JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2003, p. 5215–5223 Vol. 41, No. 11
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.11.5215–5223.2003

Characterization of Genetic Differences between *Mycobacterium avium* subsp. *paratuberculosis* Type I and Type II Isolates

Karen Dohmann,[1] Birgit Strommenger,[1]† Karen Stevenson,[2] Lucía de Juan,[3] Janin Stratmann,[1] Vivek Kapur,[4] Tim J. Bull,[5] and Gerald-Friedrich Gerlach[1]*

Department of Infectious Diseases, Institute for Microbiology, School of Veterinary Medicine Hannover, D-30173 Hannover, Germany[1]; International Research Centre, Pentlands Science Park, Moredun Research Institute, Penicuik EH26 OPZ, Scotland,[2] and Department of Surgery, St. George's Hospital Medical School, London SW17 ORE,[5] United Kingdom; Departamento de Patología Animal I (Sanidad Animal), Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain[3]; and Biomedical Genomics Center and Departments of Microbiology and Veterinary Pathobiology, University of Minnesota, St. Paul, Minnesota 55108[4]

Received 27 June 2003/Returned for modification 13 August 2003/Accepted 20 August 2003

A combination of representational difference analysis and comparative DNA sequencing revealed that four type I (sheep) isolates of *Mycobacterium avium* subsp. *paratuberculosis* were differentiated from nine type II (bovine) isolates by the presence of an 11-bp insertion in a novel *M. avium* subsp. *paratuberculosis*-specific region of genomic DNA. Further, our studies show that *M. avium* subsp. *paratuberculosis* type I isolates contain three type-specific loci that are missing in *M. avium* subsp. *paratuberculosis* type II but are present in *M. avium* subsp. *avium*. Taken together, the results are consistent with the hypothesis that *M. avium* subsp. *paratuberculosis* type I strains are an evolutionary intermediate between *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis* type II isolates or share a subset of *M. avium* subsp. *avium* type-specific loci through horizontal transfer.

Mycobacterium avium subsp. *paratuberculosis* is the causative agent of paratuberculosis (Johne's diseases), a chronic granulomatous enteritis in ruminants. The disease is prevalent in domestic and wild animals worldwide (15, 16, 24, 31) and has a considerable economic impact on the livestock industry (13). In humans, *M. avium* subsp. *paratuberculosis* has been suggested to be involved in Crohn's disease (6, 11, 20). *M. avium* subsp. *paratuberculosis* shows a DNA homology of more than 90% to *M. avium* subsp. *avium* (14, 26). It differs from other subspecies of *M. avium* by its dependence on the iron chelator mycobactin for growth in culture (9, 30) and by the presence of multiple copies of the insertion sequences IS*900* (12) and IS*Mav2* (29).

By using analyses by pulsed-field electrophoresis (PFGE) and IS*900*-restriction fragment length polymorphisms, *M. avium* subsp. *paratuberculosis* isolates have been divided into two distinct types, with type I comprising very slow-growing, mostly pigmented isolates forming smooth and uniform colonies mainly obtained from sheep and other small ruminants and type II comprising slow-growing, nonpigmented isolates forming rough and nonuniform colonies exhibiting a very broad host range (27). Both types can also be differentiated by IS*1311*-PCR (19) and by a specific multiplex PCR (8). However, the molecular differences between *M. avium* subsp. *paratuberculosis* type I and type II strains which might give important clues with respect to (i) the evolutionary relationship between the two *M. avium* subsp. *paratuberculosis* types, (ii) the different phenotypes, and (iii) the differences in host preference have not as yet been elucidated.

In this study we investigated the differences between *M. avium* subsp. *paratuberculosis* type I and type II strains by applying the technique of representational difference analysis (RDA) (18) to isolate *M. avium* subsp. *paratuberculosis* subspecies as well as type-specific DNA fragments. The isolation was followed by PCR, restriction endonuclease digest, and nucleotide sequence analyses of the respective fragments obtained from *M. avium* subsp. *paratuberculosis* isolated from various hosts.

The bacterial strains and plasmids used in this study are listed in Table 1. Mycobacteria were grown on Middlebrook 7H10 agar supplemented with a solution of oleic acid-albumin-dextrose-catalase enrichment (DIFCO, Augsburg, Germany), Tween 80 (0.05%), and mycobactin J (2 μg ml^{-1}; Synbiotics, Lyon, France). *Escherichia coli* transformants were grown in Luria-Bertani medium supplemented with ampicillin (100 μg ml^{-1}). The *M. avium* subsp. *paratuberculosis* isolates were classified as type I and type II by PFGE (27) and two *M. avium* subsp. *paratuberculosis* PCR tests (8, 19); three pigmented ovine isolates (M189, 213G, 21P) were classified as type I by all three tests, and one nonpigmented caprine isolate CAM42 was classified as type I by both PCR tests but exhibited a PFGE profile intermediate between type I and type II. All other isolates were classified as type II strains. All type I and type II isolates were independent and originated from different farms.

The RDA was performed as previously described (29). For the identification of *M. avium* subsp. *paratuberculosis*-specific DNA regions the genome of *M. avium* subsp. *avium* strain ATCC 25291 (driver) was subtracted from that of the bovine *M. avium* subsp. *paratuberculosis* strain 6783 (tester). For the

* Corresponding author. Mailing address: Tierärztliche Hochschule Hannover, Zentrum für Infektionsmedizin, Institut für Mikrobiologie. Phone: (49) 511 856-7598. Fax: (49) 511 856-7697. E-mail: gfgerlach@gmx.de.

† Present address: Robert Koch Institut, D-38855 Wernigerode, Germany.

TABLE 1. Strains and plasmids used in this study

Isolate or primer	Origin or description[a]
M. avium subsp. *paratuberculosis*	
ATCC 19698, bovine	American Type Culture Collection, Rockville, Md.
Isolate 6783 (DSM 44135), bovine	Laboratory reference strain
Isolate M189, pigmented ovine	MRI
Isolate 99PW, bovine	MRI
Isolate 51/91, nonpigmented ovine	MRI
Isolate 502038-191, nonpigmented ovine	MRI
Isolate JD143, nonpigmented ovine	MRI
Isolate 21P, pigmented ovine	MRI
Isolate 213G, pigmented ovine	MRI
Isolate M100/C, caprine	MRI
Isolate 176P, caprine	MRI
Isolate CAM42, caprine	MRI
Isolate 52291V.Veen-HPC, human	MRI
M. avium subsp. *avium*	
ATCC 35712	DSMZ
ATCC 25291 (DSM 44156)	DSMZ
DSM 44158	DSMZ
MAA-S4 (*M. avium* subsp. *avium* serotype 4)	J. Magee, Newcastle Public Health Laboratory
NCTC 8559	NCTC
Other *Mycobacterium* isolates	
M. intracellulare NCTC 10425	NCTC
M. scrofulaceum NCTC 10803	NCTC
M. smegmatis NCTC 8159	NCTC
M. phlei NCTC 8151	NCTC
M. tuberculosis H37Rv NCTC 7416	NCTC
M. bovis NCTC 10772	NCTC
M. microti NCTC 8710	NCTC
M. terrae NCTC 10856	NCTC
M. kansasii NCTC 10268	NCTC
M. xenopi NCTC 10042	NCTC
M. malmoense NCTC 11298	NCTC
M. gordonae NCTC 10267	NCTC
M. szulgai NCTC 10831	NCTC
M. flavescens NCTC 10271	NCTC
M. marinum NCTC 2275	NCTC
M. chelonae subsp. *chelonae* NCTC 946	NCTC
M. chelonae subsp. *abscessus* NCTC 10882	NCTC
M. fortuitum NCTC 10394	NCTC
M. peregrinum NCTC 10264	NCTC
M. haemophilum NCTC 11185	NCTC
E. coli TOP 10F′	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 rec A1 deoR araD139* Δ(*araleu*)7697 *galU galK rpsL* (Str^R) *endA1 nupG*; TOPO TA cloning vector (Invitrogen)
Plasmid pCR 2.1-TOPO	*E. coli* cloning vector carrying an ampicillin and kanamycin resistance determinant (Invitrogen)

[a] MRI, strain collection at the Moredun Research Institute; DSMZ, Deutsche Sammlung fuer Mikroorganismen und Zellkulturen; NCTC, National Collection of Type Cultures.

identification of *M. avium* subsp. *paratuberculosis* type-specific DNA regions the nonpigmented bovine strain 6783 was used as the tester and the pigmented ovine *M. avium* subsp. *paratuberculosis* strain M189 was used as the driver, and vice versa. Briefly, tester and driver DNA were digested simultaneously with *Bam*HI, *Bgl*II, and *Bcl*I (New England Biolabs, Bad Schwalbach, Germany). Tester fragments were ligated to the oligonucleotide adapter R*Bam*12/R*Bam*24 (Table 2). Ligation products were diluted 10- to 1,000-fold, and a constant amount of digested driver DNA was added, resulting in 4-, 40-, and 400-fold excesses of driver DNA. DNA was ethanol precipitated, dried, resuspended in 5 μl of *Taq* polymerase buffer (Invitrogen, Groningen, The Netherlands) containing 1 M NaCl, overlaid with paraffin oil, and hybridized for 20 h at 67°C. The hybridized DNA was diluted with 15 μl of H_2O, 5 μl of each hybridization reaction mixture was removed, and the overhangs were filled in by using *Taq* polymerase in the recommended buffer (Invitrogen, Groningen, The Netherlands) containing deoxynucleoside triphosphates (0.2 mM) at 72°C for 20 min in a 20-μl volume. Primer R*Bam*24 (25 pmol in a 5-μl volume) was added, and tester-specific DNA was exponentially amplified in a 25-μl volume with an initial denaturation step at 94°C for 3 min, 35 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 3 min, and a final extension at 72°C for 10

TABLE 2. Primers used in this study

Primer and/or fragment	Sequence
RDA primers[a]	
R*Bam*12	5′-GAT CCT CGG TGA-3′
R*Bam*24	5′-AGC ACT CTC CAG CCT CTC ACC GAG-3′
ISMav2 primers[b]	
IS*Mav*1	5′-GTA TCA GGC CGT GAT GGC GG-3′
IS*Mav*2	5′-CGC GAC CAG CGC TCG ATA CA-3′
IS*900* primers[c]	
MK5	5′-TTC TTG AAG GGT GTT CGG GGC C-3′
MK6	5′-GCG ATG ATC GCA GCG TCT TTG G-3′
IS*901* primers[d]	
MK7	5′-GTC TGG GAT TGG ATG TCC TG-3′
MK8	5′-CAC CAC GTG GTT AGC AAT CC-3′
RDA fragments specific for *M. avium* subsp. *paratuberculosis*[e]	
RD I130	
I 130A	5′-TGT GAG GAC ATT CGG TCG GTC-3′
I 131A	5′-TCT ACC TGC ACC CAC GAT GAG-3′
RD II60	
II 60A	5′-TGC CGA CGT GTA CGA ATC AG-3′
II 61A	5′-TCG TTC CGG TCT CTG CGC TA-3′
RD III10	
III 10B	5′-TGC ACG CCC GTT ACA TCA TCC-3′
III 11B	5′-TAG CGG CAG TCA CGA TCG AG-3′
RDA fragments specific for *M. avium* subsp. *paratuberculosis* type I strains[f]	
pig-RDA10	
p19	5′-TAG CGG TCC CGC AGT TTG GC-3′
p20	5′-TCA AGC CGA ACG AGG TGG TCG-3′
pig-RDA20	
p21	5′-TCG TCC CGT CCC GAT GCT GT-3′
p22	5′-TGA GTC CTG TCG TGC ATG CG-3′
pig-RDA30	
p23	5′-TGA AGA GCC CGG ACA AGG GG-3′
p24	5′-TAG GTC TCA GTG GTC CAC CAG C-3′

[a] From Lisitsyn et al. (18).
[b] From Stratmann et al. (28).
[c] From Doran et al. (10).
[d] From Kunze et al. (17).
[e] Annealing temperature, 60°C.
[f] Annealing temperature, 61°C.

min. PCR products were analyzed on a 1.5% agarose gel and were cloned by using a TOPO TA cloning kit (Invitrogen, Groningen, The Netherlands); *E. coli* transformants were tested for inserts in a PCR with R*Bam*24 as the primer. PCR products were analyzed on a 1.5% agarose gel, and their specificity was tested by differential Southern blot analyses using chromosomal DNA of the tester and driver as probes. Agarose gel electrophoresis, Southern blot analyses, PCR, DNA cloning, and transformation of *E. coli* were done by following standard procedures (25). Primers were purchased from Invitrogen, and DNA sequencing reactions were done by SeqLab (Göttingen, Germany). GenBank database searches were performed by using BLASTX and BLASTN (1). In addition, BLASTX alignments of DNA sequences were performed by using GenBank/EMBL as well as the *M. avium* subsp. *avium* (The Institute for Genome Research [TIGR] strain 104) and *M. avium* subsp. *paratuberculosis* (UMN strain K-10) unfinished genome sequences of TIGR (http://www.tigr.org) and the University of Minnesota *M. avium* subsp. *paratuberculosis* database (http://genome.cm.umn.edu/cgi-bin/blast/web_blast.cgi). DNA-modifying enzymes were purchased from New England Biolabs (Karlsruhe, Germany). Primers were designed for the PCR analyses of *M. avium* subsp. *paratuberculosis* subspecies- and type I-specific DNA regions (Table 2). PCR was carried out in 25-μl reaction volumes containing standard PCR buffer (Invitrogen) according to the manufacturer's instructions at annealing temperatures optimal for each primer pair (Table 2), and PCR products were analyzed on a 1.5% agarose gel.

The RDA with *M. avium* subsp. *paratuberculosis* as tester and *M. avium* subsp. *avium* as driver revealed three fragments specific for *M. avium* subsp. *paratuberculosis*, designated RDI130, RDII60, and RDIII10, ranging from 456 to 652 bp

TABLE 3. Molecular data for the *M. avium* subsp. *paratuberculosis* subspecies- and type I-specific fragments

RDA fragment	Length (bp)	Accession no.	Identity with the *M. avium* subsp. *avium* genome (%)	Position in *M. avium* subsp. *paratuberculosis* genome (UMN strain K-10, contig 16)	Position in *M. avium* subsp. *avium* genome (TIGR strain 104, contig 3294)
M. avium subsp. *paratuberculosis* specific					
RDA I130	631	AY 254383	54	2,637,820–2,638,450	
RDA I130 (pigmented)	642	AY 254384	54		
RDA II60	652	AY 254385	56	1,188,511–1,187,860	
RDA III10	456	AY 254386	58	3,987,903–3,988,358	
M. avium subsp. *paratuberculosis* type I specific					
pig-RDA10	233	AY 266300	99		5,394,822–5,395,054
pig-RDA20	197	AY 266301	98		1,986,505–1,986,701
pig-RDA30	548	AY 266302	99		3,021,830–3,022,376

(Table 3) with 54 to 58% homology to the *M. avium* subsp. *avium* and 100% homology to the *M. avium* subsp. *paratuberculosis* genome (Table 3). The specificity of these fragments was confirmed by dot blots of genomic DNA from 22 different species of mycobacteria (Table 1). Fragment RDI130 is located inside a 15-kb *M. avium* subsp. *paratuberculosis*-specific region containing the previously described F57 fragment (23) and fragments Mpt52.16 and Mpt54.16 (21). Fragment RDII60 is located on a 6-kb *M. avium* subsp. *paratuberculosis*-specific region containing fragment Mpt62.12 (21). Fragment RDIII10 overlaps a *pks* oxidoreductase-like gene adjacent to IS*900* locus 6 described by Bull and coworkers (5). Using specific primers and stringent annealing temperatures (Table 2), PCR for the fragments RDII60 and RDIII10 was positive for all 13 *M. avium* subsp. *paratuberculosis* isolates tested but was negative for all 4 isolates of *M. avium* subsp. *avium*. In addition, the nucleotide sequence was identical in all 13 strains, indicating a high degree of genetic stability among strains isolated from different hosts.

Fragment RDI130 contained identical sequences in all nine *M. avium* subsp. *paratuberculosis* type II isolates tested but included 11 additional nucleotides (AGTGACGGCTG) in all *M. avium* subsp. *paratuberculosis* type I isolates. This difference was verified by restriction digests of RDI130-specific PCR products with the endonuclease *Tsp*45I (Fig. 1). Analysis of the sequence adjacent to fragment RDI130 on the *M. avium* subsp. *paratuberculosis* genome revealed that the fragment is part of a putative polycistronic operon carrying a set of five genes. The 11-bp insertion is located in the second open reading frame (ORF) and contains an in-frame stop codon. The fourth ORF of this cluster encodes a protein with FtsK motifs that may be involved in cell division (2, 3, 4). The fifth ORF of this operon has already been identified as a *M. avium* subsp. *paratuberculosis*-specific phage integrase (GenBank accession number L39071).

To investigate *M. avium* subsp. *paratuberculosis* type II-specific DNA regions, the bovine *M. avium* subsp. *paratuberculosis* type II strain 6783 was used as tester and the pigmented ovine type I strain M189 as driver. This RDA did not result in any specific product, suggesting that *M. avium* subsp. *paratuberculosis* type II strains, unlike type I strains (see below), do not contain extended loci of type-specific DNA or that the sensitivity of the assay was not sufficient to detect these fragments.

To investigate *M. avium* subsp. *paratuberculosis* type I-specific DNA regions the RDA was repeated upon reversing tester and driver strains. This RDA resulted in three *M. avium* subsp. *paratuberculosis* type I-specific DNA fragments, designated pig-RDA10, pig-RDA20, and pig-RDA30, that showed

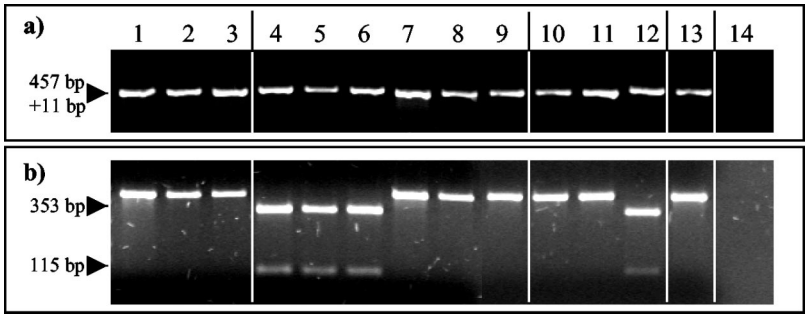

FIG. 1. PCR analysis of *M. avium* subsp. *paratuberculosis* isolates of different host groupings by using the internal primer pair I130A/131A and *Tsp*45I restriction of the PCR product RD I130. The DNA template used was derived from three nonpigmented bovine (lanes 1 to 3), three pigmented ovine (lanes 4 to 6), three nonpigmented ovine (lanes 7 to 9), three nonpigmented caprine (lanes 10 to 12), and one nonpigmented human *M. avium* subsp. *paratuberculosis* isolate (lane 13) as well as the *M. avium* subsp. *avium* ATCC 25291 isolate (lane 14). (a) The PCR with the primers I130A/I131A resulted in products of different sizes for the *M. avium* subsp. *paratuberculosis* type I and type II isolates. (b) The PCR product RD I130 of the *M. avium* subsp. *paratuberculosis* type I isolates was cut into two fragments by digestion with *Tsp*45I. The arrows to the left indicate the size of the corresponding PCR products.

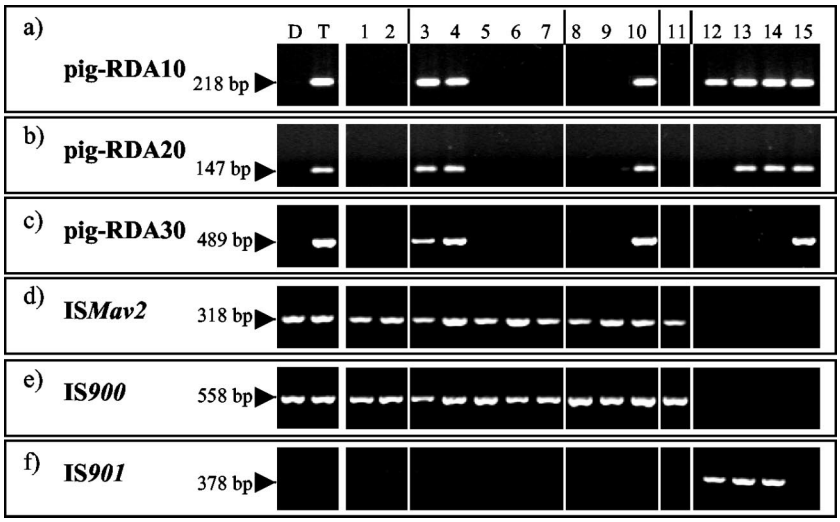

FIG. 2. PCR analyses of *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *avium* isolates with the primer pair p19/p20, specific for pig-RDA10 (a); p21/p22, specific for pig-RDA20 (b); p23/p24, specific for pig-RDA30 (c); ISMav2 I/ISMav2 II, specific for *M. avium* subsp. *paratuberculosis* (d); MK5/MK6, specific for IS*900* of *M. avium* subsp. *paratuberculosis* (e); and (f) MK7/MK8, specific for IS*901* of *M. avium* subsp. *avium*. The template DNA used was derived from the nonpigmented type II driver strain (lane D) and the pigmented type I tester strain (lane T). Other template DNA used was from two nonpigmented bovine (lanes 1 and 2), two pigmented (lanes 3 and 4), three nonpigmented ovine (lanes 5 to 7), three nonpigmented caprine (lanes 8 to 10), and one nonpigmented human (lane 11) *M. avium* subsp. *paratuberculosis* isolate as well as four *M. avium* subsp. *avium* isolates (ATCC 35712, ATCC 25291, DSM 44158, and MAA S4; lanes 12 to 15).

no homology to the *M. avium* subsp. *paratuberculosis* K-10 genome (a type II strain) but contained 98 to 99% homology to *M. avium* subsp. *avium* sequences (BLAST search in the TIGR database) (Table 3). The PCR with specific primers (Table 2) resulted in specific products in all *M. avium* subsp. *paratuberculosis* type I strains, whereas all type II strains remained negative (Fig. 2a to c). Furthermore, three *M. avium* subsp. *avium* serotype 2 isolates (ATCC 35712, ATCC 25291, and DSM 44158) showed a specific PCR product with the primer pair p19/p20 (specific for pig-RDA10), and two of them (ATCC 25291 and DSM 44158) showed a PCR product with p21/p22 specific for pig-RDA20 (Fig. 2a and b and 3a) but did not show a PCR product with the primer pair p23/p24 specific for pig-RDA30 (Fig. 2c and 3b). The *M. avium* subsp. *avium* serotype 4 strain (MAA S4) was positive in all three PCRs. The four *M. avium* subsp. *paratuberculosis* type I isolates, like all other nonpigmented isolates, were positive in an *M. avium* subsp. *paratuberculosis*-specific IS*900*- and IS*Mav2*-PCR (Fig. 2d and e, Table 2) but were negative in an IS*901*-PCR with primers MK7 and MK8 (Fig. 2f, Table 2).

Further analysis using partial alignments of the *M. avium* subsp. *avium* TIGR 104 and *M. avium* subsp. *paratuberculosis* K-10 genome sequences revealed that pig-RDA10 maps to an ORF near the end of a 16,483-bp sequence found in *M. avium* subsp. *avium* (designated 16K region) (Fig. 3a) containing genes encoding reducing and hydrolyzing enzymes. This sequence is flanked in *M. avium* subsp. *avium* by a large 0.9-Mbp region (A region) which is 98% homologous to the corresponding *M. avium* subsp. *paratuberculosis* region. However, the 16K region along with the immediately adjacent A region are inverted in *M. avium* subsp. *paratuberculosis* type I, while in *M. avium* subsp. *paratuberculosis* type II the A region is inverted but the 16K region is deleted. Flanking these inversions in both *M. avium* subsp. *paratuberculosis* type I and type II are regions homologous to *M. avium* subsp. *avium* (C region) (Fig. 3a; 4,491,358 to 4,474,594) but are interrupted by a copy of IS*900* (IS*900* in locus 15) exactly 16,483 bp away from the A region. The identical length of this C region with that of the 16K region and the proximity to a copy of IS*900* suggests that this element may have been involved in this very large genomic inversion. Our PCR analyses suggest that the entire *M. avium* subsp. *avium* 16K locus is present in *M. avium* subsp. *paratuberculosis* type I strains but is absent in type II strains (Fig. 3a, Table 4), while the IS*900* in locus 15 is present in both *M. avium* subsp. *paratuberculosis* type I and type II strains but is absent in *M. avium* subsp. *avium*. No product could be obtained with primers p1/p5 and primers p3/p5 (Fig. 3a, Table 4). This suggests that *M. avium* subsp. *paratuberculosis* type I isolates contain only part of the *M. avium* subsp. *paratuberculosis* type II C locus in this chromosomal position. However, PCR with primers p6/p7 amplifying the left-hand end of the C locus was positive (Table 4), suggesting that it may be present but is translocated to a different genomic location in these strains.

RDA fragment pig-RDA20 maps to a large 197,233-bp *M. avium* subsp. *avium*-specific region (designated 197K locus) (Fig. 3b). Sequence analyses of this region indicate that it encodes several systems involved in metabolic pathways, with fragment pig-RDA20 positioned within an ORF predicted to encode a peptide synthase. Sequences flanking the 197K locus are 98% homologous in *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*, but in *M. avium* subsp. *paratuberculosis* the 197K locus is replaced by a 19,441-bp sequence (19K locus) carrying a gene cassette containing close homologues to *mce* genes involved in mycobacterial entry into mammalian cells (7, 22). Primers specific for the left- and right-hand end of the *M. avium* subsp. *avium* 197K locus and the *M. avium* subsp. *paratuberculosis* 19K locus were designed (Table 5). PCRs with primers specific for the left- and right-hand end of the *M.*

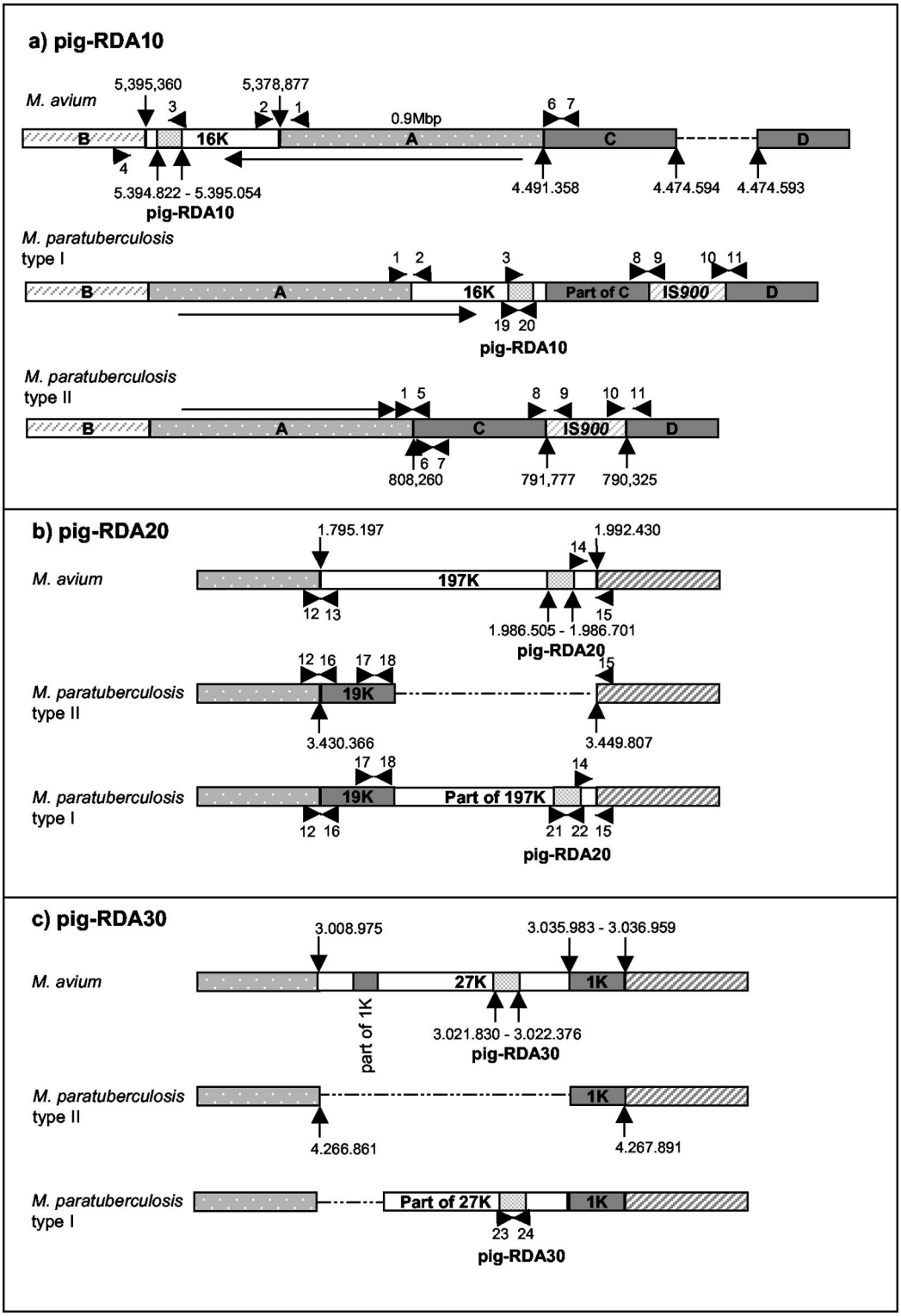

FIG. 3. Genetic organization of the loci pig-RDA10, pig-RDA20, and pig-RDA30 specific for *M. avium* subsp. *paratuberculosis* type I isolates. The numbers given in the maps relate to the positions on the *M. avium* subsp. *avium* (TIGR strain 104, contig 3294) and *M. avium* subsp. *paratuberculosis* (UMN strain K-10, contig 16) genomes. (a) pig-RDA10 locus. The sequence identified is also present in the 16K locus present in *M. avium* subsp. *avium* (top) and *M. avium* subsp. *paratuberculosis* type I (middle) but is absent from *M. avium* subsp. *paratuberculosis* type II (bottom). The 16K locus is flanked by a 0.9-Mbp sequence (designated A) present in *M. avium* subsp. *avium* and partially present in *M. avium* subsp. *paratuberculosis* but is oriented in the opposite direction (indicated by an arrow). These sequences are bounded by regions (designated B, C, and D) which are interrupted by IS*900* locus 15 in both *M. avium* subsp. *paratuberculosis* type I and type II isolates. A putative organization of the locus in *M. avium* subsp. *paratuberculosis* type I strains is depicted in the middle map. (b) pig-RDA20 locus. The sequence identified is also present in the 197K locus specific for *M. avium* subsp. *avium* (top). In *M. avium* subsp. *paratuberculosis* the same flanking regions embrace the 19K

TABLE 4. Primers for the PCR analyses of the *M. avium* subsp. *avium* 16K locus and the *M. avium* subsp. *paratuberculosis* C locus

Primer location and sequence	Position in *M. avium* subsp. *avium* genome (TIGR strain 104, contig 3294)	Position in the *M. avium* subsp. *paratuberculosis* genome (UMN strain K-10, contig 16)	PCR results for:		
			M. avium subsp. *avium* S2/S4	*M. avium* subsp. *paratuberculosis* type I	*M. avium* subsp. *paratuberculosis* type II
Left transition side of *M. avium* subsp. *avium* 16K					
p1 (5′-TCC GCA GCC AGA ACG GCG AA-3′)	5,378,644–5,378,663	808,474–808,469	+/+	+	−
p2 (5′-TCA CCG AGA CCA TGA CGC TGC-3′)	5,379,037–5,379,057				
Right transition side of *M. avium* subsp. *avium* 16K					
p3 (5′-TGT GCG AGC GCG AAA ACC GC-3′)	5,394,672–5,394,691		+/+	−	−
p4 (5′-TGA TCA GCG CGA CCT TGC CC-3′)	5,395,421–5,395,440				
Left transition side of *M. avium* subsp. *avium* subsp. *paratuberculosis* C locus					
p1 (5′-TCC GCA GCC AGA ACG GCG AA-3′)	5,378,644–5,378,663	808,474–808,469	−/−	−	+
p5 (5′-TCA GCT CCT CGA TCC CCG TC-3′)	4,491,296–4,491,315	808,191–808,210			
Left-hand end of *M. avium* subsp. *paratuberculosis* C locus					
p6 (5′-TGT TCG CGC CCA TTC TGC GG-3′)	4,491,321–4,491,340	808,235–808,216	+/+	+	+
p7 (5′-TCA GGA TCC GCT CCA CCT CG-3′)	4,491,245–4,491,264	808,140–808,159			
Right-hand end of *M. avium* subsp. *avium* 16K plus left-hand end of *M. avium* subsp. *paratuberculosis* C locus					
p3 (5′-TGT GCG AGC GCG AAA ACC GC-3′)	5,394,672–5,394,691		+/+	−	−
p5 (5′-TCA GCT CCT CGA TCC CCG TC-3′)	4,491,296–4,491,315	808,191–808,210			
M. avium subsp. *paratuberculosis* IS*900* in locus 15, left-hand end					
p8 (5′-TCG ATG GTG CCG CTG GCG TT-3′)		792,007–792,026	−/−	+	+
p9 (5′-TAC CTG TCG GCC TTG GTC AGC-3′)		791,573–791,593 (and many other hits)			
M. avium subsp. *paratuberculosis* IS*900* locus 15, right-hand end					
p10 (5′-GCG ATG ATC GCA GCG TCT TTG G-3′)		790,893–790,872 (and many other hits)	−/−	+	+
p11 (5′-TCG AAC AGC AGG GGT TCG GTC-3′)	4,474,535–4,474,555	790,267–790,287			

avium subsp. *paratuberculosis* 19K locus suggest that *M. avium* subsp. *paratuberculosis* type I isolates contain this entire locus (Fig. 3b, Table 5). Additional PCRs showed that the left-hand end of the *M. avium* subsp. *avium* 197K locus was absent, whereas the right-hand end was present in all *M. avium* subsp. *paratuberculosis* type I isolates (Fig. 3b, Table 5).

RDA fragment pig-RDA30 maps to a 27,008-bp *M. avium* subsp. *avium*-specific region (27K region) (Fig. 3c) positioned within the carboxy-terminal end of a *tetR* regulation gene that is absent in *M. avium* subsp. *paratuberculosis* type II isolates. The sequences flanking the 27K region are 98% homologous in *M. avium* subsp. *avium* and *M. avium* subsp. *paratuberculosis*. A 1,030-bp (1K region) sequence encoding a transposase is located on the right-hand side (Fig. 3c) as well as in several other locations in the *M. avium* subsp. *paratuberculosis* and the *M. avium* subsp. *avium* genomes. The presence of several copies of different insertion sequences within the left-hand end of the *M. avium* subsp. *avium* 27K region prevented the design of primers specific for this region. We were therefore unable to determine the extent to which the 27K region is present in *M. avium* subsp. *paratuberculosis* type I strains (Fig. 3c).

Our study characterizes, for the first time, genomic differences between *M. avium* subsp. *paratuberculosis* type I and type II strains by using four and nine independent isolates, respectively. It suggests that, while neither *M. avium* subsp. *paratuberculosis* type I nor type II isolates contain unique type-specific loci, type II strains appear to have undergone more deletions and rearrangements of regions than type I strains that have corresponding loci in *M. avium* subsp. *avium*. The presence of the 0.9-Mbp reversed region containing pig-RDA10 in both *M. avium* subsp. *paratuberculosis* type I and type II isolates suggests that both types of *M. avium* subsp. *paratuberculosis* strains originate from a common progenitor and that the two types are not derived from divergent *M. avium* subsp. *avium* strains that have similarly acquired IS*900*. These findings are also consistent with the hypothesis that *M. avium* subsp. *paratuberculosis* type I isolates are an evolutionary intermediate between *M. avium* subsp. *avium* and *M. avium*

locus specific for *M. avium* subsp. *paratuberculosis* (middle). The probable organization of the locus in *M. avium* subsp. *paratuberculosis* type I strains is depicted in the bottom map. (c) pig-RDA30 locus. The sequence identified is also present in the 27K locus specific for *M. avium* subsp. *avium* (top) flanked by a transposase (designated 1K) and containing an additional truncated 1K region. In *M. avium* subsp. *paratuberculosis* the 27K locus is missing (middle). The probable organization of the locus in *M. avium* subsp. *paratuberculosis* type I strains is depicted in the bottom map. The arrows are numbered to indicate the positions of the primers used (Table 4).

TABLE 5. Primers for the PCR analyses of the *M. avium* subsp. *avium* 197K locus and the *M. avium* subsp. *paratuberculosis* 19K locus

Primer location and sequence	Position in *M. avium* subsp. *avium* genome (TIGR strain 104, contig 3294)	Position in *M. avium* subsp. *paratuberculosis* genome (UMN strain K-10, contig 16)	PCR results for:		
			M. avium subsp. *avium* S2/S4	*M. avium* subsp. *paratuberculosis* type I	*M. avium* subsp. *paratuberculosis* type II
Left transition side of *M. avium* subsp. *avium* 197K					
p12 (5′-TGA TCC GGG CGA CGA TCT GG-3′)	1,794,970–1,794,989 and 2,073,405–2,073,422	3,430,140–3,430,159	−/+	−	−
p13 (5′-TGG CGT TGA TAT CGC GAC TGG-3′)	1,795,236–1,795,256				
Right-hand end of *M. avium* subsp. *avium* 197K					
p14 (5′-TCG TCC AGG TAG CCG TTC AAC TC-3′)	1,991,916–1,991,938		+/+	+	−
p15 (5′-TGC AGC AGG TGT TCG GGA TGG-3′)	1,992,400–1,992,420 and 2 other hits, 2,449,453–2,449,472 and 4,813,981–4,814,000				
Left transition side of *M. avium* subsp. *paratuberculosis* 19K					
p12 (5′-TGA TCC GGG CGA CGA TCT GG-3′)	1,794,970–1,794,989	3,430,140–3,430,159	−/−	+	+
p16 (5′-TGT CGT CAA CGC GGT TGG CG-3′)		3,430,532–3,430,551			
Right-hand end of *M. avium* subsp. *paratuberculosis* 19K					
p17 (5′-TAC TAC GCC TAC GCA CCG CG-3′)		3,448,574–3,448,593	−/−	+	+
p18 (5′-TAG ATC AGG ATG TGC CCG GCC-3′)		3,448,995–3,448,975			
Right-hand end of *M. avium* subsp. *paratuberculosis* 19K plus left-hand end of *M. avium* subsp. *avium* 197K					
p17 (5′-TAC TAC GCC TAC GCA CCG CG-3′)		3,448,574–3,448,593	−/−	−	−
p13 (5′-TGG CGT TGA TAT CGC GAC TGG-3′)	1,795,236–1,795,256				

subsp. *paratuberculosis* type II strains. Alternatively, since many of the fragments map to regions that are likely to be carried by phage or to be adjacent to mobile genetic elements, the results may be indicative of the fact that type I isolates and some isolates of *M. avium* subsp. *avium* are more likely to share some of these elements through horizontal transfer. Further studies based on large-scale comparative sequence analysis as well as a larger number of isolates will be needed to reconstruct the evolutionary history of this closely related group of animal-pathogenic mycobacteria.

This study was funded by the EU project Assess Mptb Risk QLK2-CT-2001-01420, by Action Research, United Kingdom, by the Scottish Executive Environment Rural Affairs Department, and by a mobility grant jointly financed by the Academic Research Council (ARC) and the Deutscher Akademischer Austauschdienst (DAAD). *M. avium* subsp. *paratuberculosis* genome sequencing in the laboratory of V.K. is supported by competitive grants from the USDA-CSREES.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. H. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:** 3389–3402.
2. **Barre, F. X., M. Aroyo, S. D. Colloms, A. Helfrich, F. Cornet, and D. J. Sherratt.** 2000. FtsK functions in the processing of a Holliday junction intermediate during bacterial chromosome segregation. Genes Dev. **14:**2976–2988.
3. **Begg, K. J., S. J. Dewar, and W. D. Donachie.** 1995. A new *Escherichia coli* cell division gene, *ftsK*. J. Bacteriol. **177:**6211–6222.
4. **Boyle, D. S., D. Grant, G. C. Draper, and W. D. Donachie.** 2000. All major regions of FtsK are required for resolution of chromosome dimers. J. Bacteriol. **182:**4124–4127.
5. **Bull, T. J., J. Hermon-Taylor, I. Pavlik, F. El Zaatari, and M. Tizard.** 2000. Characterization of IS*900* loci in *Mycobacterium avium* subsp. *paratuberculosis* and development of multiplex PCR typing. Microbiology **146:**2185–2197.
6. **Chamberlin, W., D. Y. Graham, K. Hulten, H. M. El Zimaity, M. R. Schwartz, S. Naser, I. Shafran, and F. A. El Zaatari.** 2001. Review article: *Mycobacterium avium* subsp. *paratuberculosis* as one cause of Crohn's disease. Aliment. Pharmacol. Ther. **15:**337–346.
7. **Chitale, S., S. Ehrt, I. Kawamura, T. Fujimura, N. Shimono, N. Anand, S. W. Lu, L. Cohen-Gould, and L. W. Riley.** 2001. Recombinant *Mycobacterium tuberculosis* protein associated with mammalian cell entry. Cell. Microbiol. **3:**247–254.
8. **Collins, D. M., M. De Zoete, and S. M. Cavaignac.** 2002. *Mycobacterium avium* subsp. *paratuberculosis* strains from cattle and sheep can be distinguished by a PCR test based on a novel DNA sequence difference. J. Clin. Microbiol. **40:**4760–4762.
9. **De Voss, J. J., K. Rutter, B. G. Schroeder, and C. E. Barry III.** 1999. Iron acquisition and metabolism by mycobacteria. J. Bacteriol. **181:**4443–4451.
10. **Doran, T. J., J. K. Davies, A. J. Radford, and A. L. Hodgson.** 1994. Putative functional domain within ORF2 on the *Mycobacterium* insertion sequences IS*900* and IS*902*. Immunol. Cell Biol. **72:**427–434.
11. **El Zaatari, F. A., M. S. Osato, and D. Y. Graham.** 2001. Etiology of Crohn's disease: the role of *Mycobacterium avium* subsp. *paratuberculosis*. Trends Mol. Med. **7:**247–252.
12. **Green, E. P., M. L. Tizard, M. T. Moss, J. Thompson, D. J. Winterbourne, J. J. McFadden, and J. Hermon-Taylor.** 1989. Sequence and characteristics of IS*900*, an insertion element identified in a human Crohn's disease isolate of *Mycobacterium paratuberculosis*. Nucleic Acids Res. **17:**9063–9073.
13. **Harris, N. B., and R. G. Barletta.** 2001. *Mycobacterium avium* subsp. *paratuberculosis* in veterinary medicine. Clin. Microbiol. Rev. **14:**489–512.
14. **Imaeda, T., G. Broslawski, and S. Imaeda.** 1988. Genomic relatedness among mycobacterial species by nonisotopic blot hybridization. Int. J. Syst. Bacteriol. **38:**151–156.
15. **Johnston, W. S., and G. K. MacLachlan.** 1986. Johne's disease in sheep. Vet. Rec. **7:**75.
16. **Kreeger, J. M.** 1991. Ruminant paratuberculosis - a century of progress and frustration. J. Vet. Diagn. Investig. **3:**373–382.
17. **Kunze, Z. M., S. Wall, R. Appelberg, M. T. Silva, F. Portaels, and J. J. McFadden.** 1991. IS*901*, a new member of a widespread class of atypical insertion sequences, is associated with pathogenicity in *Mycobacterium avium*. Mol. Microbiol. **5:**2265–2272.
18. **Lisitsyn, N., N. Lisitsyn, and M. Wigler.** 1993. Cloning the differences between two complex genomes. Science **259:**946–951.
19. **Marsh, I., R. Whittington, and D. Cousins.** 1999. PCR-restriction endonuclease analysis for identification and strain typing of *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* based on polymorphisms in IS*1311*. Mol. Cell Probes **13:**115–126.
20. **McFadden, J. J., P. D. Butcher, R. Chiodini, and J. Hermon-Taylor.** 1987. Crohn's disease-isolated mycobacteria are identical to *Mycobacterium paratuberculosis*, as determined by DNA probes that distinguish between mycobacterial species. J. Clin. Microbiol. **25:**796–801.
21. **Nielsen, K., and P. Ahrens.** 2002. Putative in vitro expressed gene fragments unique to *Mycobacterium avium* subspecies *paratuberculosis*. FEMS Microbiol. Lett. **214:**199–203.

22. **Parker, S. L., Y. L. Tsai, and C. J. Palmer.** 1995. Comparison of PCR-generated fragments of the *mce* gene from *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium intracellulare*, and *Mycobacterium scrofulaceum*. Clin. Diagn. Lab. Immunol. **2:**770–775.
23. **Poupart, P., M. Coene, H. Van Heuverswyn, and C. Cocito.** 1993. Preparation of a specific RNA probe for detection of *Mycobacterium paratuberculosis* and diagnosis of Johne's disease. J. Clin. Microbiol. **31:**1601–1605.
24. **Power, S. B., J. Haagsma, and D. P. Smyth.** 1993. Paratuberculosis in farmed red deer (*Cervus elaphus*) in Ireland. Vet. Rec. **132:**213–216.
25. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **Saxegaard, F., I. Baess, and E. Jantzen.** 1988. Characterization of clinical isolates of *Mycobacterium paratuberculosis* by DNA-DNA hybridization and cellular fatty acid analysis. Acta Pathol. Microbiol. Immunol. Scand. **96:**497–502.
27. **Stevenson, K., V. M. Hughes, L. de Juan, N. F. Inglis, F. Wright, and J. M. Sharp.** 2002. Molecular characterization of pigmented and nonpigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis*. J. Clin. Microbiol. **40:**1798–1804.
28. **Stratmann, J., B. Strommenger, K. Stevenson, and G. F. Gerlach.** 2002. Development of a peptide-mediated capture PCR for detection of *Mycobacterium avium* subsp. *paratuberculosis* in milk. J. Clin. Microbiol. **40:**4244–4250.
29. **Strommenger, B., K. Stevenson, and G. F. Gerlach.** 2001. Isolation and diagnostic potential of IS*Mav2*, a novel insertion sequence-like element from *Mycobacterium avium* ssp. *paratuberculosis*. FEMS Microbiol. Lett. **196:**31–37.
30. **Thorel, M. F., M. Krichevsky, and V. V. Levy-Frebault.** 1990. Numerical taxonomy of mycobactin-dependent mycobacteria, emended description of *Mycobacterium avium*, and description of *Mycobacterium avium* subsp. *avium* subsp. nov., *Mycobacterium avium* subsp. *paratuberculosis* subsp. nov., and *Mycobacterium avium* subsp. *silvaticum* subsp. nov. Int. J. Syst. Bacteriol. **40:**254–260.
31. **Weber, A., R. Gürke, K. Bauer, and K. Schreyer.** 1992. Bacterial studies on the occurrence of *Mycobacterium paratuberculosis* in fecal samples of zoo ruminants. Berl. Munch. Tierarztl. Wochenschr. **105:**161–164.