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Molecular Characterization of Pigmented and Nonpigmented Isolates of *Mycobacterium avium* subsp. *paratuberculosis*

Karen Stevenson,[1]* Valerie M. Hughes,[1] Lucía de Juan,[2] Neil F. Inglis,[1] Frank Wright,[3] and J. Michael Sharp[1]

Moredun Research Institute, Penicuik EH26 0PZ,[1] and Biomathematics and Statistics Scotland, Scottish Crop Research Institute, Dundee DD2 5DA,[3] Scotland, United Kingdom, and Departamento de Patología Animal I (Sanidad Animal), Facultad de Veterinaria, Universidad Complutense de Madrid, 28040 Madrid, Spain[2]

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Five pigmented isolates of *Mycobacterium avium* subsp. *paratuberculosis* were examined by pulsed-field gel electrophoresis (PFGE), IS*900* restriction fragment length polymorphism (IS*900*-RFLP), and IS*1311* polymorphism analysis using PCR. All of the pigmented isolates exhibited one of three distinct PFGE profiles with *Sna*BI, designated 9, 10, and 11, and with *Spe*I, designated 7, 8, and 9, which generated three multiplex profiles designated [9-7], [10-8], and [11-9]. All of the pigmented isolates had the same IS*900*-RFLP *Bst*EII and *Pvu*II profiles. The IS*900*-RFLP *Bst*EII profile was new, but the IS*900*-RFLP *Pvu*II profile corresponded to *Pvu*II type 6 of a sheep strain described by Cousins and colleagues (D. V. Cousins, S. N. Williams, A. Hope, and G. J. Eamens, Aust. Vet. J. 78:184-190, 2000). IS*1311*-PCR analysis typed all of the pigmented isolates as sheep (S) strains. The genetic relationship between pigmented and nonpigmented isolates was investigated by using multiplex PFGE data from the analysis of both the 5 pigmented isolates and 88 nonpigmented isolates of *M. avium* subsp. *paratuberculosis* from a variety of host species and geographic locations. It was possible to classify the isolates into two distinct types designated type I, comprising the pigmented isolates, and type II, comprising the nonpigmented isolates, which exhibit a very broad host range.

Mycobacterium avium subsp. *paratuberculosis* is the etiological agent of paratuberculosis (Johne's disease), a fatal chronic granulomatous enteritis affecting principally ruminants. The organism is distinguished from other subspecies of *M. avium* by its mycobactin requirement for in vitro growth (22) and by the presence of the insertion sequence IS*900* (9). Molecular typing procedures such as pulsed-field gel electrophoresis (PFGE) and restriction fragment length polymorphism (RFLP) coupled with hybridization to IS*900* (IS*900*-RFLP) have shown that in comparison with other pathogens, there is relatively little genetic variability within this subspecies. However, there is a small subset of isolates distinguished by their prominent yellow or orange pigment that have not been characterized fully.

Pigmented *M. avium* subsp. *paratuberculosis* has been isolated in cases of lepromatous ovine paratuberculosis (18–20). In such cases, the affected intestine appears yellow or orange, probably due to the very large number of organisms present in the lesions. The organisms are difficult to culture, and few isolates have been maintained in collections. The pigmentation appears to be an inherent characteristic, as it is present at all stages of growth and is not altered by animal passage. The pigmented strains appear to have a host preference for sheep, although they can produce disease in cattle following experimental infection (21) and there has been one report of a naturally occurring bovine case (24). The purpose of this study was to analyze available pigmented isolates and investigate their genetic relatedness to other, nonpigmented *M. avium* subsp. *paratuberculosis* isolates.

* Corresponding author. Mailing address: Moredun Research Institute, Pentlands Science Park, Bush Loan, Penicuik EH26 0PZ, Scotland. Phone: 44 131 445 5111. Fax: 44 131 445 6111. E-mail: stevk@mri.sari.ac.uk.

MATERIALS AND METHODS

***M. avium* subsp. *paratuberculosis* isolates.** Five pigmented and 88 nonpigmented strains of *M. avium* subsp. *paratuberculosis* were analyzed in this study. Four of the pigmented strains were isolated at the Moredun Research Institute, Penicuik, Scotland, and Finn Saxegaard (National Veterinary Institute, Oslo, Norway) kindly provided pigmented strain 21P from the Faroe Islands. Nonpigmented isolates were obtained from the Veterinary Sciences Division of the Scottish Agricultural College, Perth, Scotland, the Veterinary Laboratories Agency, Starcross, England, and Queens University of Belfast, Belfast, Northern Ireland. *M. avium* subsp. *paratuberculosis* reference strains ATCC 19698 and NCTC 8578 were also included in the study. The nonpigmented isolates were obtained from a variety of tissues or fecal samples from infected animals, and 13 bovine isolates were cultured from milk samples as part of a survey funded by the Food Standards Agency (B08001). Nonpigmented isolates originated from different host species including cattle, sheep, goat, and deer; they also included isolates collected in a previous study by Beard et al. (2) from a number of wildlife species including rabbit (*Oryctolagus cuniculus*), hare (*Lepus europaeus*), fox (*Vulpes vulpes*), stoat (*Mustela erminea*), weasel (*Mustela nivalis*), badger (*Meles meles*), rat (*Rattus norvegicus*), wood mouse (*Apodemus sylvaticus*), rook (*Corvus frugilegus*), and crow (*Corvus corone*). The nonpigmented isolates were from different geographical areas within the United Kingdom: seven regions in Scotland, seven counties in England, and Northern Ireland (five isolates).

All isolates were propagated on slopes of Middlebrook 7H11 agar medium supplemented with 20% (vol/vol) heat-inactivated newborn calf serum, 2.5% (vol/vol) glycerol, 2 mM asparagine, 10% (vol/vol) Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment medium (Becton Dickinson, Oxford, Oxfordshire, United Kingdom), 2 Selectatabs (code MS 24; MAST Laboratories Ltd., Merseyside, United Kingdom) per liter, and 2 μg of mycobactin J (Allied Monitor, Fayette, Mo.) ml^{-1} for 8 to 10 weeks for nonpigmented isolates and as long as 6 months for pigmented isolates at 37°C. All isolates examined were fresh or low-passage-number isolates. Isolates were stored as glycerol stocks at −70°C.

PCR analysis. All *M. avium* subsp. *paratuberculosis* isolates were screened for the presence of IS*900* and IS*1311* insertion sequences. Briefly, a single bacterial colony was suspended in 200 μl of sterile distilled water. Mycobacteria were lysed

by using a Hybaid (Ashford, Middlesex, United Kingdom) RiboLyser at 5.5 m s^{-1} for 20 s, with cooling on ice before and after treatment. DNA was extracted by using guanidine hydrochloride (4) and was analyzed by PCR. Alternatively, DNA was prepared in low-melting-point agarose plugs as described below. Amplified products were detected on agarose gels. PCR analysis for IS*900* was carried out by using the primers and PCR conditions described by Sanderson et al. (17). The IS*900* PCR product was verified by hybridization to a 2,4-dinitrophenyl-labeled oligonucleotide probe complementary to the amplified sequence (2) and by restriction analysis with *Alw*I as reported by Cousins et al. (6). PCR analysis for IS*1311* and restriction analysis to discriminate between other *M. avium* subspecies and *M. avium* subsp. *paratuberculosis* and between cattle and sheep strains were performed according to the work of Marsh et al. (12).

IS*900* sequencing. IS*900* was amplified from pigmented strain M189 as described above. The PCR product was excised from the agarose gel and extracted by using AgarACE (Promega, Southampton, United Kingdom) according to the manufacturer's instructions. The DNA was cloned into pGEM-T (Promega) and was sequenced on an ABI 377 sequencer. Sequence analysis was performed by using the software package Vector NTI (InforMax Inc., Oxford, United Kingdom).

PFGE analysis. DNA for PFGE analysis was prepared from stirred broth cultures of *M. avium* subsp. *paratuberculosis* isolates as described previously (10). Briefly, 10 ml of Middlebrook 7H9 broth (supplemented with 0.4% [wt/vol] Tween 80, 10% [vol/vol] OADC, and 2 μg of mycobactin J ml^{-1}) was inoculated with a single colony of *M. avium* subsp. *paratuberculosis* and incubated at 37°C with continuous stirring to prevent clumping of cells. When the cell density was at least approximately McFarland standard 2, cell suspensions were quantified by optical density by using a Densimat (BioMerieux, Lyon, France) and then harvested by centrifugation, washed, and resuspended in modified spheroplasting buffer. Bacterial suspensions (6×10^9cells ml^{-1}) were mixed with an equal volume of 1.5% (wt/vol) low-melting-point agarose and poured into plug molds. Plugs were incubated in Tris-EDTA (pH 8) containing 1 to 2 mg of lysozyme ml^{-1} for at least 18 h at 37°C and then in 500 mM EDTA containing 1%(wt/vol) sarcosine and 1 to 2 mg of proteinase K ml^{-1} for 7 days at 55°C.

Slices (inserts) 3 to 5 mm thick were cut from the plugs, washed six times in 5 ml of Tris-EDTA (pH 8), preequilibrated in commercial restriction buffer containing bovine serum albumin, and then incubated with a restriction reaction mixture containing 10 U of restriction endonuclease ml^{-1}. After overnight incubation, fresh restriction reaction mixture was added and incubation was continued for at least another 3 h to ensure complete digestion of the sample. Restricted samples were loaded onto a 1% (wt/vol) agarose gel and electrophoresed in 0.5× TBE (44.5 mM Tris, 44.5 mM boric acid, 1 mM EDTA). *Sna*BI and *Spe*I restriction enzymes were purchased from New England Biolabs (Hitchin, Hertfordshire, United Kingdom).

Electrophoresis was conducted using a CHEF Mapper (Bio-Rad, Hemel Hempstead, Hertfordshire, Hertfordshire, United Kingdom). Parameters for electrophoresis were as follows. For *Sna*BI restriction fragments, initial and final switch times were 6.75 and 26.29 s, respectively, and with switch times ramped linearly; the gradient was 6 V cm^{-1}; the included angle was 120°; and electrophoresis was carried out at 14°C for 40 h. For *Spe*I restriction fragments, initial and final switch times were 2.16 and 35.38 s, respectively, and with switch times ramped linearly; the gradient was 6 V cm^{-1}; the included angle was 120°; and electrophoresis was carried out at 14°C for 23 h. Lambda midrange markers (catalog no. 355-2; New England Biolabs) were loaded onto gels as molecular size standards.

Nomenclature. The different PFGE profiles for each enzyme were assigned numbers, with the ATCC 19698 type strain designated profile 1 in each case. This system was used to distinguish the nomenclature for PFGE from the alphabetical and numerical system used for IS*900*-RFLP (14). The different *Sna*BI-*Spe*I multiplex PFGE profiles have been expressed as the *Sna*BI and *Spe*I profile numbers joined by a hyphen and enclosed in square brackets. For example, the multiplex PFGE profile of an isolate that has a *Sna*BI profile of 2 and a *Spe*I profile of 3 is expressed as [2-3]. This system was chosen because it gives some indication of the genetic relationship between isolates, and adding new profiles is easy. A database of the PFGE profiles is available at www.mri.sari.ac.uk/Bacteriology/PFGE-mycobacteria.

Phylogenetic analysis. Electronic images of PFGE profiles were initially analyzed by using the computer software package Imagemaster, version 3.0 (Amersham Pharmacia Biotech). Phylogenetic analysis was carried out on the PFGE data from this study, encompassing a total of 16 multiplex PFGE profiles. The PFGE data from the *Spe*I and *Sna*BI restriction enzyme digestions were combined to form a matrix of the 16 multiplex PFGE profiles and the presence or absence of 84 bands. The PHYLIP Phylogeny Inference Package version 3.6 2001 was used for the phylogenetic analysis, and the data were analyzed by two approaches; a distance-based approach and the maximum parsimony approach. Differences in band presence or absence are due to nucleotide substitution or processes that result in length differences (e.g., insertion, deletion, or duplication events) in the nucleotide sequences. If nucleotide substitution is the only evolutionary process acting, it is possible to infer changes at the nucleotide level from the band presence or absence data and to construct phylogenetic trees with branch lengths proportional to the genetic change (nucleotide substitutions per position). Modelling both nucleotide substitution and insertion or deletion events for PFGE data is not currently possible, so a simple parsimony analysis is a reasonable approach. Both approaches were used, since the *M. avium* subsp. *paratuberculosis* genome is known to contain a number of insertion sequences.

(i) Distance-based approach. Genetic distances were calculated by using the RESTDIST program (PHYLIP package, version 3.6) with plausible values for the nucleotide substitution process (transition/transition ratio = 2; gamma rate heterogeneity parameter alpha = 4) based on the Jin/Nei model. The distance matrix was then analyzed by the Fitch-Margoliash method (with the FITCH program in the PHYLIP package).

(ii) Maximum parsimony approach. The data were analyzed by using the PHYLIP DOLLOP program (Dollo parsimony method for binary data). Statistical testing of the clusters within the phylogenetic tree for both distance-based and parsimony analysis was carried out by using bootstrapping with 100 trials.

IS*900*-RFLP analysis. DNA for RFLP analysis was prepared in low-melting-point agarose plugs, washed, and treated with restriction endonucleases as described for PFGE analysis. DNA was restricted with *Bst*EII (catalog no. R6641; Promega UK Ltd.) or *Pvu*II (catalog no. 642 690; Roche) by using the conditions recommended by the manufacturer. Following digestion, inserts were melted in a water bath at 70°C for 5 min and then loaded onto a 0.8% (wt/vol) agarose gel. DNA fragments were separated by conventional gel electrophoresis in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 20 V overnight. Lambda-*Hin*dIII and 1-kb DNA ladders (catalog no. G5711; Promega UK Ltd.) were used as molecular size markers.

After electrophoresis, the gel was treated with denaturation solution (1.5 M sodium chloride–0.5 M sodium hydroxide) for 1 h followed by neutralizing solution (1.5 M sodium chloride–0.5 M Tris-HCl adjusted to pH 7.5) for 1 h. All incubations were carried out at room temperature with constant gentle agitation. DNA fragments were transferred to Hybond-N nylon membranes (Amersham Pharmacia) by standard blotting techniques (11) and heat fixed onto the membranes for 2 h at 80°C. DNA was hybridized overnight at 65°C to a probe derived from IS*900* and randomly labeled by using the Gene Images random prime-labeling module (catalog no. RPN 3540; Amersham Pharmacia). The membrane was washed once for 5 min in 1× SSC (0.15 M NaCl plus 0.015 M sodium citrate [pH 7])–0.1% (wt/vol) sodium dodecyl sulfate at 42 to 65°C, followed by three 5-min washes in 0.1× SSC–0.1% (wt/vol) sodium dodecyl sulfate at 65°C, and then wiped with a tissue soaked in 0.1× SSC at 65°C. Detection of the fluorescein-labeled probe was carried out by using the Gene Images CDP-Star detection module (catalog no. 3510; Amersham Pharmacia) according to the manufacturer's instructions.

RESULTS

All of the pigmented isolates analyzed in this study were obtained from lepromatous cases of ovine paratuberculosis, and the intestines were characteristically pigmented (Fig. 1). The organisms grew very slowly in culture and were acid fast as determined by Ziehl-Neelsen staining. Single colonies were smooth, convex, and glistening with a yellow pigment. By contrast, colonies of nonpigmented isolates of *M. avium* subsp. *paratuberculosis* were rough and had a creamy color. The pigmented organisms were coccobacillary as opposed to the bacillary nonpigmented isolates, as determined by light microscopy.

All of the pigmented isolates and nonpigmented isolates of *M. avium* subsp. *paratuberculosis* analyzed in this study were found to be positive for IS*900* by PCR. Since IS*900*-like sequences have been detected by PCR in a few other species of mycobacteria (6), the PCR products were verified as IS*900* by restriction analysis and hybridization to a probe. In addition, the PCR product from pigmented isolate M189 was se-

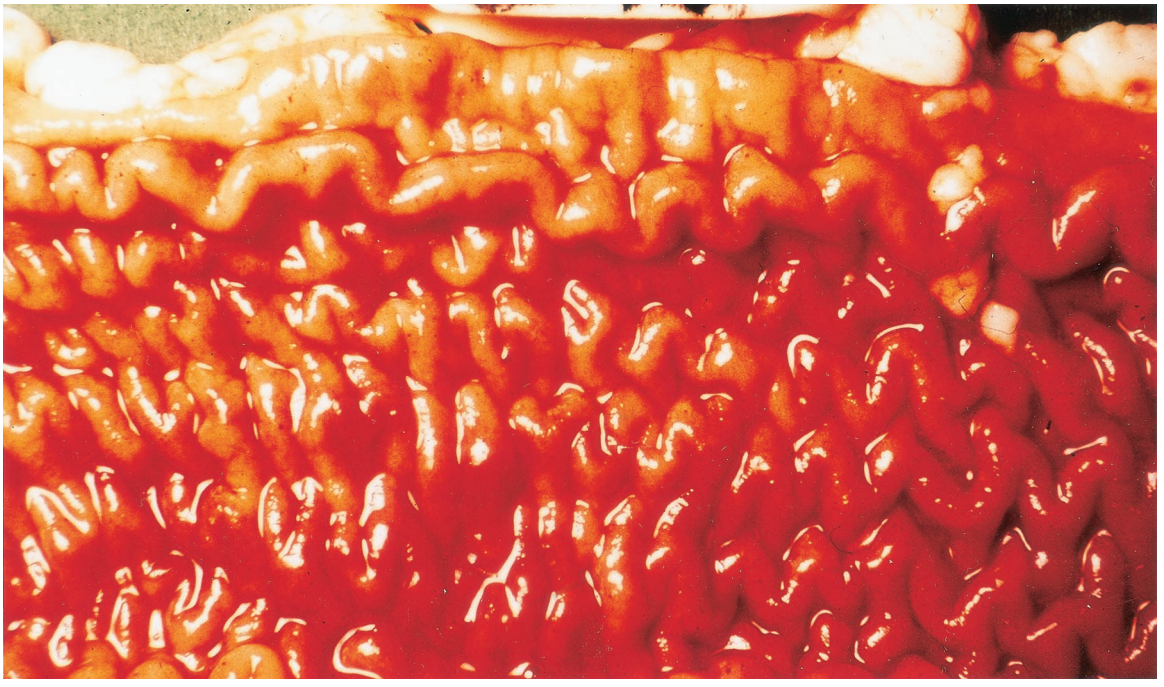

FIG. 1. Opened segment of ileum from a case of pigmented ovine paratuberculosis.

quenced, and the sequence was found to be identical to that reported for *M. avium* subsp. *paratuberculosis* (GenBank accession number X16293). All isolates analyzed in this study possessed IS*1311* sequences and were confirmed as *M. avium* subsp. *paratuberculosis* by restriction of the PCR product with *Mse*I (26). Restriction of the IS*1311* PCR product with *Hin*fI detects a point mutation at base position 223 in C strains, isolated mainly from cattle, that is absent in S strains, isolated mainly from sheep. Restriction analysis of the isolates revealed that the pigmented isolates were of the S type and the nonpigmented isolates, including those isolated from sheep, were of the C type.

The polymorphisms that define all of the pulsed-field types are clearly visible under the specified running conditions. Combining the results of *Sna*BI and *Spe*I generates 16 multiplex PFGE profiles (Table 1). All of the isolates were analyzed by multiplex PFGE using the enzymes *Sna*BI and *Spe*I. Restriction of the pigmented isolates with these enzymes yielded profiles distinct from those of the nonpigmented isolates. Three distinct profiles, designated 9, 10, and 11, were identified with

TABLE 1. PFGE and RFLP profiles of *M. avium* subsp. *paratuberculosis* reference isolates

Isolate[a]	Host species	Geographic origin	Multiplex PFGE profile	*BstE*II RFLP profile[b]	*Pvu*II RFLP profile[c]
ATCC 19698	Bovine	Unknown	[1-1]	C1	1
99PW	Bovine	Staffordshire, England	[2-1]	C5	N_{pa}[d]
JD146	Ovine	Perth & Kinross, Scotland	[2-2]	C17	3
JD29	Ovine	Angus, Scotland	[3-1]	C17	3
R197	Leporine	Perth & Kinross, Scotland	[3-2]	C17	3
F76	Ovine	Perth & Kinross, Scotland	[3-3]	C17	3
377PS	Bovine	Shropshire, England	[4-6]	N_{ca}	1
JD18	Bovine	Aberdeenshire, Scotland	[5-1]	C17	3
JD143	Ovine	Perth & Kinross, Scotland	[5-2]	C17	3
307R	Bovine	Lancashire, England	[6-5]	C5	N_{pa}
808R	Bovine	Northern Ireland	[7-1]	C5	N_{pa}
743PSS	Bovine	Midlothian, Scotland	[7-5]	C1	1
377PW	Bovine	Shropshire, England	[8-4]	N_{cb}	2
M189	Ovine	Midlothian, Scotland	[9-7]	N_{cc}	6
213G, 208G, 235G	Ovine	Shetland, Scotland	[10-8]	N_{cc}	6
21P	Ovine	Faroe Islands	[11-9]	N_{cc}	6

[a] Pigmented isolates are boldfaced.
[b] RFLP designations according to the work of Pavlik et al. (15).
[c] RFLP designations according to the work of Whipple et al. (25).
[d] N_{ca}, N_{cb}, N_{cc}, N_{pa}, new profiles not previously published.

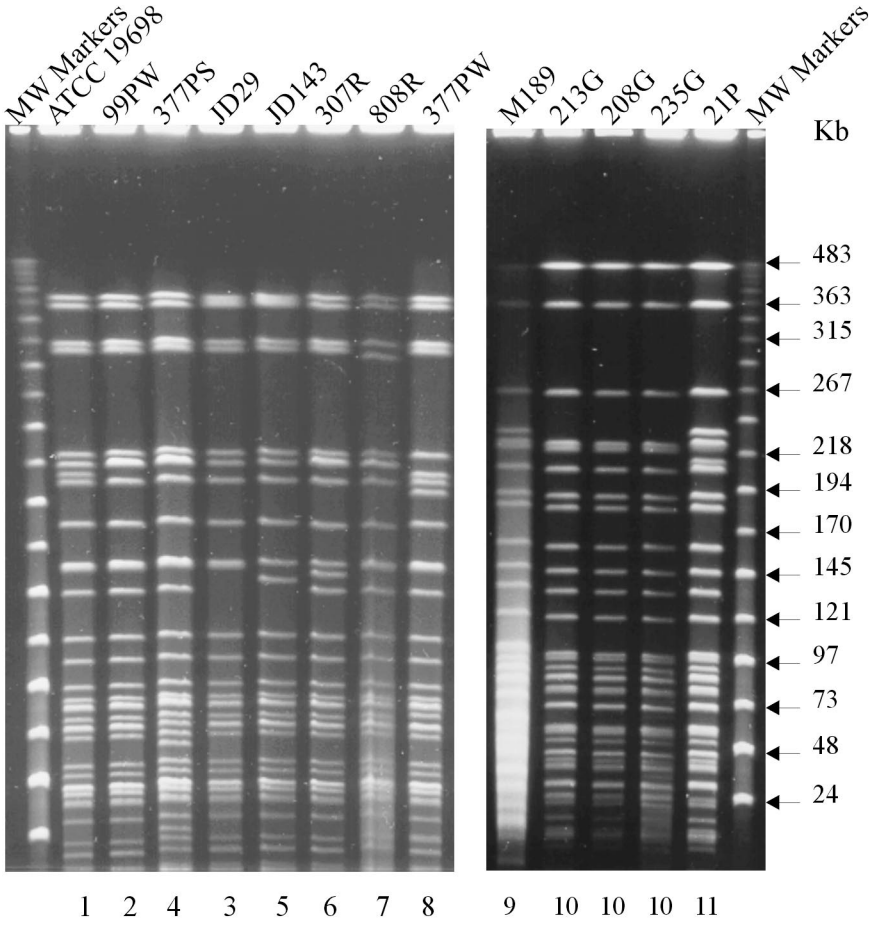

FIG. 2. PFGE profiles of *M. avium* subsp. *paratuberculosis* isolates generated with *Sna*BI. (Left) Profiles of nonpigmented isolates; (right) profiles of pigmented ovine isolates. Numbers above the lanes are designations of isolates, details for which are given in Table 1. Numbers below the lanes represent the different PFGE *Sna*BI reference types. PFGE running conditions are described in Materials and Methods.

*Sna*BI (Fig. 2), and three distinct profiles, designated 7, 8, and 9, were identified with *Spe*I (Fig. 3), which generated three multiplex profiles designated [9-7], [10-8], and [11-9] (Table 1). A total of 88 nonpigmented isolates of *M. avium* subsp. *paratuberculosis* were analyzed by multiplex PFGE. By use of *Sna*BI, eight PFGE types were detected, which were designated 1 to 8, with ATCC 19698 designated as type 1. The PFGE profiles of eight representative strains are presented in Fig. 2. When the nonpigmented isolates were typed with *Spe*I, six PFGE profiles were detected. The PFGE profiles of six representative strains are presented in Fig. 3. Of the nonpigmented isolates, 18 were from an ovine host. Thirteen of these ovine isolates were found to have multiplex profile [2-1], two isolates had [5-2], and one isolate each had multiplex profile [2-2], [3-1], or [3-3].

All of the pigmented isolates and one representative nonpigmented strain for each of the multiplex PFGE profiles were analyzed by IS*900*-PCR using *Bst*EII and *Pvu*II. All of the pigmented isolates had the same IS*900*-RFLP *Bst*EII profile (Fig. 4). This profile appears to be similar to the IS*900*-RFLP *Bst*EII S profiles previously described by Collins et al. (5), Whittington et al. (27), and Cousins et al. (7). The pigmented isolates had identical IS*900*-RFLP *Pvu*II profiles (Fig. 5), which appeared to correspond to *Pvu*II type 6 (described by Cousins et al. [7]). In contrast, nonpigmented isolates representative of the different multiplex PFGE profiles were found to have *Bst*EII profiles corresponding to C1, C5, and C17 (defined by Pavlik et al. [15]) and two new profiles not previously published (Table 1) and *Pvu*II profiles corresponding to types 1, 2, and 3 (defined by Whipple et al. [25]) and one new profile not previously described.

Both a distance-based approach and the maximum parsimony approach were used to estimate the genetic relatedness of pigmented to nonpigmented isolates. The results of the distance-based approach are given below and in Fig. 6. The consensus best tree and the bootstrap support values obtained by the parsimony approach were very similar to those obtained by the distance-based approach (data not shown).

The phylogenetic tree in Fig. 6 is resolved into two significant clusters: a minor cluster of 3 profiles (containing profiles [9-7], [10-8], and [11-9] representing the pigmented isolates) and a major cluster of 13 profiles (containing all the profiles of nonpigmented isolates, including bovine, ovine, cervine, caprine, and wildlife *M. avium* subsp. *paratuberculosis* isolates). Bootstrap support values greater than 50% are shown in the figure, although only those greater than 70% are considered

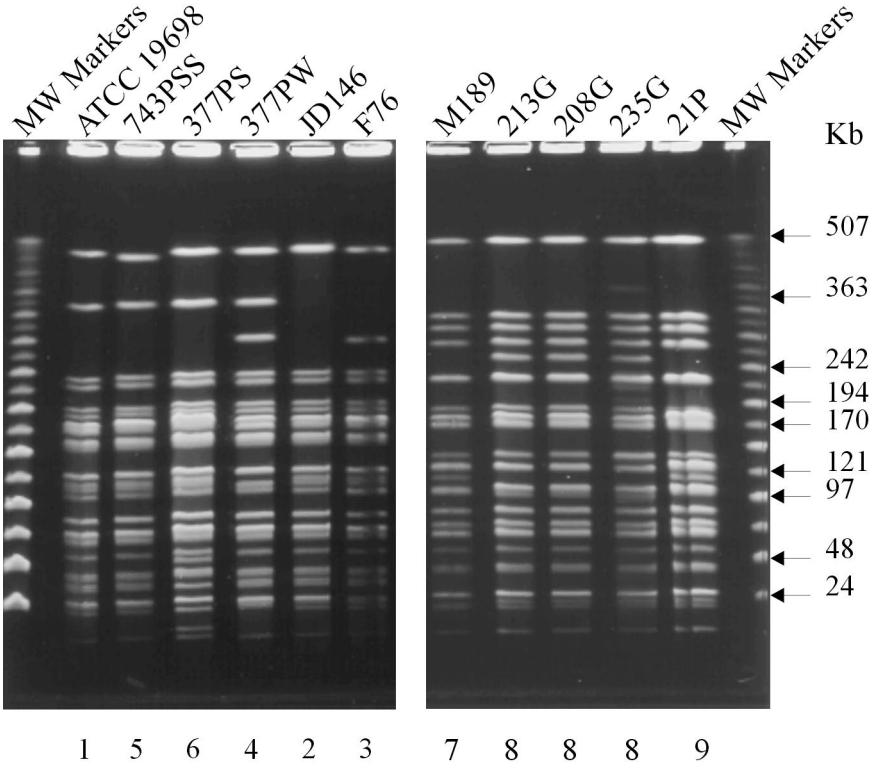

FIG. 3. PFGE profiles of *M. avium* subsp. *paratuberculosis* isolates generated with *Spe*I. (Left) Profiles of nonpigmented isolates; (right) profiles of pigmented ovine isolates. Numbers above the lanes are designations of isolates, details for which are given in Table 1. Numbers below the lanes represent the PFGE *Spe*I reference types. PFGE running conditions are described in Materials and Methods.

statistically significant. The branching order within the clusters is not statistically significant.

If the difference between profiles is due only to nucleotide substitution events, then the branch lengths from the distance-based analysis give an idea of the divergence in the nucleotide sequences. The long branch between the two clusters is 0.027 nucleotide substitutions per position, representing approximately 2.7% change.

DISCUSSION

This is the first report of the molecular characterization of pigmented isolates of *M. avium* subsp. *paratuberculosis* and the most comprehensive multiplex PFGE study of *M. avium* subsp. *paratuberculosis* isolates to date. Molecular typing by PFGE, IS*900*-RFLP, and IS*1311*-PCR has revealed that these isolates are genetically closely related to the S strains described by Collins et al. (5), de Lisle et al. (8), Bauerfeind et al. (1), Cousins et al. (7), and Whittington et al. (27). *M. avium* subsp. *paratuberculosis* isolates analyzed by PFGE to date appear to segregate into two distinct clusters; cluster I encompasses the pigmented ovine isolates, and cluster II comprises isolates from a wider host range. Isolates within a cluster share many phenotypic and genetic characteristics but also show a small amount of genetic heterogeneity. This division into two clusters supports the earlier observation that two forms of ovine paratuberculosis exist in Britain, one caused by a strain of the organism prevalent in cattle and one by a slow-growing pigmented strain (19, 20). The data are also consistent with the

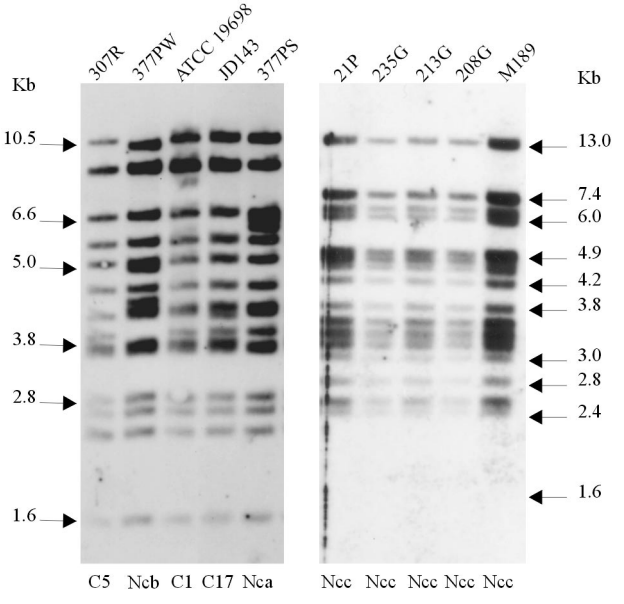

FIG. 4. IS*900*-RFLP profiles of *M. avium* subsp. *paratuberculosis* isolates generated with *Bst*EII. (Left) Profiles of nonpigmented isolates; (right) profiles of pigmented ovine isolates. Numbers above the lanes are designations of isolates, details for which are given in Table 1. Numbers below the lanes represent the IS*900*-RFLP types as defined by Pavlik et al. (15). N_{ca}, N_{cb}, and N_{cc}, new profiles not previously published.

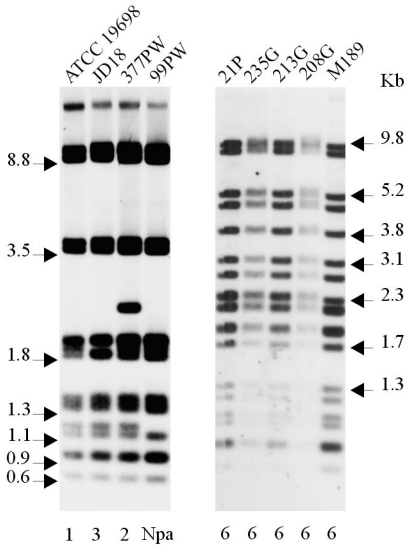

FIG. 5. IS*900*-RFLP profiles of *M. avium* subsp. *paratuberculosis* isolates generated with *Pvu*II. (Left) Profiles of nonpigmented isolates; (right) profiles of pigmented ovine isolates. Numbers above the lanes are designations of isolates, details for which are given in Table 1. Numbers below the lanes represent the IS*900*-RFLP types as defined by Whipple et al. (25).

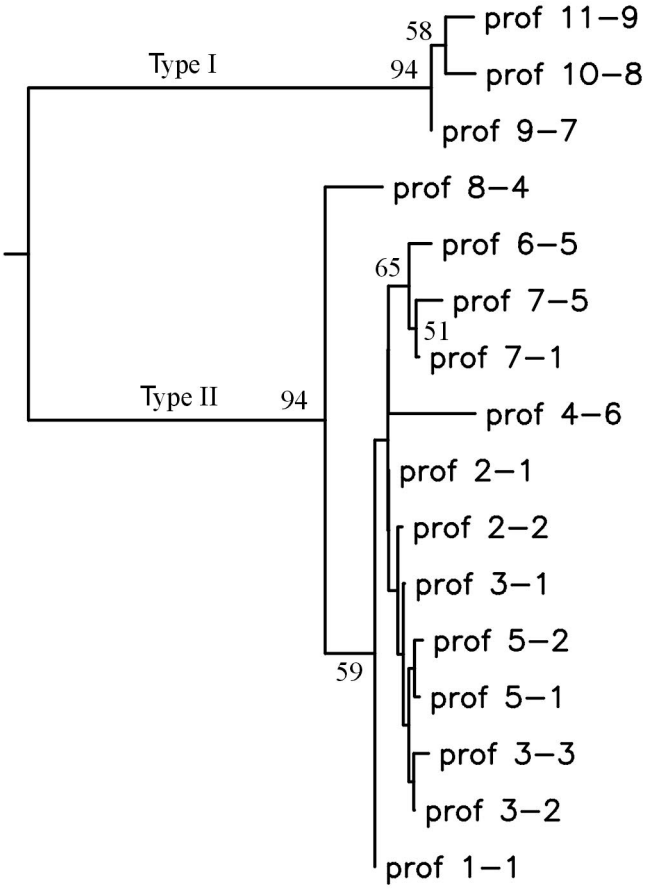

FIG. 6. Dendrogram of DNA convergence generated from the 16 multiplex PFGE profiles (prof) of pigmented and nonpigmented isolates of *M. avium* subsp. *paratuberculosis*. Bootstrap support values greater than 50% are shown, although only those greater than 70% are considered statistically significant.

division of isolates into two types of strains, S and C, as reported by Collins et al. (5) and Bauerfeind et al. (1). The nomenclature used for this strain classification is confusing and misleading, as it suggests that there is an exclusive correlation between strain type and host species of origin. While there may be circumstantial evidence for strong host preferences, there is no evidence for host-specific strains of *M. avium* subsp. *paratuberculosis*. In Australia and New Zealand, S strains are the predominant strains isolated from sheep (7, 27). However, in Europe, where paratuberculosis is endemic, C strains are more commonly isolated from sheep as well as from cattle and nonruminant hosts (13, 16). Furthermore, pigmented isolates can infect and produce disease in cattle (21, 24; B. Hanusson, personal communication), and S strains from the Faroe Islands, Iceland, and Australia have been isolated from cattle (28). Therefore, we propose that the two genetically distinct clusters of isolates be referred to as type I and type II. Type I comprises the slow-growing strains that appear to have a strong host preference for sheep and may be more virulent for sheep (18). These include the pigmented strains, ovine isolates from Iceland (28), Morocco (1), South Africa (1, 8), Australia (7, 27), and New Zealand (5), and a few Norwegian caprine strains (5, 8, 23). Type II includes the faster-growing strains commonly isolated from cattle and a broad host range including humans and a variety of wildlife species (2).

The geographic distribution of pigmented strains is intriguing. In Britain, cases of pigmented paratuberculosis have been reported in Scotland and England (18, 19). Based on records kept by the Scottish Agricultural College Veterinary Sciences Division in Scotland, between 1996 and 2000 approximately 19% of confirmed cases of ovine paratuberculosis were caused by pigmented isolates (A. Greig, personal communication). Pigmented ovine paratuberculosis appears to be more prevalent in southern Scotland. No cases of pigmented bovine paratuberculosis were reported during this period, and no type I nonpigmented strains have been isolated as far as is known. However, in Scotland most cases of paratuberculosis are diagnosed on the basis of serological testing or fecal smears, and cases of pigmented paratuberculosis are likely to be diagnosed only at a postmortem examination or at the abattoir. Elsewhere in Europe, pigmented ovine paratuberculosis has been reported in the Faroe Islands (F. Saxegaard, personal communication; B. Hanusson, personal communication) and Spain (R. A. Juste, personal communication). Outside Europe, there are no reported cases of pigmented paratuberculosis except in Morocco, where 3 of 56 ovine isolates were found to be pigmented (3). Despite the prevalence of nonpigmented type I strains in sheep in Australia and New Zealand, there has been no known case of pigmented paratuberculosis in these countries (D. Kennedy, personal communication; G. W. de Lisle, personal communication). Why the distribution of pigmented strains should be restricted to certain geographic regions is a matter for conjecture. There does not appear to be any correlation with breeds of sheep, and there are no known environmental factors that could explain the distribution.

This study has demonstrated that PFGE can detect more

genetic diversity than IS*900*-RFLP alone. In the panel of reference isolates, 16 multiplex PFGE profiles versus 6 multiplex IS*900*-RFLP profiles were detected. Multiplex PFGE clearly has the ability to subdivide IS*900*-RFLP types. For example, multiplex PFGE subdivides *Bst*EII profile C1 into two types ([1-1] and [7-5]), *Bst*EII profile C5 into three types ([2-1], [6-5], and [7-1]), and *Bst*EII profile C17 into six types ([2-2], [3-1], [3-2], [3-3], [5-1], and [5-2]). Although IS*900*-RFLP currently is the most widely used technique for discriminating strains of *M. avium* subsp. *paratuberculosis*, it has some important limitations. IS*900*-RFLP does not detect sufficient genetic diversity, and in many countries a single IS*900*-RFLP type predominates, limiting its use for epidemiological monitoring and surveillance. Furthermore, the discriminatory power of IS*900*-based typing techniques depends on the number of copies and insertion loci of IS*900* in any one strain; such techniques, therefore, are inherently limited in their ability to detect genetic polymorphisms. Multiplex PFGE analysis explores the whole genome and may be more appropriate and successful for discriminating organisms with limited genetic heterogeneity. In this study we looked only at the IS*900*-RFLP types of the 16 PFGE reference strains. Testing a larger number of isolates of the same multiplex PFGE profile may identify more IS*900*-RFLP types. Combining both techniques may achieve even greater discrimination of *M. avium* subsp. *paratuberculosis*, which will greatly assist epidemiological studies on a national or global scale.

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