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Region of Difference 4 in Alpine *Mycobacterium caprae* Isolates Indicates Three Variants

Janina Domogalla,[a] Wolfgang M. Prodinger,[b] Helmut Blum,[c] Stefan Krebs,[c] Susanne Gellert,[a] Matthias Müller,[a] Erdmute Neuendorf,[a] Florian Sedlmaier,[a] Mathias Büttner[a]

Bavarian Health and Food Safety Authority, Oberschleissheim, Germany[a]; Division of Hygiene and Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria[b]; Laboratory for Functional Genome Analysis (LAFUGA), Gene Center, Ludwig-Maximilians-University Munich, Munich, Germany[c]

The lack of complete genome sequence information for *Mycobacterium caprae* complicates a robust differentiation of *M. caprae* and *Mycobacterium bovis*. In this study, the presence or absence of *M. caprae*-specific single nucleotide polymorphisms in *lepA* and *gyrB* genes was assessed. The region of difference 4 (RD4) was analyzed for the identification and characterization of *M. caprae*. Molecular characteristics were evaluated in 12 recent *M. caprae* isolates from livestock and wildlife collected over a 3-year period in Bavaria, Germany. Conventional PCR strategies, sequence analysis of PCR fragments, and data from a next-generation sequencing approach together with variable-number tandem-repeat genotyping were utilized. Single nucleotide polymorphisms in the *lepA* and *gyrB* genes indicating the presence of *M. caprae* were detected in all the isolates. At least three different RD4 variants were found for Alpine *M. caprae* isolates. The results demonstrate that the RD4 region is rather heterogeneous in *M. caprae* genomes. As assumed by others, the presence of RD4 is critical for PCR-based differentiation of *M. caprae* from *M. bovis*, but in addition, the observed variability of RD4 allows the identification of *M. caprae* genotypes and may be indicative of a geographical-type appearance.

Mycobacterium caprae was first described in samples from goats in Spain (1) and was established in 2003 as a unique species in the *Mycobacterium tuberculosis* complex (MTC) (2) based on the presence or absence of regions of difference (RDs) and single nucleotide polymorphisms (SNPs) (3). *M. bovis* and *M. caprae* are the most relevant pathogens for livestock and wildlife large animal hosts. In some countries bovine tuberculosis (TB) is still a problem, particularly when a wildlife reservoir exists and TB transmission from wildlife to cattle and cattle to wildlife on the pasture is a permanent danger (4). Three wildlife reservoirs of TB caused by *M. bovis* have been described extensively (4–7). To date a wildlife reservoir harboring *M. caprae* has not been described. However, there have been several reports about *M. caprae* infections in red deer (*Cervus elaphus*) (8–12) and wild boars (*Sus scrofa*) (8, 9, 13) from continental European countries, mainly from Spain (8, 9) and from countries with Alpine wildlife habitats (10–13).

During the years 2007 and 2012 in the Bavarian Alpine "Allgäu" region, an area with plenty of cattle summer pastures also habituated by red deer, infections in cattle have been found to be mainly caused by *M. caprae*, whereas *M. bovis* was more frequent among animals found in the northern part of Germany, according to international unpublished findings of the national animal infectious disease reporting system, TierSeuchenNachrichten 3.0 (TSN 3.0).

The majority of molecular strategies for *M. caprae* differentiation refer to sporadically examined *M. caprae* isolates (3, 14, 15). In addition, a complete genome sequence of *M. caprae* is not available in the NCBI GenBank so far, in contrast to genome sequences of *M. tuberculosis* and *M. bovis*. Comparative genomic studies with the complete DNA sequence of *M. tuberculosis* strain H37Rv have identified several *Mycobacterium tuberculosis*-specific genomic RDs which have been deleted in most other members of the MTC (14, 15). *M. caprae* isolates displayed the same RD deletions as *M. bovis* strains except for RD4, a 12.7-kb-spanning genomic region that is absent in *M. bovis* but present in *M. caprae* (14). However, to our knowledge, only very few *M. caprae* isolates, including most isolates originating from Spain, have been tested with regard to the presence of the complete RD4 sequence; 4 *M. caprae* isolates originate from Spain (14, 16) and 10 isolates are without detailed origin information (15). Rodriguez et al. (8) confirmed the existence of 545-bp RD4-specific sequences in 62 of 63 *M. caprae* isolates. Notably, the detection of an RD4 deletion has been used as a major criterion for distinguishing *M. bovis* from the other members of the MTC, especially from *M. caprae* (16–21).

One *M. caprae*-specific T-to-G substitution at position 1311 in the *gyrB* sequence has been described in several *M. caprae* isolates from central Europe (12, 22, 23) and Spain (3, 8). However, Kubica et al. (24) reported that 3 of 55 German *M. caprae* isolates from human patients did not show this *M. caprae*-specific SNP. To date, the only commercially available DNA strip-based assay for the differentiation of MTC species, the GenoType MTBC line probe assay (Hain Lifescience GmbH, Nehren, Germany), takes advantage of this SNP to differentiate *M. caprae* from other members of the MTC (25).

Reddigton et al. (26) described a novel *M. caprae*-specific C-to-T substitution at position 690 in the *M. tuberculosis* H37Rv *lepA* gene, which was conserved in all five tested *M. caprae* isolates originating from Germany and the Netherlands.

However, little is known about the genomic features of *M.*

Received 15 November 2012 Returned for modification 12 December 2012
Accepted 23 January 2013
Published ahead of print 13 February 2013
Address correspondence to Janina Domogalla, janina.domogalla@lgl.bayern.de.

doi:10.1128/JCM.02966-12

TABLE 1 Origins of *Mycobacterium* DNA samples

Species	Isolate identification no.	Host, yr of origin[a]
M. caprae	09/RW044	Red deer isolate, 2009
M. caprae	10/RW314	Red deer isolate, 2010
M. caprae	10/RW079	Red deer isolate, 2010
M. caprae	11/D028	Red deer isolate, 2011[b]
M. caprae	11/D133	Red deer isolate, 2011[b]
M. caprae	11/D102	Red deer isolate, 2011[b]
M. caprae	11/Roe010	Roe deer isolate, 2011[b]
M. caprae	V-09-425	Cattle isolate, 2009
M. caprae	D6431	Cattle isolate, 2009
M. caprae	11/Rd152461	Cattle isolate, 2011
M. caprae	12/Rd52799	Cattle isolate, 2012
M. caprae	V-09-274	Sheep isolate, 2009
Other MTC members		
M. tuberculosis	ATCC 25177	NA
M. africanum	TT70/03	Nilgai isolate, 2004
M. bovis	V207	Cattle isolate, 2009
M. bovis	DSM 43990	NA
M. bovis BCG	ATCC 27289	NA
NTM members		
M. arupense	V-09-1049	Red deer isolate, 2009
M. bohemicum	V-09-1241	Red deer isolate, 2009
M. vanbaalenii	RW444	Red deer isolate, 2010
M. nonchromogenicum	RW712	Red deer isolate, 2010
M. interjectum	RW871	Red deer isolate, 2010
M. avium subsp. *paratuberculosis*	10016571500101	Cattle isolate, 2010
M. avium subsp. *paratuberculosis*	10016792400101	Cattle isolate, 2010

[a] All the isolates originated in Bavaria, Germany. NA, not applicable.
[b] Isolates were obtained during the Tuberculosis in Alpine Wildlife project, carried out by 17 partners of the four Alpine countries (Austria, Germany, Switzerland, and Italy) and funded by their respective national research funding organizations and supported by the EMIDA ERA-Net (http://tb-alpine-wildlife.org).

caprae concerning markers for genotyping. Rapid and robust diagnostic differentiation of *M. caprae* is complicated by this fact.

We used different PCR protocols to determine whether genetic characteristics can be found to accurately differentiate *M. caprae* from the other MTC members. These PCR protocols were sequence analysis of PCR fragments and two standard PCR-based genotyping methods, spoligotyping (27) and mycobacterial interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR) typing (28, 29). We assessed the presence or absence of *M. caprae*-specific SNPs in *lepA*, *gyrB* gene, and RD4 sequences in the genomes of 12 recent Alpine *M. caprae* isolates from livestock and wildlife. In addition, we compared the results of a next-generation sequencing approach used to analyze DNA from seven *M. caprae* isolates. PCR and whole-genome sequencing revealed that three different RD4 variations exist for *M. caprae* isolates originating from delimited Alpine regions of Bavaria.

MATERIALS AND METHODS

Bacterial strains and DNA preparation. A total of 12 *M. caprae* isolates from domestic and wildlife hosts in different parts of Bavaria, Germany, were analyzed (Table 1). For standardization of PCR setups, five isolates of MTC and seven isolates from nontuberculosis mycobacteria (NTM) species were used (Table 1). Clinical isolates were characterized using GenoType MTBC, GenoType mycobacterium AS, and GenoType mycobacterium CS (all from Hain Lifescience GmbH). The DNA of the NTM strains was obtained from the strain collection of the Bavarian Health and Food Safety Authority, Oberschleissheim, Germany.

MTC isolates were grown in liquid medium (Middlebrook 7H9 broth; Becton Dickson, Heidelberg, Germany) supplemented with 800 μl polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin (BBL MGIT PANTA) (Becton Dickinson) and were incubated at 37°C for 4 weeks. After two washing steps and heat inactivation (30 min at 95°C), genomic DNA from MTC isolates for PCR and whole-genome sequencing was extracted from 1.5 ml liquid culture using the cetyltrimethylammonium bromide method and purification with phenol-chloroform. DNA concentrations were determined with a Nanodrop ND-1000 device (Thermo Fisher Scientific, Schwerte, Germany).

Molecular typing. Two PCR-based genotyping methods were applied for the molecular characterization of *M. caprae* isolates, spoligotyping (27) and MIRU-VNTR typing (28, 29). The latter determines the copy numbers in 24 or 25 VNTR loci and is regarded as the actual gold standard for comparison of MTC strains (29). DNA for PCR amplification was obtained by the boiled-prep method (11). Spoligotyping was performed as described by Kamerbeek et al. (27). MIRU-VNTR typing was carried out using single PCRs for each locus and DNA fragments were analyzed by gel electrophoresis, as described earlier (11). Fingerprint patterns were digitally compared using BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium).

Whole-genome sequencing. Seven isolates of *M. caprae* were sequenced on a MiSeq-Illumina instrument with paired-end sequencing and a read length of 150 bp in each direction. For the isolates 09/RW044, 10/RW079, 11/D028, and 11/D102, sequencing libraries were prepared from 100 ng of fragmented DNA sonicated with a Bioruptor (NextGen, Diagenode, Liege, Belgium) (for 25 cycles with 30 s on/30 s off) and the Rapid Library preparation kit (NuGen Inc., San Carlos, CA) and were amplified by 6 cycles of PCR with Illumina P5 and P7 primers (P5, AAT GATACGGCGACCACCGA; P7, CAAGCAGAAGACGGCATACGA). After the PCR step, libraries were purified with AMPure XP beads (Beckman Coulter, Brea, CA) and quantified with a Bioanalyzer (Agilent, Santa Clara, CA). From the isolates 11/Rd152461, 12/Rd52759, and 11/Roe010, respectively, 1 ng of AMPure XP-purified DNA was used to prepare fragmented libraries with the XT kit (Illumina, San Diego, CA) according to the manufacturer's instructions.

The raw sequencing data were imported to a locally installed instance of the Galaxy platform (30). Raw reads were demultiplexed, adapter and quality trimmed, and mapped to the *M. tuberculosis* reference genome H37Rv (GenBank database, accession number CP003248) with the program BWA (31). Regions of interest were inspected on the Integrative genomics viewer (IGV), a lightweight visualization tool that enables intuitive real-time exploration of diverse, large-scale genomic data sets on standard desktop computers (32) in order to find large deletions.

Primer and probe design. The primers and the probe are listed in Table 2. Nucleotide sequence data for the primers and the probe were deduced from various reports (14, 16, 18, 26, 33) or were generated in this study using the NCBI/Primer-BLAST program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) based on the sequences derived from whole-genome sequencing analysis.

All primers and probes were purchased from Primer-Management (Eurofins MWG Operon, Ebersberg, Germany).

Real-time PCR. The reactions were run in a Stratagene Mx3005P real-time thermocycler (Agilent Technologies, Waldbronn, Germany). Thermocycling, fluorescent data collection, and data analysis were performed with MxProTM quantitative PCR (qPCR) software (Agilent Technologies).

***lepA*-based species-specific real-time PCR for *M. caprae*.** Each reaction consisted of 1 U *Taq* DNA polymerase recombinant (Invitrogen, Life Technologies, Darmstadt, Germany), 1× PCR buffer, 5 mM $MgCl_2$, 1 mM deoxynucleoside triphosphate (dNTP), 450 nM forward (MTC_Fw)

TABLE 2 Probe and primers for *lepA* specific real-time PCR and primers for PCR detection of the *gyrB* gene and RD4 region

Primer	PCR type	Nucleotide sequence (5′ to 3′)	Reference no.
MTC_Fw	*lepA*-based species specific	AGA CCG TGC GGA TCT TG	26
MTC_Rv	*lepA*-based species specific	CAT GGA GAT CAC CCG TGA	26
M. caprae-specific probe	*lepA*-based species specific	FAM TAT CGG GTA CAC AAA GAC GA-BHQ1	26
MTUBf	*gyrB*-based species specific	TCG GAC GCG TAT GCG ATA TC	33
MTUBr	*gyrB*-based species specific	ACA TAC AGT TCG GAC TTG CG	33
RD4F	RD4 internal left part	CCA CGA CTA TGA CTA GGA CAG CAA	18
RD4R	RD4 internal left part	AAG AAC TAT CAA TCG GGC AAG ATC	18
Rv1510F	RD4 internal central part	GTG CGC TCC ACC CAA ATA GTT GC	16
Rv1510R	RD4 internal central part	TGT CGA CCT GGG GCA CAA ATC AGT C	16
RD4-flankF	RD4 5-kb deletion flanking PCR	CTC GTC GAA GGC CAC TAA AG	14
RD4-5kbDelR	RD4 5-kb deletion flanking PCR	TTC GTT AGC CGC ACA TCC ATG A	
RD4-DelF	RD4 38-kb deletion flanking PCR	GAA GGA AGC GGC TGC GAT TGG TGC	
RD4-DelR	RD4 38-kb deletion flanking PCR	TGG TTA CAC GCT CGG CGC AGT CA	

and reverse (MTC_Rv) primers, 125 nM *M. caprae*-specific probe, 0.5 μl of internal control (IC)-primer-probe mix (2.5 pmol primer/μl plus 1.25 pmol probe/μl), 1 μl ruminant IC DNA sample (200 ng/μl), 5 μl of template (0.1 to 0.3 ng/μl), and RNase-free water to a final volume of 25 μl. The IC for the detection of the beta-actin mRNA was added to each setup in order to assess *Taq* polymerase inhibition (34). The cycling parameters were: 5 min incubation at 95°C and 45 cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 30 s.

Conventional PCR. PCR amplifications were performed in a T3000 thermocycler (Biometra, Goettingen, Germany). The PCR-amplified DNA fragments were separated by electrophoresis in 0.8 to 2% agarose gels and visualized under UV after ethidium bromide staining.

***lepA*-based species-specific conventional PCR for the MTC.** The PCR mixture was prepared with 0.05 U of HotStarTaq DNA polymerase (Qiagen, Hilden, Germany), 1× reaction buffer, 1.25 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM each primer (MTC_Fw/MTC_Rv), 1 μl template, and RNase-free water to 50 μl per reaction to amplify a 155-bp *lepA* fragment. The cycling parameters were denaturation for 15 min at 95°C, 35 cycles of 95°C for 1 min, 60°C for 30 s, and 72°C for 30 s and a final elongation step of 10 min at 72°C (program 1).

***gyrB*-based species-specific PCR for the MTC.** Primers MTUBf/MTUBr were used to amplify a 1,020-bp *gyrB* fragment that comprises the discriminative regions from six *M. caprae* isolates. The PCR mixture contained 0.05 U of Phusion high-fidelity DNA polymerase (Biozym Scientific GmbH, Hessisch Oldendorf, Germany), 1× reaction buffer, 0.2 mM dNTP, 0.5 μM each primer, 1 μl template, and RNase-free water to 50 μl. The cycling parameters were denaturation for 30 s at 98°C, 35 cycles of 10 s at 98°C and 30 s at 72°C, and a final elongation step of 10 min at 72°C.

RD4-based species-specific PCR for MTC detection. Primers chosen to amplify an internal fragment of RD4 (Fig. 1) were used in two PCRs with DNA from all *M. caprae* isolates. The two fragments that were amplified applied to the left and central parts of RD4 of the *M. tuberculosis* H37Rv genome in the 5′-to-3′ direction.

For the RD4 internal left part, an 88-bp fragment of the left part of RD4 (Fig. 1) was amplified in a 50-μl reaction mixture containing 0.05 U of HotStarTaq DNA polymerase (Qiagen), 1× reaction buffer, 1 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM each primer (RD4F/RD4R), 1 μl template, and RNase-free water (PCR mixture 1). The cycling parameters were those for program 1 but with an annealing of 55°C for 30 s.

For the RD4 internal central part, a 1,031-bp fragment of the central part of RD4 (Fig. 1.) was amplified using PCR mixture 1 but with 1× Q solution and primer Rv1510F/Rv1510R. The cycling parameters were those for program 1 but with an annealing of 60°C for 1 min and an extension step of 72°C for 1 min.

RD4 5-kb deletion-flanking PCR. Previous results from contig sequences in the next-generation sequencing approach suggested a 5-kb deletion present in the left part of the RD4 sequence in some *M. caprae* genomes. Hence, flanking primers specific to the deletion were chosen for a PCR setup with DNA from the *M. caprae* isolates (Fig. 1). To amplify a calculated DNA fragment of approximately 700 bp, we used PCR mixture 1 but with 1× Q solution and primer (RD4-flankF/RD4-5kbDelR). Instead of dGTP, 7-deaza-2′-deoxy-GTP (Roche, Mannheim, Germany) for GC-rich templates was used. The cycling parameters were program 1 but with an annealing step of 55°C for 1 min and an extension step of 72°C for 1 min.

In addition, PCR mixture 1 but with 1× Q solution and primer (RD4-flankF/Rv1510R) was used for amplification of a 2.5-kb deletion-flanking fragment from the *M. caprae* isolates 11/D028 and 10/RW079. The cycling parameters were program 1 but with an annealing step of 55°C for 1 min and an extension step of 72°C for 2 min.

For amplification of the 6.9-kb DNA fragment that comprised the left and central parts of the RD4 sequence in *M. caprae* isolates without deletion, the PCR master mix contained 0.05 U of Phusion high-fidelity DNA polymerase (Biozym Scientific, GmbH), 1× GC buffer, 0.2 mM dNTP, 0.5 μM each primer (RD4-flankF/Rv1510R), 1.5 μl dimethyl sulfoxide (DMSO), 1 μl template, and RNase-free water to 50 μl. The cycling parameters were denaturation for 1 min at 98°C followed by 35 cycles of 10 s at 98°C, 30 s at 65°C, and 260 s at 72°C and a final elongation step of 10 min at 72°C.

RD4 38-kb deletion-flanking PCR. The results of next-generation sequencing suggested that a 38-kb deletion spanning the whole RD4 region might be present in some *M. caprae* genomes (Fig. 1). Hence, flanking primers adjacent to the suspected deletion were designed for amplification of a small approximately 250-bp fragment representing flanking sequences. PCR mixture 1 was used but with 1× Q solution and primer (PDelF/PDelR). The cycling parameters were program 1 but with an annealing step of 55°C for 1 min and an extension step of 72°C for 1 min.

Sequencing and sequence analysis. DNA fragments were prepared for sequencing by the QIAquick gel extraction kit (Qiagen) and sequencing was performed by Eurofins MWG Operon. The obtained sequences were compared with each other and with genomic sequences of members of the MTC, available from the genome database of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genome/browse/). The sequence data were aligned by using the Clustal W2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (35).

Nucleotide sequence accession numbers. Sequence reads obtained from genomic sequencing of the *M. caprae* isolates RW/044, RW/079, 11/D028, and 11/D102 have been deposited in SRA (http://trace.ncbi.nlm.nih.gov/Traces/sra/). Bam files of these isolates mapped to H37Rv are available under the accession numbers SRS386245, SRS386249, SRS386254, and SRS386255. The obtained PCR sequences of the 5-kb and 38-kb deletion-flanking regions and the fragment of the *gyrB* gene amplification were deposited at NCBI GenBank under the accession numbers listed in Table 3.

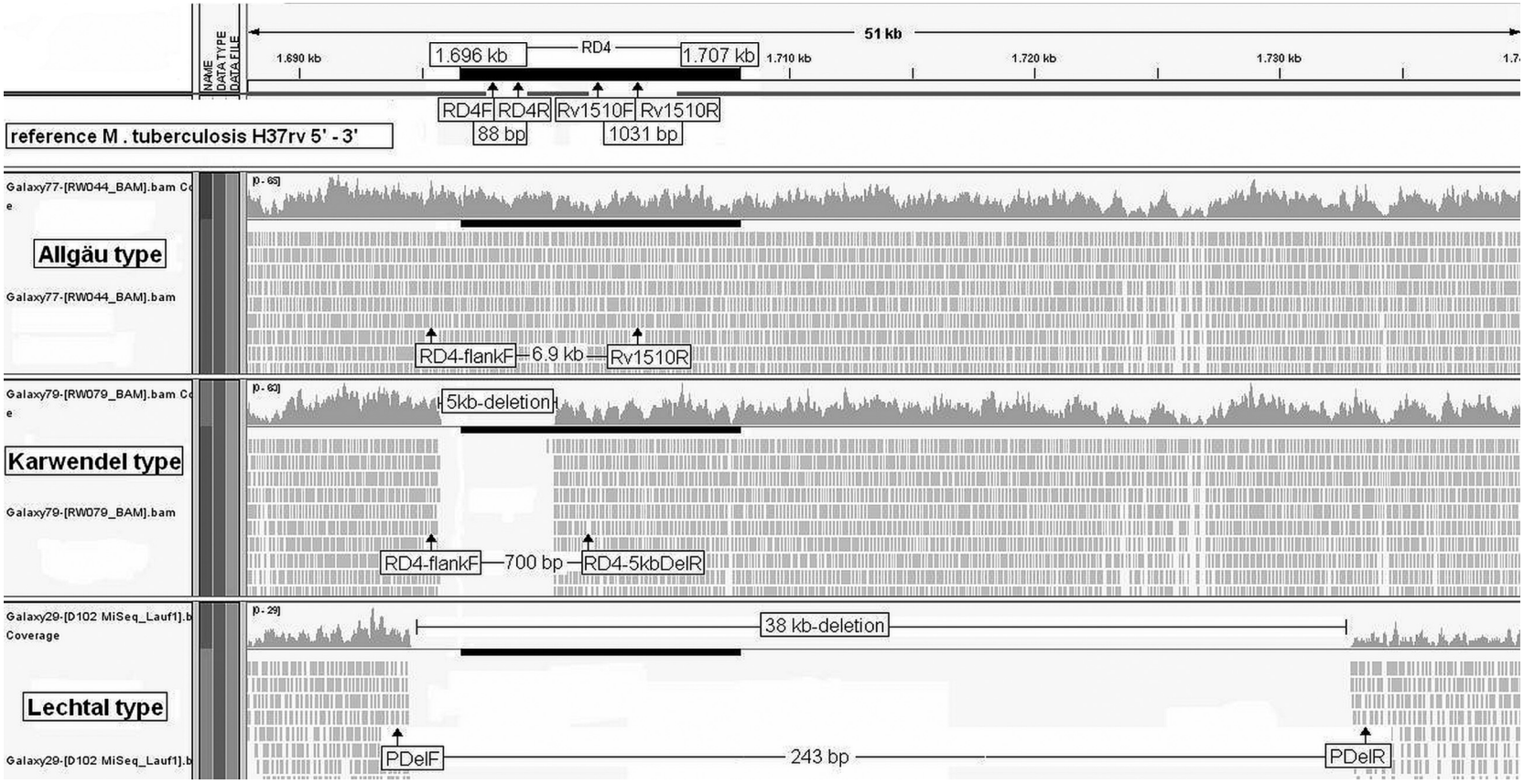

FIG 1 Screenshot of the mapping of the *M. caprae* whole-genome sequencing data to the *M. tuberculosis* reference genome. RD4 nucleotide sequence variations for *M. caprae* isolates from red deer living in the Alpine region of Bavaria, Germany, are shown. The graph was constructed by the Integrative Genomics Viewer (IGV) using Illumina MiSeq paired-end reads of isolate numbers 09/RW044, 10/RW079, and 11/D102. RD4 is represented as a bold black line. The gray histograms indicate the sequence coverage, and the small gray bars indicate individual reads mapped to the corresponding genomic positions. Absence of coverage indicates the respective deletions (marked by solid lines in the histograms) in the RD4 region that were confirmed by PCR with primers (positions shown by boxes and arrows) flanking the deletions and yielding product sizes (shown between the arrows) consistent with the positions and sizes of the respective deletions.

TABLE 3 RD4 mapping and genotypes of 12 representative *M. caprae* isolates from southern Bavaria and accession numbers of amplified *gyrB* and RD4 deletion-adjacent sequences

Isolate no.	PCR result		Accession no.		Genotyping		
	RD4 internal left portion	RD4 internal central portion	Amplified *gyrB* sequence[a]	RD4 deletion-adjacent sequence	MIRU-VNTR code[b]	Spoligotype[c]	Type
11/D028	−	+		[d]	**2**3642**1**2535222534133**3**63**2**2	SB2174	Karwendel
10/RW079	−	+	JX420304	KC007398[d,e]	**2**3642**1**2535222534133**3**63**2**2	SB2174	Karwendel
V-09-425	−	+		[d]	**2**3642**1**2535222534133**3**63**2**2	SB2174	Karwendel
11/D133	−	+		[d]	**2**3642**1**2535222534133**3**63**2**2	SB2174	Karwendel
V-09-274	−	−		JX420311	22642424332245341325533 23	SB0418	Lechtal
11/D102	−	−	JX308291	JX420308	22642424332245341325533 23	SB0418	Lechtal
11/Roe010	−	−	JX420306	JX420309	22642424332245341325533 23	SB0418	Lechtal
12/Rd52799	−	−	JX420307	JX420310	22642424332245341325533 23	SB0418	Lechtal
09/RW044	+	+	JX420305		**2**3**5**42425**3**42245341**2**2**2**43**2**2	SB0418	Allgäu
10/RW314	+	+	JX420303		**2**3**5**42425**3**42245341**2**2**2**43**2**2	SB0418	Allgäu
11/Rd152461	+	+			**2**3**5**42425**3**42245341**2**2**2**43**2**2	SB0418	Allgäu
D6431	+	+			**2**3**5**42425**3**42245341**2**2**2**43**2**2	SB0418	Allgäu

[a] In addition to the GenoType MTBC assay, sequences provide information about the SNPs in the *gyrB* gene.
[b] Copy numbers in loci MIRU2-4-10-16-20-23-24-26-27-31-39-40 and VNTR424-577-1982-2401-3690-4156-1955-2163b-2165-2347-2461-3171-4052. Copy numbers differing from the Lechtal genotype are in bold type.
[c] According to www.mbovis.org.
[d] A 700-bp fragment was obtained, but the nucleotide sequence could not be determined because of high guanine-cytosine content.
[e] Results from contig sequences in the next-generation sequencing of the 2.5-kb PCR approach produced a 2,224-bp fragment spanning the 5-kb deletion.

RESULTS

SNP analysis. The *M. caprae*-specific *gyrB* gene SNPs were detected using GenoType MTBC (Hain Lifescience GmbH) in all 12 *M. caprae* isolates. Additionally, these SNPs of the *gyrB* gene (22) were confirmed by the determined nucleotide sequences of six *M. caprae* isolates (Table 3).

Using real-time PCR with an *M. caprae*-specific probe (26), we identified the *M. caprae* characteristic C-to-T substitution at position 690 of the *lepA* gene in all 12 isolates. Sequences indicative of other members of the MTC and NTM, respectively, were not detected. A 155-bp PCR fragment of the *lepA* gene was obtained from all MTBC members in a conventional PCR setup, but not from NTM members. Hence the data confirm that the *M. caprae*-specific probe binding within the *lepA* gene allows the identification of *M. caprae* isolates.

RD4 and genotyping analysis. A MIRU-VNTR and spoligotyping code was obtained for *M. caprae* samples (Table 3), and two RD4-based PCR setups were chosen to amplify an internal fragment of the left and central parts in the RD4 sequence. The results of the cluster analysis by MIRU-VNTR genotyping and the RD4-based PCR setups allowed a discrimination of three groups of *M. caprae* isolates (Table 3). The geographic origin of the *M. caprae* isolates supported the diversity of genotyping and RD4 pattern results (Fig. 2). According to the RD4 differences and the MIRU-VNTR code, we chose the origin-related designations Allgäu, Lechtal, and Karwendel types for the three groups of our Alpine *M. caprae* isolates (Table 3).

The complete sequence of RD4 proved to be conserved in the Alpine *M. caprae* isolates of the Allgäu type (Fig. 1). The results of analysis of the next-generation sequencing data sets of eight *M. caprae* isolates performed using the program IGV suggested the presence of two different deletions of 5 kb in the genome of the Karwendel type and of 38 kb in the genome of the Lechtal type (Fig. 1). To confirm this finding, we designed flanking primers specific for deletion-adjacent sequences.

The sequence analysis of amplified deletion-flanking fragments showed that the 38-kb deletion includes the nucleotide sequences from position 1.694595 to 1.732924 in the *M. tuberculosis* H37Rv genome encompassing the whole RD4.

Using flanking primers specific for the 5-kb deletion, we obtained an approximately 700-bp PCR fragment for all *M. caprae* isolates of the Karwendel type. The nucleotide sequence of the PCR fragment could not be determined because of a high guanine-cytosine content. However, results from contig sequences in the Illumina sequencing of the 2.5-kb PCR approach of isolate 10/RW079 resulted in a 2,224-bp fragment spanning the 5-kb deletion. In this fragment, the 5-kb deletion contains the sequence stretch from position 1.695834 to 1.700422 spanning the left part of RD4. Of particular interest is the finding that the deletion is located 188 bp upstream of the previously assumed RD4 deletion in the genome of *M. bovis* subsp. *bovis* strain AF2122/97 (14, 21, 36). Notably, these genomic characteristics of the Allgäu, Lechtal, and Karwendel types isolated from red deer were also present in isolates from cattle from the respective geographic regions (Table 2; Fig. 2).

DISCUSSION

Twelve *M. caprae* isolates originating from wildlife and livestock in the Bavarian Alpine region of Germany were evaluated for genomic traits. The presence or absence of *M. caprae*-specific SNPs in the *lepA* and *gyrB* genes and of RD4-specific sequence peculiarities were assessed for identification and characterization of the *M. caprae* isolates.

The *M. caprae*-specific T-to-G substitution at position 1331 of the *gyrB* sequence and the C-to-T substitution at position 690 of the *lepA* sequence were confirmed to be conserved in all tested *M. caprae* isolates. The presence of these single-nucleotide changes was shown to be a stable marker in our Alpine *M. caprae* isolates as well as for *M. caprae* infections of different host species. However, it remains to be tested whether the sensitivity of the PCR is sufficient when DNA is prepared directly from native tissue samples.

Interestingly, three different RD4 variations were found in Ba-

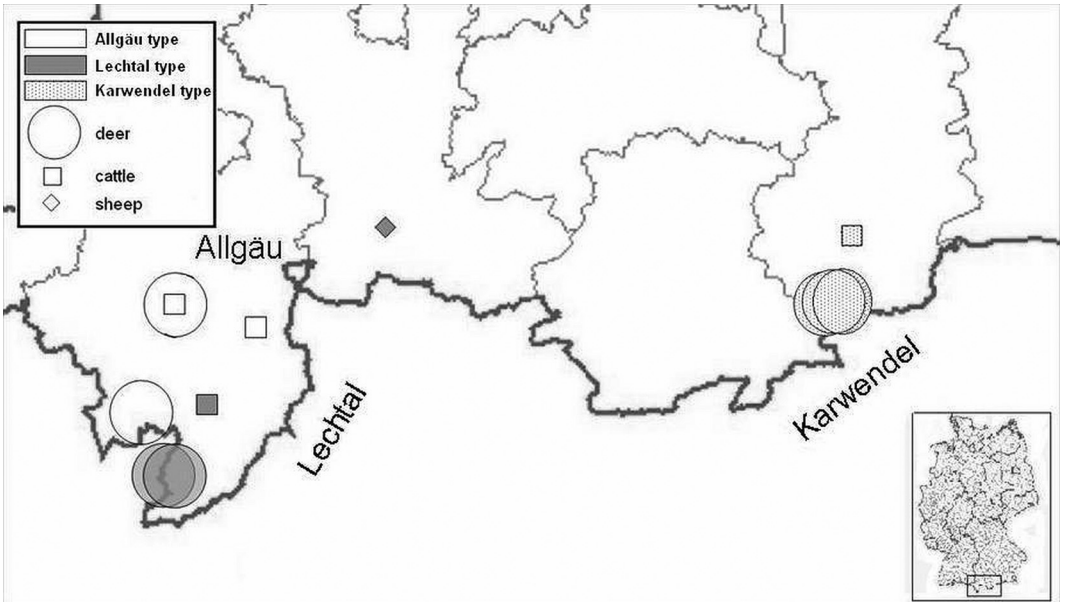

FIG 2 Geographic distribution of the three types of Bavarian *M. caprae* isolates included in this study. Each symbol indicates the host species. The *M. caprae* types are represented by shading, and the number of symbols reflects the number of tuberculosis cases. (Primary artwork reproduced with permission of TSN 3.0.)

varian Alpine *M. caprae* isolates which corresponded with their geographic origins and genotypes (Fig. 1 and Fig. 2). The absence of RD4 has previously been described as a marker for *M. bovis* (14–21). However, only a few *M. caprae* isolates have been tested so far with refined sequence analysis of RD4, and most isolates originated from Spain (14–16). Rodríguez et al. (8) verified the existence of RD4-specific sequences in 62 Spanish *M. caprae* isolates without giving detailed information about the primers used. Hence it is unknown which part of RD4 was detected and it has not been confirmed that the whole RD4 sequence is conserved. The results of our study demonstrate that RD4 is not uniformly present in Alpine *M. caprae* genomes as assumed to date. Due to this heterogeneity, RD4 cannot be recommended for use in effective differentiation of *M. caprae* from *M. bovis*. It cannot be excluded that further genomic deletions may be found in other *M. caprae* isolates.

The 5-kb deletion in the Karwendel type includes the genes Rv1505c to Rv1509, whereas the 38-kb deletion in the Lechtal type has led to the loss of the genes Rv1504c to Rv1531. This finding is of particular interest because RD4 is predicted to encode proteins involved in membrane degradation (Rv1508c) and exopolysaccharide synthesis (Rv1516c) (37). In addition, Al-Khodari et al. (38) identified in TB-infected persons immunodominant peptides reactive with antibodies recognizing amino acid (aa) epitopes 241 to 265 of Rv1508c and aa 325 to 336 of Rv1516c. Thus, some peptide sequences eliciting an antibody-mediated immune response are not well conserved in Alpine *M. caprae* isolates, and their loss may be a result of long-term host interactions.

During the past 10 years, a low but consistent incidence of *M. caprae* was registered in the Alpine part of Bavaria, Germany, according to TSN 3.0 reports. Efficient TB control should include epidemiological surveillance systems based on mycobacterial species identification to gain a better knowledge about the regional occurrence and host range of MTC members and other mycobacterial species. DNA fingerprinting methods have proven to be powerful epidemiologic tools for the differentiation of the MTC, the current gold standard being MIRU-VNTR typing (29). The presence of three types of the Bavarian Alpine *M. caprae* isolates, all three found in both cattle and red deer, clearly demonstrates tuberculosis transmission between livestock and wildlife (Fig. 2). However, the primary host of the strains cannot be predicted. Notably, the three types have shown a remarkable genetic stability for at least 3 years (Table 1).

To date, the Allgäu type has not been described. The region-specific types, genotype Lechtal and genotype Karwendel, have been found in bordering regions in Austria and were characterized by spoligotyping and MIRU-VNTR for 12 loci (10–12). The genotype Lechtal has been identified in a "hot spot" area of TB in red deer (prevalence 23.1%) in the Lechtal, Austria (12). Because of the local high incidence in the red deer population, a high red deer transmission rate of the Lechtal type can be assumed. A spillover of the Lechtal type to the German Allgäu region seems likely. On the other hand, the Allgäu type exists in the same area of Bavaria and contains the complete RD4. This raises the question of whether the RD4 deletion in the Lechtal type might have evolved from the Allgäu type by animal passages. It became evident that a detection of the three variants of RD4 sequences was not possible using spoligotyping or MIRU-VNTR based on repetitive DNA elements. However, informative SNPs are valuable markers for classifying MTC members into phylogenetic lineages because of very low degrees of homoplasy (39, 40). Whole-genome sequencing provides complete information for the identification of a sufficient number of reliable SNPs and the genomic characteristics of *M. caprae* (41).

ACKNOWLEDGMENTS

We thank the EMIDA ERA-Net coordination board for helpful discussions and the consortium for agreement with publication of the manuscript. We are grateful to Eva Maria Gerstmair, Ludmilla Naumann, and

the Tuberculosis Culture Collection Laboratory of the Bavarian Health and Food Safety Authority for providing isolates.

This work was funded by the EMIDA ERA-Net Project Tuberculosis in Alpine Wildlife (BMBF-FK0315857A) and the Bavarian State Ministry of Environment and Health.

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