversion. *, there is a premature stop codon in *omp2a* of *B. ovis* 63/290.

quence information available for *B. abortus* bv. 1 strain 9-941. BLASTN analysis of the nucleotide sequences of the 1,065-bp PCR fragments generated confirmed specific amplification of the *recA* gene in all strains. The uniform *recA* sequence of the *B. microti* strains under study was 100% identical to the consensus sequence of *Brucella* spp.

omp2 gene sequences were also 100% identical among *B. microti* isolates. Genetic diversity in *Brucella* spp. at the *omp2* locus is driven mainly by gene conversion between the two *omp2* gene copies, *omp2a* and *omp2b*, which are in opposite orientations at this locus and are separated by about 900 bp. Interestingly, the highest divergence (about 15%) between the two copies is found in *B. microti*, which appears to be ancestral to the classical *Brucella* species at the genomic level. In other species, *omp2* gene conversion has led to exchange of either *omp2a*-specific or *omp2b*-specific motifs in the respective *omp2* sequence position of each gene copy, sometimes resulting in the homogenization of the gene copies. This is, for example, the case in *B. ovis*, with two gene copies almost identical to the *omp2a* consensus, or in *B. ceti*, with two gene copies almost identical to the *omp2b* consensus (Fig. 2). The *omp2* sequences closest to those of *B. microti* are those of *B. suis* bv. 5 (strain 513), another wild rodent isolate. There are indeed only minimal differences between *omp2a* and *omp2b* in *B. microti* CCM 4915^T and *B. suis* bv. 5 strain 513 (1 nucleotide and 2 nucleotides, respectively). However, in terms of *omp2* gene conversion, *B. microti* and *B. suis* bv. 5 are identical, as shown in Fig. 2.

(iii) MLSA and MLVA-16. By use of MLSA based on 21 distinct genomic fragments equating to >10.2 kb of conserved sequence, the allelic pattern identified was unique and consistent in all *B. microti* isolates under study. Hence, *B. microti* is represented by a single and specific sequence type (ST), which forms a genotypic cluster distinct from those of all other brucellae.

In the comparative multilocus variable-number tandem repeat (VNTR) analysis, *B. microti* strains also grouped together, forming a separate cluster within the genus *Brucella*, most closely related to the *B. neotomae* reference strain 5K33 (Fig. 3). *B. inopinata* and *B. suis* bv. 5 were more distantly related. By use of panel 1 VNTRs, the 11 *B. microti* isolates clustered into 3 different genotypes (genotypes 67, 68, and 81 [http://mlva.u-psud.fr/]) that have not yet been described in other *Brucella* spp., including rodent strains, i.e., *B. neotomae* and *B. suis* bv. 5. On the basis of the complete MLVA-16 data, each of the *B. microti* strains showed an individual genotype (Fig. 4 and 5). The *B. microti* isolates grouped neither according to their hosts nor according to their geographical origins. However, the soil isolates, BMS 10, BMS 17, and BMS 20 (genotype 67 with panel 1), were found in the same cluster, because they showed only minimal differences in the highly variable VNTR markers of panel 2B (Fig. 4 and 5).

Analysis of phenotypic characteristics. (i) Bacterial cultures. All isolates could be easily cultured on *Brucella* agar at 28°C and 37°C without supplementary CO_2. The *B. microti* strains were characterized by nonfastidious, rapid growth. The bacterial colonies usually appeared transparent to whitish, and were 1 to 2 mm in diameter, after 1 to 2 days of incubation. After 72 h of growth at 37°C, most of the colonies developed a markedly brownish pigmentation. However, the pigmentation intensity and rate differed between individual strains, e.g., strain IBM 284 had already developed brownish colonies after 24 h.

(ii) Differential phenotyping by classical microbiological methods and metabolic activity testing. Except for strain IBM

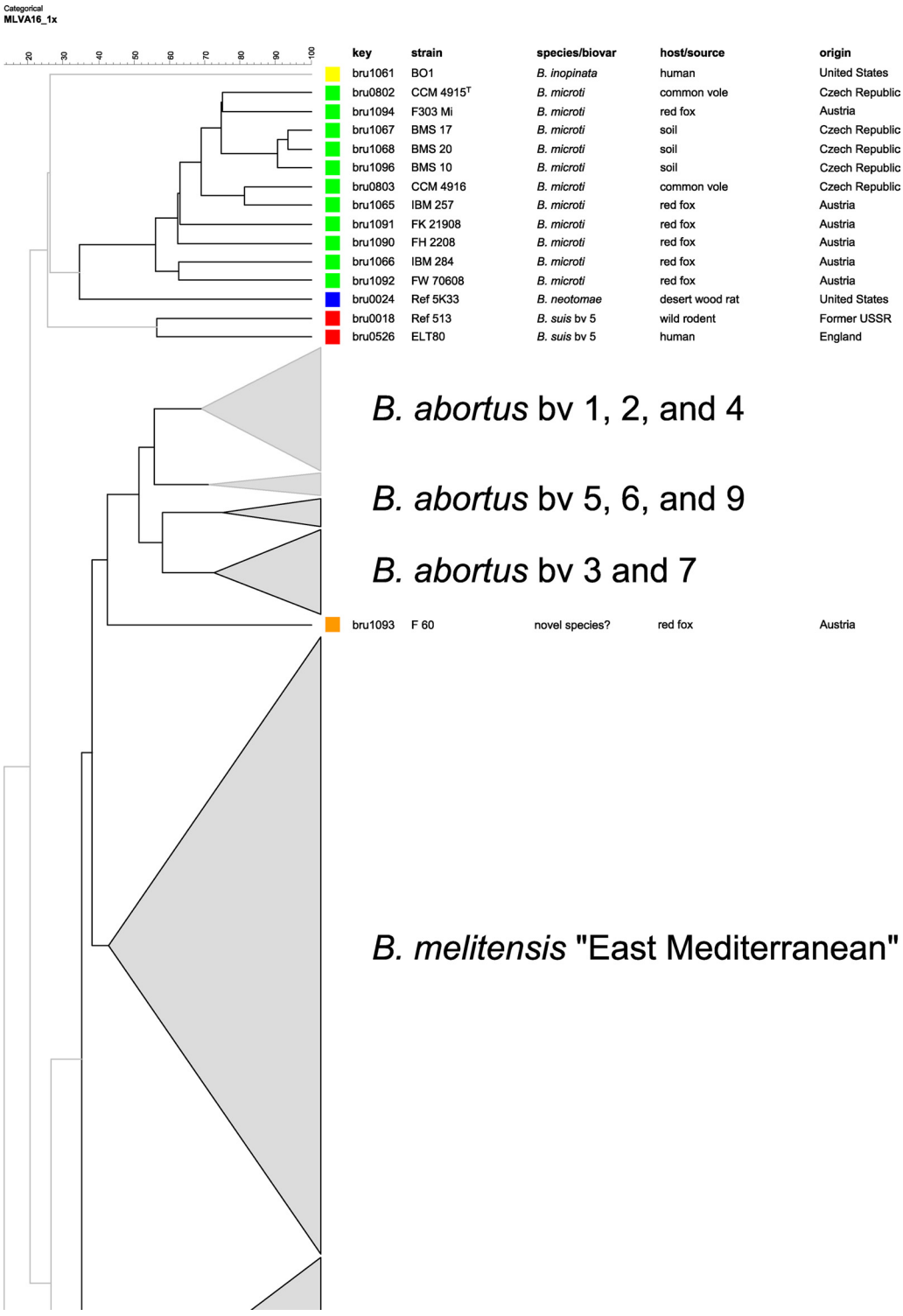

FIG 3 Condensed dendrogram of clustered MLVA-16 genotypes of *Brucella* spp. A total of 344 *Brucella* isolates revealed 340 different genotypes. The bars reflect the percentages of divergence. The cluster of the *B. microti* isolates and closely related species, such as *B. neotomae*, *B. inopinata*, and *B. suis* bv. 5, are presented in more detail. Two atypical isolates are separately marked.

257, *B. microti* isolates did not produce H_2S. Urea was regularly hydrolyzed within 90 to 105 min. All isolates grew in the presence of thionine at dilutions of 1/25,000, 1/50,000, and 1/100,000 and in the presence of basic fuchsin at dilutions of 1/50,000 and 1/100,000. Rough (R) colonies were ruby colored after staining with crystal violet, and trypaflavine led to spontaneous agglutination of rough strains (22).

Isolates from animal sources were lysed by phages F1, F25, and

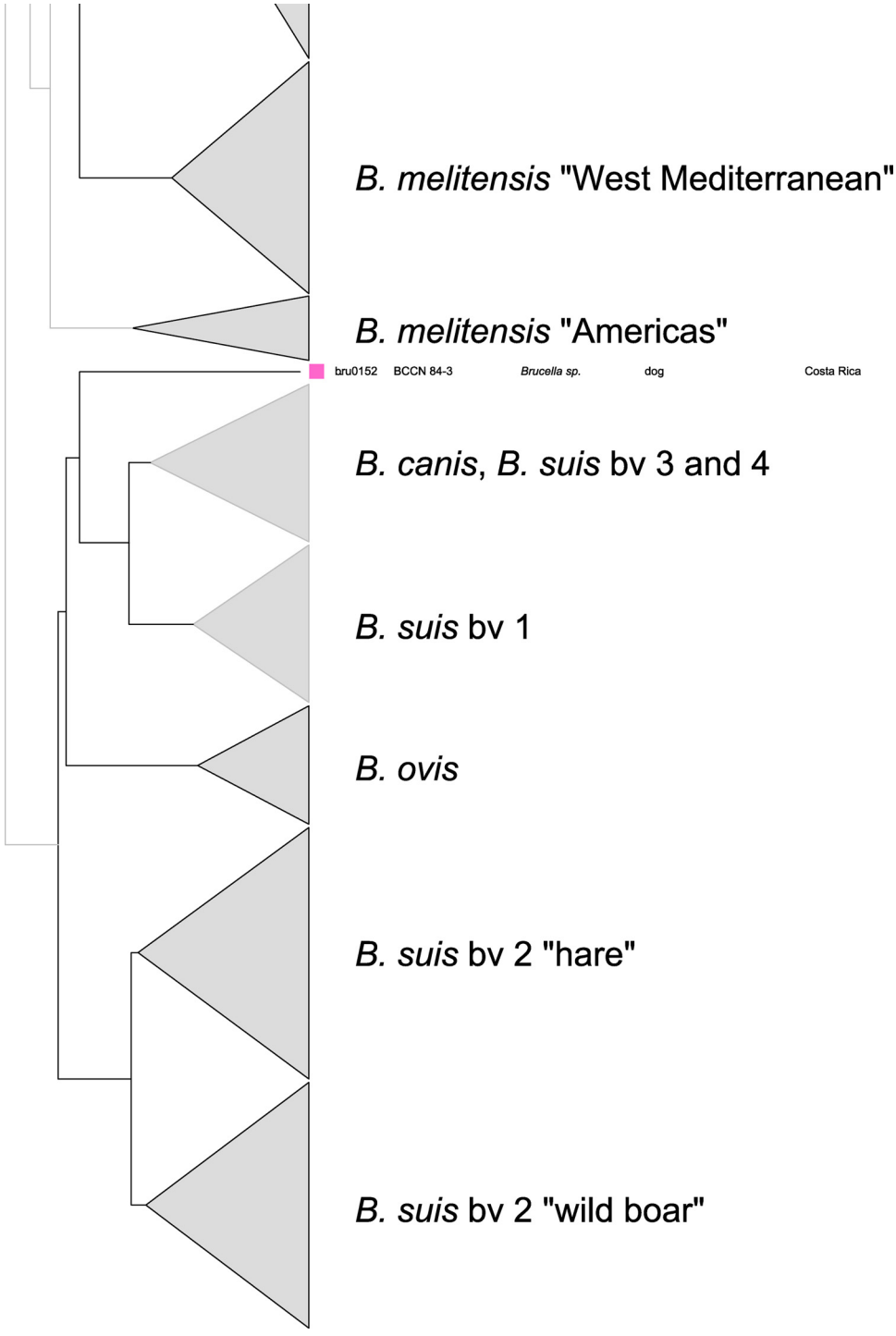

FIG 3 continued

Tb at $10^4\times$ RTD (10^4 times the routine test dilution) but not at the RTD, except for the fox isolates FH 2208, FK 21908, FW 70608, and F 303 Mi, which showed plaques when phage F25 was used at the RTD. Strain F 303 Mi also showed plaques at the RTD of phage Tb. The smooth strains were all lysed by phages BK2, Iz, and Wb at both dilutions, whereas the rough strains of environmental origin were not. In contrast, the rough strains were lysed by phage R/C at $10^4\times$ RTD but not at the RTD, whereas the smooth strains were not lysed at all.

The *B. microti* strains were M antigen dominant except for a single fox isolate. Strain FW 70608 did not agglutinate with monospecific anti-M serum but only with *Brucella* anti-A monospecific serum. The soil isolates (rough strains) agglutinated with anti-A and anti-M, as well as with anti-R, monospecific sera. One of the

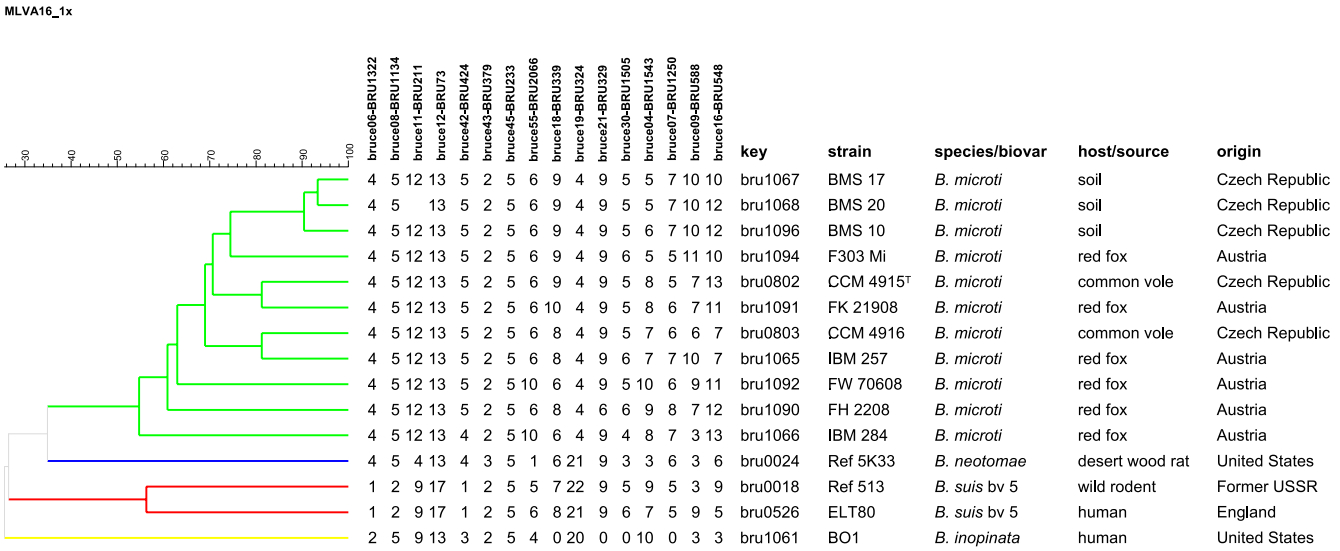

FIG 4 Dendrogram of clustered MLVA-16 genotypes (panels 1 and 2). The 11 *B. microti* isolates were clustered into 11 different genotypes based on the differences in the numbers of repeat units at 16 VNTR loci. Key, DNA batch.

soil strains (BMS 10) revealed both smooth and rough lipopolysaccharide phenotypes. The smooth BMS 10 strain agglutinated only with monospecific anti-M serum.

Independently of the animal or environmental source and of geographical origin, all *B. microti* strains exhibited high biochemical and enzymatic activities (3). The key reactions typical for brucellae, i.e., Glu(pNA)-OH (ENAOH), Pyr-pNA (PYRNA) (consistently negative reactions), and *H*-hydroxyproline-βNA (HP) (consistently strong positive reactions), clearly identified the isolates as members of the genus *Brucella*. Thirteen of the 93 substrates tested in the metabolic activity assay (4 aminopeptidase reactions, 1 monosaccharide, 2 organic acids, 2 amino acids, 1 amino acid derivate, 1 peptide, and 2 classical reactions) showed differing results within the species. Despite the overall similar biochemical profiles among the *B. microti* strains, individual isolates differed markedly in their metabolic activities (Table 2).

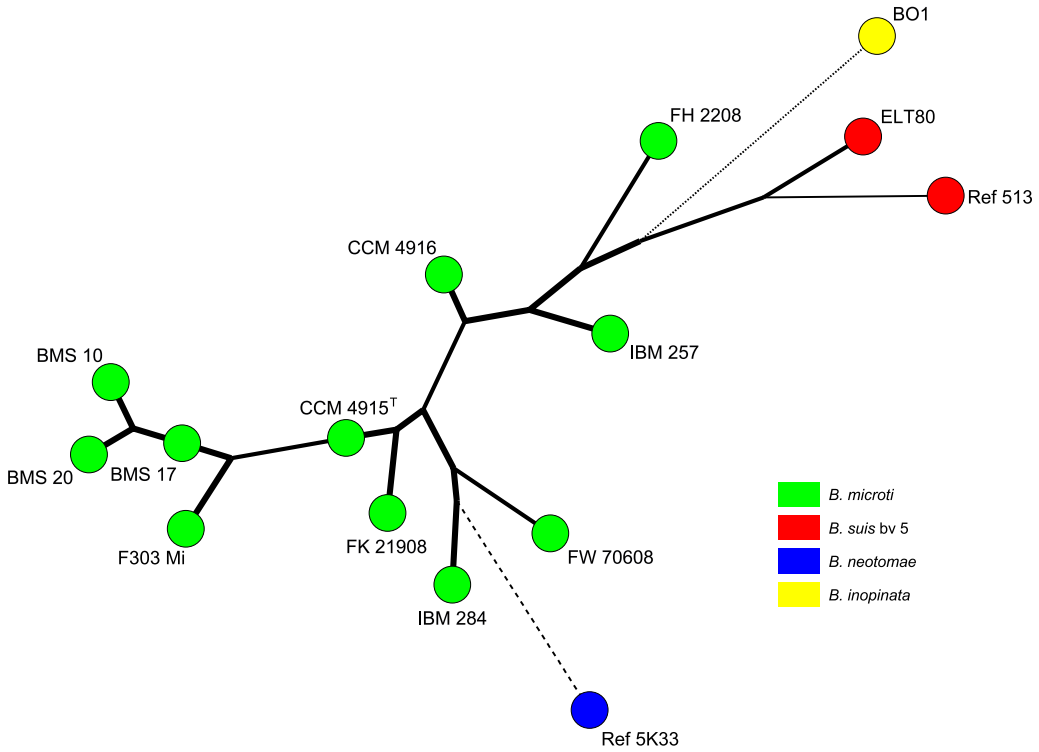

FIG 5 Maximum-parsimony analysis of 11 *B. microti* isolates and the closely related strains *B. neotomae* 5K33, *B. inopinata* BO1, and *B. suis* bv. 5 (513 and ELT80), based on MLVA-16 data. Species are distinguished by different colors.

TABLE 2 Metabolic activities of 11 *Brucella microti* strains[a]

Substrate or reaction	Abbreviation	Substrate class	Activity of the following *Brucella microti* strain: CCM 4915^T	CCM 4916	IBM 284	IBM 257	FH 2208	FK 21908	FW 70608	F 303 Mi	BMS 10	BMS 17	BMS 20
Leu-pNA	LNA	Aminopeptidases with pNA	+	+	++	+	+	++	+	+	+	++	++
Ac-Gly-Lys-β	AcGK	Aminopeptidases with βNA	+	+	−	−	−	−	−	−	−	−	−
Ala-Phe-Pro-Ala-β	AFPA	Aminopeptidases with βNA	+	++	+	+	+	+	+	+	+	+	+
Asn-β	N	Aminopeptidases with βNA	−	+	−	−	−	−	−	−	−	−	−
Ac-Lys-Ala-β	AcKA	Aminopeptidases with βNA	+	+	−	−	−	−	−	−	−	−	−
D-Ala-D-Ala-β	dAdA	Aminopeptidases with βNA	++	+	+	+	+	+	++	+	+	+	+
Val-Tyr-Ser-β	VTS	Aminopeptidases with βNA	++	+	+	−	−	−	+	−	−	+	−
p-Nitrophenyl-α-D-maltoside	aMAL7	Glucosidases	+	++	+	+	+	+	+	+	+	+	+
p-Nitrophenyl-α-D-xylopyranoside	aXYL7	Glucosidases	+	++	+	+	+	+	+	+	+	+	+
D(−)-Ribose	D-RIB	Monosaccharides	+	++	++	++	++	+	++	++	++	+	++
D(−)-Arabinose	D-ARA	Monosaccharides	++	−	++	−	++	++	++	++	++	++	++
D(+)-Xylose	D-XYL	Monosaccharides	+	++	+	++	++	+	++	++	++	+	++
D-Threitol	D-TOL	Sugar derivates	++	++	+	++	++	++	++	+	++	+	++
DL-Lactic acid	DLLac	Organic acids	+	++	+	++	++	+	++	+	+	+	+
L-Asparagine	L-Asn	Amino acids	+	++	+	++	++	++	++	++	++	+	++
L-Glutamic acid	L-Glu	Amino acids	+	++	++	++	++	++	++	++	++	++	++
D-Alanine	D-Ala	Amino acids	++	++	++	++	++	++	++	++	++	−	−
Propionic acid	Propn	Organic acids	−	−	++	++	++	++	++	++	++	−	++
DL-β-Hydroxybutyric acid	βHBut	Organic acids	++	−	++	++	++	++	++	−	++	−	++
Nα-Acetyl-L-arginine	AcArg	Amino acid derivates	++	++	−	++	++	−	++	−	−	−	−
Hippuryl-Arg	HipArg	Peptides	++	++	−	++	++	−	++	−	−	−	−
Glycine-free base	Gly	Amino acids	−	++	++	++	++	++	++	++	++	++	−
Nitrite reduction	NTI	Classical reactions	−	+	+	+	+	++	++	+	++	++	+
Voges-Proskauer	VP	Classical reactions	++	+	+	+	++	++	++	++	++	+	+
Nitrate reduction	NTA	Classical reactions	−	+	+	+	+	++	++	+	++	++	+
Glu(pNA)-OH	**ENAOH**	**Aminopeptidases with pNA**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**
Pyr-pNA	**PYRNA**	**Aminopeptidases with pNA**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**	**−**
***H*-Hydroxyproline-βNA**	**HP**	**Aminopeptidases with βNA**	**++**	**++**	**++**	**++**	**++**	**++**	**++**	**++**	**++**	**++**	**++**

[a] A representative selection of the 93 substances tested in the *Brucella*-specific Micronaut assay has been merged (3). The quality of each biochemical reaction is presented as follows: −, no metabolic activity; +, moderate metabolic activity; ++, strong metabolic activity. *Brucella*-specific traits are shown in boldface, and clearly distinctive features of the isolates are shaded.

DISCUSSION

With the eradication of brucellosis in domestic animals in many parts of the developed world, the impact of wildlife brucellosis becomes increasingly relevant as a potential reservoir of the etiologic agent and a source for the reemergence of this zoonotic disease (2, 15, 16). Although rats and mice are synanthropic species living in close contact with humans and domestic animals, knowledge of the epidemiology of brucellosis in wild rodents and its impact on human health is still limited. There are some historical reports about the isolation of classical brucellae from rodents (32, 35). However, only two rodent-specific *Brucella* species have been described to date. In the 1960s, *B. neotomae* was isolated from desert wood rats (*Neotoma lepida*) in Utah (31), and just recently, *B. microti* was isolated from the common vole (*Microtus arvalis*) (17, 28). The significance of these findings became obvious when additional *Brucella* strains (non-*B. microti*) isolated from wild rodents in North Queensland, Australia (33), revealed notable genetic similarity with two human isolates, strains BO1 (10) and BO2 (34).

The isolation of *B. microti* from different animal species and also from soil (26, 27) in Austria and in the Czech Republic throughout a whole decade proves the endemic persistence of the pathogen within a restricted geographical region. It is possible that enzootic transmission cycles including rodents, carnivores, and

the natural environment maintain the long-term survival of *B. microti* in this region. Soil might be the primary reservoir of infection, but other vectors cannot be excluded. Although the pathogenicity of *B. microti* for livestock and humans has not yet been confirmed, new foci of *Brucella* infections potentially posing a public health threat have to be monitored (24). The natural environment may play a key role in the reemergence of the disease.

To gain deeper insight into the composition of the species *B. microti*, all currently available isolates have been characterized using a comprehensive combined molecular and classical microbiological approach.

The molecular techniques used in this study revealed that *B. microti* is a species with a high degree of genetic relatedness. With respect to one of its closer relatives, *B. suis* 1330, the genome sequence of *B. microti* CCM 4915^T was found to be 99.84% identical in aligned regions. In contrast, the virulence and pathogenicity of *B. microti* are totally different, e.g., intramacrophagic replication proceeds much faster, and the mortality rate in murine models of infection is much higher, than in *B. suis* (18).

The 100% identity of the 16S rRNA gene sequence among the *B. microti* strains tested and with the consensus sequence of the genus is consistent with previous studies of the six classical *Brucella* species (14). 16S rRNA and *recA* sequence analysis, as well as MLSA (based on nine genes: *gap*, *aroA*, *glk*, *dnaK*, *gyrB*, *trpE*, *cobQ*, *omp25*, and *int-hyp*), also revealed a high degree of homology among the Australian rodent strains most recently described (33). However, the 16S rRNA sequence of these strains was unique in the genus *Brucella* (comparable to those of *B. inopinata* [BO1] and strain BO2), showing 99.2% identity to the consensus sequence.

The *recA* sequence in *B. microti* strains was identical to the corresponding consensus sequence of *Brucella* spp. In contrast, *recA* shows high genetic diversity in *Ochrobactrum* spp., which are closely related to members of the genus *Brucella* (30).

Interestingly, *B. microti* and *B. suis* bv. 5 (strain 513), another wild rodent strain, had almost identical *omp2* genes. Hence, the position of strain 513, or, in more general terms, that of bv. 5, within the species *B. suis* currently appears aberrant (38).

Based on MLSA of 21 genetic loci, *B. microti* strains were found to represent a single clone, i.e., only a single sequence type (ST) could be identified, a pattern comparable to those of *B. ovis* and *B. neotomae* (37). However, *B. microti* could be clearly distinguished from the currently known *Brucella* species, although the global alignment of *B. microti* CCM 4915^T and *B. suis* 1330 chromosomes revealed an almost perfect colinearity (5). Because of the wide ecological niche of *B. microti*, intraspecies polymorphisms might have been expected. For the marine mammal brucellae, isolated from different animal species, a much higher level of intragroup diversity, supporting a host-specific classification, has been described (37).

By use of MLVA-16, each *B. microti* strain exhibited a distinct genotype (Fig. 4), mainly due to diverging panel 2B VNTR markers. Since different *Brucella* isolates originating from the same outbreak usually show identical MLVA-16 genotypes (1), the *B. microti* strains under study have to be regarded as a heterogeneous population. IBM 284 and FW 70608 also showed aberrant panel 1 genotypes because of minor differences at the bruce55 and bruce42 loci. In both strains, these deviant genotypes were associated with unusual phenotypic characteristics. IBM 284 rapidly developed brownish colonies, and FW 70608 agglutinated with monospecific anti-A serum but not with anti-M serum, in contrast to all the other *B. microti* strains. A similar association of VNTR genotypes with phenotypic features has been described for fuchsin-sensitive *B. melitensis* strains (1).

Both molecular typing and biotyping are essential for full understanding of the population structure of the genus *Brucella* and the intraspecies variability of its closely related members. In the *B. microti* population, genotypic and phenotypic features diverged. The minimal genetic differences found in *B. microti* strains do not readily account for the differences in phenotype and host specificity or habitat. Furthermore, the pathogenicity of *B. microti* differs significantly from that of other *Brucella* species (18). *B. microti* showed an enhanced capacity for intramacrophagic replication and exhibited a highly lethal, septicemia-like course of infection in murine models. The resistance of *B. microti* to acidic pHs might explain its ability to survive in soil (18). In contrast to most of the other *Brucella* species currently known, *B. microti* is a fast-growing and metabolically very active microorganism (3). This metabolic activity is a feature shared by all *B. microti* strains with their closest phylogenetic relative, *Ochrobactrum*. That is the reason why brucellae, including *B. microti*, are commonly misidentified as *Ochrobactrum* species by commercially available identification systems, such as the API 20 NE system (11, 17). Mechanisms involving gene regulation and altered expression may have contributed to the adaptation of the bacteria to environmental stress, and the differing metabolic activities of the isolates may mirror such regulatory processes. The outstanding metabolic activity of *B. microti* compared to that of other members of the genus also justified its designation as a distinct species (3, 28).

In summary, the competence of *B. microti* to adapt its lifestyle so as to survive in mammals and in soil suggests that *B. microti* represents an ancestral *Brucella* species whose phenotype is more closely related to that of the soil-associated facultative human pathogen *Ochrobactrum* than to that of the classical *Brucella* species. It has been suggested that the classical *Brucella* species evolve intracellularly as isolated units in their preferred hosts, with recombination restricted by ecological isolation (23). In contrast, the ecological niche of *B. microti* in soil theoretically allows many interactions with bacteria of other genera, facilitating horizontal gene transfer. On the basis of the genetic markers investigated, however, the *B. microti* isolates under study revealed a clonal population structure. Since the number of *B. microti* isolates characterized in this study was low and their geographical distribution was rather limited, further surveillance on a global scale is urgently needed in order to obtain a deeper understanding of distribution, ecology, zoonotic potential, genomic organization, and relatedness to other rodent strains.

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