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# Sequence Polymorphisms in a Surface PPE Protein Distinguish Types I, II, and III of *Mycobacterium avium* subsp. *paratuberculosis*▽

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**In the last 2 decades, a variety of different molecular typing methods have been developed to differentiate strains of *Mycobacterium avium* subsp. *paratuberculosis*. The most successful techniques are based on insertion sequences, repetitive loci, comparative genomics, or single nucleotide polymorphisms. In the present study, we chose to examine whether a single *M. avium* subsp. *paratuberculosis* gene could serve as a means of differentiation of a variety of isolates. The MAP1506 gene locus encodes a member of the polymorphic PPE protein family that has putative roles relevant to *M. avium* subsp. *paratuberculosis* pathogenicity. The MAP1506 locus was sequenced from a collection of 58 *M. avium* subsp. *paratuberculosis* isolates from different sources, hosts, and typing profiles. Following sequence alignment and analysis, it was found that bovine (type II) strains of *M. avium* subsp. *paratuberculosis* consistently differed from ovine (type I) and intermediate (type III) strains in seven and eight nucleotides, respectively. Polymorphic regions of the MAP1506 locus were selected for analysis by denaturing gradient gel electrophoresis, allowing visual discrimination of the three subtypes of *M. avium* subsp. *paratuberculosis* isolates. This is the first report describing the use of PCR and denaturing gradient gel electrophoresis on a single gene as a method to distinguish types I, II, and III of *M. avium* subsp. *paratuberculosis*.**

*Mycobacterium avium* subsp. *paratuberculosis* is the causative organism of Johne's disease (or paratuberculosis), a debilitating chronic gastroenteritis in ruminants (50). Animals typically become infected by the fecal-oral route in the first few months of life. The chronic wasting and profuse diarrhea that characterize clinical paratuberculosis are not usually observed until three or more years following infection (12). Paratuberculosis is prevalent in domestic animals worldwide and has a significant impact on the global economy, including the Canadian economy (10, 38). *M. avium* subsp. *paratuberculosis* has also been implicated in Crohn's disease in humans (22).

Molecular techniques for strain typing based on mobile genetic elements, repetitive elements, and single nucleotide polymorphisms (SNPs) of *M. avium* subsp. *paratuberculosis* have been well explored in the last decade (reviewed by Motiwala et al. [39]). The most widely used method to type *M. avium* subsp. *paratuberculosis* isolates is restriction fragment length polymorphism (RFLP) analysis, with detection of polymorphisms by hybridization to an IS*900* probe (IS*900* RFLP) (49). This technique is able to distinguish bovine (type II), ovine (type I), and intermediate (type III) isolates (42) but is slow, technically demanding, and applicable only to cultivable strains of *M. avium* subsp. *paratuberculosis*, as it requires a considerable amount of genomic DNA. Moreover, IS*900* RFLP requires analysis of complex banding patterns and has limited discriminatory power (39). Another technique for typing *M. avium* subsp. *paratuberculosis* is pulsed-field gel electrophoresis of digested genomic DNA (21, 48, 49); however, this technique suffers from many of the same limitations associated with IS*900* RFLP. IS*1311* PCR-restriction endonuclease analysis (REA), described by Marsh et al. (32), is also used to type *M. avium* subsp. *paratuberculosis*. While this technique has the benefit of employing a PCR step to reduce the need for a large quantity of starting DNA, it suffers in its low discriminatory power, as only the bovine (C) and ovine (S) types can be differentiated. Thus, rapid molecular typing methods with discriminatory power greater than that seen for methods in current use need to be assessed as alternatives when studying the genetic diversity in *M. avium* subsp. *paratuberculosis*.

Mycobacteria contain two unique polymorphic protein families, the PE and PPE proteins, which are unknown for any other species. These families are particularly expanded in the pathogenic mycobacterial species. The names PE and PPE are derived from the motifs Pro-Glu and Pro-Pro-Glu, respectively, found in conserved domains near the N termini of these proteins. Although no precise function is known for any member of these families, some members in *M. tuberculosis* have been found to associate with the cell wall (7, 19) and to influence interactions with other cells (7). It has been suggested that some PE/PPE proteins play a role in immune evasion and antigenic variation (4, 6, 13, 18). Members of the PE and PPE families have also been linked to virulence (31, 44), and some PPE proteins have been found to be immunodominant antigens (11). There are 10 PE and 37 PPE genes in the *M. avium* subsp. *paratuberculosis* genome (comprising 1% of the genome) (26, 30), but no information on their putative roles exists.

The aim of this study was to examine the polymorphic vari-

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ability of a PPE protein family gene locus, namely, the MAP1506 locus, in a collection of *M. avium* subsp. *paratuberculosis* isolates from different hosts and geographic locations and of different IS*900* RFLP profiles. The MAP1506 locus was chosen, as it is one of the few PPE gene loci that was previously discovered to be expressed in vitro and has its protein product surface exposed on the cell wall of *M. avium* subsp. *paratuberculosis* (J. De Buck, unpublished results). We hypothesized that SNPs would be present in the MAP1506 gene locus and allow differentiation of *M. avium* subsp. *paratuberculosis* isolates. A secondary aim of this study was to apply denaturing gradient gel electrophoresis (DGGE), a widely used method for mutation analysis (25) and studies of microbial diversity in multispecies communities (41), for the visualization of SNPs in the MAP1506 locus.

## MATERIALS AND METHODS

***M. avium* subsp. *paratuberculosis* isolates.** A set of seven type II, three type I, and three type III *M. avium* subsp. *paratuberculosis* strains (15, 42) (Table 1) with different IS*900* RFLP profiles (M. Behr [McGill University, Canada] and D. Collins [AgResearch, New Zealand], personal communication) was provided by D. Collins. Another 45 isolates from 11 countries on 3 continents (Table 1) and from a variety of hosts were kindly provided by S. Sreevatsan (Veterinary Population Medicine Department, College of Veterinary Medicine, University of Minnesota), S. Naser (Department of Molecular Biology and Microbiology, University of Central Florida), S. Nielsen (The Royal Veterinary and Agricultural University, Denmark), R. Juste (Animal Health and Production Department, Neiker Tecnalia, Spain), K. Stevenson (Moredun Research Institute, Scotland), M. Behr (McGill University, Canada), M. Ngeleka (Prairie Diagnostics Services, Canada), and D. Collins (AgResearch, New Zealand).

**Genomic DNA extraction and MAP1506 locus PCR.** *M. avium* subsp. *paratuberculosis* cultures were grown in Middlebrook 7H9 broth (Becton Dickinson, Oakville, ON, Canada) supplemented with 10% oleic acid albumin dextrose complex (Becton Dickinson), 0.5% glycerol, and 2 mg/liter mycobactin J (Allied Monitor, MO). Lysis was performed by boiling for 30 min in 10 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 1% Triton X-100. Genomic DNA was purified with the DNeasy blood and tissue kit (Qiagen, Mississauga, Ontario, Canada). MAP1506 gene loci (1,224 bp) were amplified by PCR from all isolates (Map1506F, 5′-GAGTCAATGATGTTGGATTATGG-3′; Map1506R, 5′-CAA TTCCGGATGACACTGG-3′). PCRs (50-μl volumes) were performed with high-fidelity platinum *Taq* polymerase (Invitrogen) and contained 2 μl template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 20 pmol of each primer, 5 μl of the manufacturer's PCR buffer containing $MgCl_2$ (final concentration of $MgCl_2$, 1.5 mM), and 1.75 U of *Taq* polymerase. PCR conditions were denaturation at 94°C for 5 min followed by 30 cycles of PCR with denaturation at 94°C for 45 s, annealing at 55°C for 1 min, and extension at 72°C for 2 min. The final extension time was 10 min at 72°C. The PCR products were sequenced and aligned with the MAP1506 gene locus sequence of the K10 strain (nucleotides 1653138 to 1654361; NCBI accession number AE016958.1) by use of DNAMAN 5.2.9 (Lynnon Bio-Soft, Quebec, Canada).

**IS*1311* PCR-REA.** Genomic DNA (prepared as described above) was used as the template in the PCR amplification of a 1,259-bp fragment of the IS*1311* insertion sequence (40, 48). The PCR product was digested with the restriction endonuclease HinfI (Invitrogen) and separated on a 1.5% agarose gel as previously described (40, 48). Some copies of IS*1311* from C strains carry a point mutation creating a recognition site for the restriction endonuclease HinfI, resulting in an extra band on the gel (32).

**DGGE.** Two pairs of primers were used to amplify different regions of the MAP1506 locus by PCR. The template for these reactions was the entire MAP1506 gene locus, which had been previously amplified using a high-fidelity *Taq* polymerase. A 288-bp fragment of the MAP1506 gene locus (comprising nucleotides 1653357 to 1653644 of the *M. avium* subsp. *paratuberculosis* K10 strain; NCBI accession number AE016958.1) was amplified with a GC-clamp (shown in bold) on the forward primer (DGGE1F, 5′-**CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG**GTGGGGTGGATGAGTACGAC-3′; DGGE1R, 5′-CTGAAACGGCGTCACCTTAC-3′). A 259-bp fragment of the MAP1506 gene locus (comprising nucleotides 1653482 to 1653740 of the *M. avium* subsp. *paratuberculosis* K10 strain; NCBI accession number AE016958.1) was amplified with a GC-clamp (shown in bold) on the reverse primer (DGGE2F, 5′-GATGCAGGTCTCGCAGTTG-3′; DGGE2R, 5′-**CGCCCGGG GCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG**CTTGGTGGTGTTGG TCGAG-3′). PCRs (50-μl volumes) were performed with the Roche Expand high-fidelity PCR system and contained 2 μl template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, 15 pmol of each primer, 4% (vol/vol) dimethyl sulfoxide, 5 μl of the manufacturer's PCR buffer containing $MgCl_2$ (final concentration of $MgCl_2$, 1.5 mM), and 1.75 U of *Taq* polymerase. PCR conditions were denaturation at 94°C for 4 min followed by 30 cycles of PCR with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min. The final extension time was 10 min at 72°C. The products were separated by DGGE using a DCode universal mutation detection system (Bio-Rad, Ontario, Canada) and 16- by 16-cm by 1-mm gels. Eight-percent polyacrylamide gels were prepared and electrophoresed with 1× Tris-acetate-EDTA buffer. The gels contained a 55 to 65% (DGGE1 product) or a 50 to 65% (DGGE2 product and DGGE1/2 products combined) denaturing gradient of urea and formamide that increased in the direction of electrophoresis. A 100% denaturing solution contained 40% (vol/vol) formamide and 7.0 M urea. Electrophoresis was performed at a constant 75 V for 19 h at 60°C. Gels were stained in 0.1% ethidium bromide for 30 min and visualized with UV light.

**Nucleotide sequence accession numbers.** MAP1506 gene locus sequences for types I and III were deposited in GenBank under accession numbers EU200348 and EU200350, respectively.

## RESULTS

**IS*1311* PCR-REA and sequence analysis of MAP1506 gene loci from various isolates.** IS*1311* PCR-REA was performed on all *M. avium* subsp. *paratuberculosis* isolates (Table 1) to confirm C and S subtypes by use of a validated methodology.

Sequencing of the MAP1506 gene loci from the first 13 isolates shown in Table 1 (7 type II, 3 type I, and 3 type III) revealed a number of consistent nucleotide substitutions (nucleotides 293, 328, 411, and 542) between the C and S subtypes (Table 1). The transition of nucleotide 344 was not present in all S strains but was restricted to those with IS*900* RFLP profiles s2, s4, and s6 (Table 1), corresponding to IS*900* RFLP type III. A complete codon comprising nucleotides 944 to 946 was absent from all S isolates. All SNPs were nonsynonymous, leading to conservative amino acid substitutions. The MAP1506 gene locus was sequenced in an additional collection of 45 *M. avium* subsp. *paratuberculosis* isolates from different origins, comprising 37 C types, 1 B type (47), and 7 S types (according to IS*1311* PCR-REA typing). These sequencing results reliably confirmed the presence of the SNPs that differentiate between type I, type II, and type III isolates.

**DGGE.** The sequencing data were used to select polymorphic regions of the MAP1506 locus that would be suitable for subsequent PCR and DGGE analysis. A region of the MAP1506 locus encompassing the SNPs at nucleotide positions 293, 328, 344, and 411 (DGGE1 amplicon) was first selected for analysis but did not possess the discriminatory power to distinguish all C and S subtypes. A second region of the MAP1506 locus encompassing T→G (nucleotide 411) and T→C (nucleotide 542) transitions (DGGE2 amplicon) allowed discrimination of the C and S subtypes. DGGE1 and DGGE2 PCR amplicons were combined and electrophoresed on a single DGGE to demonstrate the discrimination of C isolates from two subtypes of S isolates (Fig. 1), corresponding to IS*900* RFLP types II, I, and III, respectively.

## DISCUSSION

Genomic differences between C and S subtypes of *M. avium* subsp. *paratuberculosis* have been previously demonstrated. Large-scale genomic deletions have been described for S

TABLE 1. SNPs in the MAP1506 gene locus of 58 *M. avium* subsp. *paratuberculosis* isolates, including three type I, seven type II, and three type III isolates with different IS*900* RFLP profiles

| Isolate | Host animal | Country | IS*1311* PCR-REA type[a] | IS*900* RFLP type[b] | IS*900* RFLP minor pattern differences[c] | SNP at: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | bp 293 | bp 328 | bp 344 | bp 411 | bp 542 | bp 944 | bp 945 | bp 946 |
| TC1613 | Bovine | United States | C | II | b1 | T | G | G | T | T | T | G | G |
| 6772 | Caprine | New Zealand | C | II | b2 | T | G | G | T | T | T | G | G |
| 5979 | Cervine | New Zealand | C | II | b3 | T | G | G | T | T | T | G | G |
| 7428 | Bovine | New Zealand | C | II | b4 | T | G | G | T | T | T | G | G |
| 87/8880 | Bovine | Australia | C | II | b5 | T | G | G | T | T | T | G | G |
| 6601 | Bovine | Australia | C | II | b6 | T | G | G | T | T | T | G | G |
| 316F | Bovine | United Kingdom | C | II | b7 | T | G | G | T | T | T | G | G |
| 6756 | Ovine | New Zealand | S | I | s1 | A | T | G | G | C | -[d] | - | - |
| 86-45 | Ovine | Canada | S | III | s2 | A | T | A | G | C | - | - | - |
| P133/79 | Ovine | Faeroe Islands | S | I | s3 | A | T | G | G | C | - | - | - |
| P465 | Ovine | Iceland | S | III | s4 | A | T | A | G | C | - | - | - |
| 6759 | Ovine | New Zealand | S | I | s5 | A | T | G | G | C | - | - | - |
| 85/14 | Ovine | Canada | S | III | s6 | A | T | A | G | C | - | - | - |
| LN20 | Porcine | Canada | S | III | s2 | A | T | A | G | C | - | - | - |
| 269ov | Ovine | Spain | S | ND[e] | ND | A | T | A | G | C | - | - | - |
| 11G | Ovine | Spain | S | ND | ND | A | T | A | G | C | - | - | - |
| Ovicap18 | Caprine | Spain | S | ND | ND | A | T | A | G | C | - | - | - |
| 311 | Caprine | Spain | S | ND | ND | A | T | A | G | C | - | - | - |
| M173/04 | Cervine | Netherlands | S | ND | ND | A | T | A | G | C | - | - | - |
| M214/04 | Cervine | Czech Republic | S | ND | ND | A | T | A | G | C | - | - | - |
| 571 | Leporine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| 834 | Bovine | Spain | C | ND | ND | T | G | G | T | T | T | G | G |
| 791 | Bovine | Spain | C | ND | ND | T | G | G | T | T | T | G | G |
| 808 | Bovine | Spain | C | ND | ND | T | G | G | T | T | T | G | G |
| Bison10.3 | Bison | United States | B[f] | ND | ND | T | G | G | T | T | T | G | G |
| ATCC19698[g] | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| K10 | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| D060162 | Bovine | Canada | C | ND | ND | T | G | G | T | T | T | G | G |
| D0616257 | Bovine | Canada | C | ND | ND | T | G | G | T | T | T | G | G |
| D0624010 | Bovine | Canada | C | ND | ND | T | G | G | T | T | T | G | G |
| D0635848 | Bovine | Canada | C | ND | ND | T | G | G | T | T | T | G | G |
| R0629132 | Bovine | Canada | C | ND | ND | T | G | G | T | T | T | G | G |
| R0632107 | Bovine | Canada | C | ND | ND | T | G | G | T | T | T | G | G |
| R0636342 | Bovine | Canada | C | ND | ND | T | G | G | T | T | T | G | G |
| F76 | Ovine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| JD143 | Ovine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| JD146 | Ovine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| JD18 | Bovine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| JD29 | Ovine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| M48/04 | Ovine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| M212/04 | Cervine | Czech Republic | C | ND | ND | T | G | G | T | T | T | G | G |
| M153/C | Caprine | Scotland | C | ND | ND | T | G | G | T | T | T | G | G |
| Map99 | Bovine | Denmark | C | ND | ND | T | G | G | T | T | T | G | G |
| Map103 | Bovine | Denmark | C | ND | ND | T | G | G | T | T | T | G | G |
| V20683474 | Bovine | Denmark | C | ND | ND | T | G | G | T | T | T | G | G |
| V20683587 | Bovine | Denmark | C | ND | ND | T | G | G | T | T | T | G | G |
| 9319 | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| 9346 | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| 7300 | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| 9286 | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| 9287 | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| 9354 | Bovine | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| Ben | Human | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| Map3 | Human | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| Linda | Human | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| Map4 | Human | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| Map5 | Human | United States | C | ND | ND | T | G | G | T | T | T | G | G |
| 04-4531 | Bovine | Canada | C | II | ND | T | G | G | T | T | T | G | G |

[a] IS*1311* PCR-REA according to the method of Motiwala et al. (40).
[b] IS*900* RFLP typing according to Pavlik et al. (42) and Collins et al. (15).
[c] Discrimination based on IS*900* RFLP minor pattern differences (M. Behr, McGill University, Canada, and D. Collins, AgResearch, New Zealand, personal communication).
[d] -, nucleotide deletion compared to the full genome sequence of *M. avium* subsp. *paratuberculosis* K10 (30).
[e] ND, not determined.
[f] IS*1311* type B (48).
[g] American Type Culture Collection (www.atcc.org).

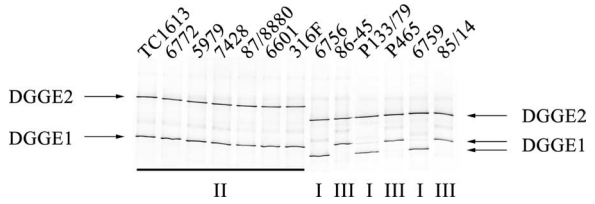


FIG. 1. DGGE1 and DGGE2 PCR products separated by DGGE. Using this technique, types I, II, and III of *M. avium* subsp. *paratuberculosis* can be differentiated. Isolate names are shown and identified as type I, II, or III.

strains (33, 34) and three smaller deletions totaling 978 bp were found in C strains (20). In a recent study comparing a C and an S isolate of *M. avium* subsp. *paratuberculosis*, 11 SNPs were discovered in a region covering 12 kb (35). Another study examined polymorphisms in the *gyrA* (2,233 bp) and *gyrB* (1,838 bp) genes in multiple type I, II, and III strains and found four and five SNPs, respectively (9). In the current study, we found four SNPs and one codon deletion in a 1,224-bp region of the *M. avium* subsp. *paratuberculosis* PPE gene, the MAP1506 locus, consistently differing between all C ($n = 45$) and S ($n = 13$) isolates that were examined. The results from our study and the other recent studies (17, 35) comparing the C and S strains of *M. avium* subsp. *paratuberculosis* suggest that SNPs may be quite common between the two subtypes. Clearly, a more accurate understanding of the number of SNPs between strains or subspecies will be achieved when larger regions or whole genomes are compared.

All of the conserved MAP1506 locus polymorphisms that were found in this study in the ovine subtype S are also present in two MAP1506 locus homologs (MAV_2924 and MAV_2926) in the genetically similar (3) *M. avium* subsp. *avium*. This finding is in agreement with a previous suggestion that the S strain is an evolutionary intermediate between *M. avium* subsp. *avium* and the C strain of *M. avium* subsp. *paratuberculosis* based on SNPs in IS*1311* (52) and by the discovery of three polymorphic regions unique to the S strain of *M. avium* subsp. *paratuberculosis* (20). Several studies (35, 51) show that SNPs are a major source of genotypic variation within the *M. avium* complex, and as demonstrated by Semret et al. (47), SNPs can be used in conjunction with large sequence polymorphisms to identify possible evolutionary paths within the *M. avium* complex. Further detailed investigations of a larger number of *M. avium* subsp. *avium* isolates are required to understand the phylogenetic and ancestral relationship of the *M. avium* complex.

Previously, a correlation of SNPs with phenotypic diversity has been demonstrated in some mycobacterial species, thereby encouraging its use for bacterial strain differentiation (1, 8, 23, 35). Moreover, SNPs are frequently used in epidemiological and evolutionary studies to differentiate between closely related species, subspecies, and strains of bacteria without knowledge of what effect the SNP may have on gene function or protein activity (1, 24, 27, 28, 49). However, SNPs in the *rpoV* and *mma3* genes of *M. bovis* have a marked impact on virulence and cellular functions, and SNPs in *M. tuberculosis* are thought to be responsible for altered phenotypes (5, 16). Targeting functional coding genes for strain typing purposes, as recently done with the *gyrA* and *gyrB* genes of *M. avium* subsp. *paratuberculosis* (9), might have advantages over targeting noncoding insertion sequences in *M. avium* subsp. *paratuberculosis* DNA to discover differences that are linked with host specificity.

We reasoned that selecting a surface-exposed PPE protein would increase our chances of locating SNPs. Although it has not been proven that SNPs occur more frequently in genes coding for surface-exposed proteins, it is tempting to hypothesize that selection pressures occurring specifically at the cell surface, e.g., host immune reactions (2), environmental interactions, and bacteriophage binding (43), favor certain mutations. As demonstrated in this study, sequence polymorphisms in the MAP1506 gene locus are detectable by DGGE and allow discrimination of *M. avium* subsp. *paratuberculosis* isolates corresponding to known IS*900* RFLP subtypes I, II, and III. Discrimination of C and S subtypes is possible by PCR (14), IS*1311* PCR-REA (32), or specific-locus PCR (20), but the additional differentiation of subtypes I and III within the S isolates illustrates the usefulness of DGGE as a new method to subtype *M. avium* subsp. *paratuberculosis* isolates. Moreover, DGGE, being a PCR-based technique, will be of great advantage in the subtyping of difficult-to-culture or nonculturable *M. avium* subsp. *paratuberculosis* organisms as well as *M. avium* subsp. *paratuberculosis* DNA extracted from tissues, blood, or milk (both human and veterinary origin). In short, MAP1506 locus DGGE is a valuable tool to characterize isolates when there is not enough bacterial growth to perform pulsed-field gel electrophoresis or IS*900* RFLP.

In this study, the resolving power of DGGE was used to separate and differentiate amplicons of a gene containing SNPs. While DGGE is usually used to characterize multispecies bacterial communities (41), single-band DGGE has previously only been used with *Mycobacterium* sp. to analyze and detect polymorphisms in genes associated with antibiotic resistance in *M. tuberculosis* (36, 37, 45, 46) or in the 16S rRNA gene (29). The DGGE technique developed in this study has added discriminatory power because it can discriminate two types within the S type, while IS*1311* PCR-REA cannot discriminate any subtypes within the S type. In this respect, this technique complements other rapid molecular techniques and may have future use as additional informative SNPs are discovered in other *M. avium* subsp. *paratuberculosis* genes that have significance in host specificity.

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