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Comparison of a New Insertion Element, IS*1407*, with Established Molecular Markers for the Characterization of *Mycobacterium celatum*

M. PICARDEAU,[1] T. J. BULL,[2] G. PROD'HOM,[1] A. L. POZNIAK,[2] D. C. SHANSON,[3] AND V. VINCENT[1]*

Laboratoire de Référence des Mycobactéries, Institut Pasteur, 75724 Paris Cedex 15, France,[1] and Kings College School of Medicine and Dentistry, London SE 9RS,[2] and Microbiology Department, Charing Cross and Westminster Medical School, London W6 8RF,[3] United Kingdom

Genomic analyses of 18 *Mycobacterium celatum* strains obtained from different patients in three countries (United States, United Kingdom, and France) were performed; the methods used in this study were restriction fragment length polymorphism (RFLP) analysis, pulsed-field gel electrophoresis (PFGE) analysis, and PCR restriction analysis (PRA) of the *hsp-65* gene. A new insertion sequence, IS*1407* (GenBank accession no. X97307), belonging to the IS*256* family, was identified in *M. celatum* type 1 strains and was characterized by sequencing. When a probe for *Mycobacterium xenopi* IS*1395*-like sequences was used, the RFLP analysis of *M. celatum* type 1 strains revealed that they contained three or four copies of IS*1407* in identical genomic positions, while this element was absent in all *M. celatum* type 2 strains. PFGE performed with three different endonucleases revealed a unique large restriction fragment (LRF) pattern for *M. celatum* type 1 strains, whereas the LRF patterns obtained for *M. celatum* type 2 strains were polymorphic. Moreover, PFGE of nondigested genomic DNA revealed extrachromosomal elements in *M. celatum* type 2. The type strain of *M. celatum* type 3 could not be differentiated from *M. celatum* type 1 strains on the basis of the results of the RFLP analysis, the PFGE analysis, and the PRA of IS*1407*. In this study we confirmed that *M. celatum* types 1 and 2 represent distinct genomic clusters and that the molecular markers in *M. celatum* type 2 exhibit greater polymorphism than the molecular markers in *M. celatum* type 1.

In 1993, Butler et al. described *Mycobacterium celatum*, a new slowly growing nonphotochromogenic species that has clinical importance (7). The first description of this species was based on the results of a study of clinical isolates recovered from bronchopulmonary specimens, as well as stool, cerebrospinal fluid, and blood specimens from human immunodeficiency virus-positive and -negative patients. The strains were obtained from diverse geographic areas in the United States. Two additional cases of infection by *M. celatum* have been reported in AIDS patients in Italy (20).

M. celatum strains have most of the phenotypic characteristics of *Mycobacterium xenopi*, *Mycobacterium avium*, and the recently described species *Mycobacterium branderi* (13). Gas-liquid chromatography allows workers to differentiate *M. celatum* from both *M. avium* and *M. xenopi* on the basis of the presence of 2-eicosanol associated with tetracosanoic and hexacosanoic acids, which are the major mycolic acid cleavage products. However, this lipid pattern is also found in *M. branderi*. Genetic analysis of the 16S rRNA gene is the only method which has been described which can be used to reliably differentiate *M. celatum* from all other species. The 16S rRNA sequences of *M. celatum* strains confirmed the close phylogenetic relationship of this organism to *M. branderi* and *M. xenopi* (level of similarity, 95%) (7, 13). Moreover, sequence analysis has revealed that there is a 10-nucleotide difference between the sequences of *M. celatum* types 1 and 2. Phenotypically, the two types could be differentiated only by multilocus enzyme electrophoresis, which resulted in two electrophoretic patterns based on the mobilities of 14 enzymes (7). Recently, 16S rRNA sequencing of additional *M. celatum* strains provided additional sequence information and revealed the existence of a new sequence type that is distinct from but very similar to the type 1 and type 2 sequences; this new sequence type is designated type 3 (5).

A previous investigation of the host range of *M. xenopi* IS*1395* revealed cross-hybridization with an unidentified repeated element in *M. celatum* type 1 (15). In order to investigate the molecular epidemiology of *M. celatum* further, we attempted to characterize this element and to evaluate the differences found in a group of *M. celatum* strains from diverse origins by using three molecular methods.

(This work is part of a doctoral thesis in microbiology presented by M.P.)

MATERIALS AND METHODS

Mycobacterial strains. The *M. celatum* reference strains and clinical isolates used in this study are listed in Table 1. Strains from the United States were kindly provided by W. R. Butler (Centers for Disease Control and Prevention, Atlanta, Ga.). *M. celatum* strains from the United Kingdom were provided by T. J. Bull (Kings College School of Dentistry and Medicine, London, United Kingdom). The *M. branderi* type strain (ATCC 51789) was a kind gift from M. L. Katila, Kuopio University Hospital, Kuopio, Finland. Other *M. celatum* isolates and two *M. xenopi* clinical isolates used as controls were from one of our laboratories (Laboratoire de Référence des Mycobactéries, Institut Pasteur, Paris, France).

Identification by rRNA gene sequencing. The 16S rRNA gene was amplified by using the PCR technique, and the sequence was determined as described previously with primers 244 and 259 (18). Sequencing reactions were performed with a model 373 stretch DNA analysis system (Applied Biosystems).

PCR restriction analysis (PRA). Approximately 1 μg of mycobacteria was removed from Löwenstein-Jensen slants and suspended in 100 μl of TE (10 mM Tris, 1 mM EDTA; pH 8.0) containing 1% Triton X-100 and incubated for 30 min at 100°C. The resulting lysates were used as DNA sources without further purification. A two-step assay was then performed; this assay consisted of PCR amplification of a 439-bp fragment of the *hsp-65* gene, followed by a restriction analysis performed with *Bst*EII and *Hae*III as previously described (19). *M. branderi* and *M. xenopi* DNAs were used as controls.

Molecular cloning and library screening. Genomic DNA was prepared as previously described (15). A 3-μg portion of *M. celatum* type 1 DNA was digested with *Eco*RI and separated on a 0.8% (wt/vol) agarose gel. Restriction fragments that were approximately 2.5 to 5 kb long were recovered from the agarose gel by using a GeneClean II kit (Bio 101, Inc., La Jolla, Calif.) and were ligated into the

* Corresponding author. Phone: (33) (1) 45 68 83 60. Fax: (33) (1) 40 61 31 18. E-mail: vvincent@pasteur.fr.

TABLE 1. *M. celatum* strains used in this study

Strain	Designation	Source[a]	Type
1	CDC 911438	Butler	1
2	CDC 920541	Butler	1
3	CDC 900251	Butler	1
4	ATCC 51131	Butler	1[b]
5	CDC 892293	Butler	1[b]
6	CDC 890446	Butler	2
7	CDC 890694	Butler	2
8	CDC 920522	Butler	2[b]
9	ATCC 51130	Butler	2[b]
10	CDC 920605	Butler	2
11	CDC 890639	Butler	2
12	960251	Bull	1[b]
13	960252	Bull	1[b]
14	960253	Bull	1[b]
15	NCTC 12882	Bull	3[b]
16	941179	Our laboratory	1[b]
17	940952	Our laboratory	1[b]
18	951976	Our laboratory	1[b]

[a] Butler, W. R. Butler, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Ga.; Bull, T. J. Bull, Kings College School of Medicine and Dentistry, London, United Kingdom.

[b] The type was confirmed or established in this study by 16S rRNA sequencing (see Materials and Methods).

*Eco*RI site of alkaline phosphatase-treated plasmid pBluescript II KS (Stratagene, La Jolla, Calif.). Ligation mixtures were transformed into *Escherichia coli* DH5α by electroporation with a Gene Pulser unit (Bio-Rad Laboratories, Richmond, Calif.). Recombinant colonies were selected on Luria-Bertani solid medium (10 g of Bacto Tryptone [Difco] per liter, 5 g of yeast extract per liter, 10 g of NaCl per liter; pH 7.5) supplemented with 100 μg of ampicillin per ml, 2 mM isopropyl-β-D-thiogalactopyranoside, and 0.04% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Colonies were then transferred to a nylon filter (N^+ Hybond; Amersham International, Amersham, United Kingdom) by standard techniques (16).

A probe for IS*1395*-like sequences was prepared as described below and was hybridized to the filters overnight at 65°C in Rapid hybridization buffer (Amersham). This was followed by stringent washes at 65°C; the preparations were washed twice (10 min each) in 2× SSC (1× SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) containing 0.1% (wt/vol) sodium dodecyl sulfate (SDS), twice (10 min each) in 1× SSC–0.1% (wt/vol) SDS, and once for 10 min in 0.1× SSC–0.1% (wt/vol) SDS.

Plasmid pMP02, which was used in later subcloning steps, was purified by using Qiagen minicolumns (Qiagen Midi kit; Qiagen, Inc., Hilden, Germany). The DNA insert was partially digested with *Sau*3A and was subcloned into the pBluescript II KS$^-$ *Bam*HI site. Recombinant plasmids were extracted from *E. coli* by using Qiagen minicolumns for sequencing.

Sequencing and analysis of IS*1407*. The sequences of double-stranded plasmid DNAs were determined by the dideoxy chain termination method (17) by using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems), a model 9600 GeneAmp PCR system (Perkin-Elmer), and a model 373 stretch DNA analysis system (Applied Biosystems). Similarities to the IS*256* family were identified in databases by using the BLAST (1) and FASTA (14) algorithms.

Restriction fragment length polymorphism (RFLP). The IS*1395* probe was amplified by PCR from an *M. xenopi* strain with primers XNA and XNB as previously described (15). The 897-bp probe in IS*1395* was purified from an agarose gel by the GeneClean procedure and was labeled with [α-^{32}P]dCTP by using a Megaprime DNA labeling kit (Amersham).

A 2-μg portion of mycobacterial DNA was digested with 10 U of *Pvu*II in a 30-μl reaction mixture. DNA fragments were resolved by overnight electrophoresis on a 1% (wt/vol) agarose gel at 1.5 V/cm and were transferred to a nylon membrane (N^+ Hybond; Amersham) (16). The membrane was hybridized overnight at 65°C with the probe in Rapid hybridization buffer (Amersham) and then washed as described above.

Pulsed-field gel electrophoresis (PFGE). *M. celatum* plugs were prepared as described previously (15). Since in the genus *Mycobacterium* the G+C contents of the DNAs are very high, we used enzymes with AT-rich sites. Restriction endonucleases *Ase*I, *Xba*I, and *Dra*I were used to generate a few restriction fragments having lengths suitable for analysis. Large restriction fragments were separated in a 0.8% (wt/vol) agarose gel by zero-integrated-field electrophoresis with the AutoBase system (Vysis, Les Ulis, France) for 65 h at room temperature with 8- to 200-kb and 8- to 500-kb ROM cards.

Nucleotide sequence accession number. The GenBank accession number for the IS*1407* DNA and amino acid sequences is X97307.

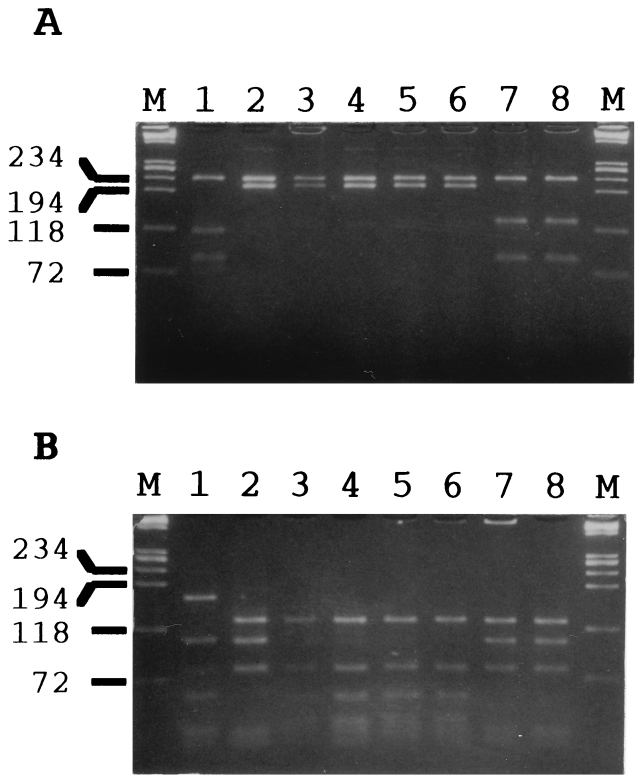

FIG. 1. (A) RFLP patterns generated by digestion with *Bst*EII. (B) RFLP patterns generated by digestion with *Hae*III. Lanes M, molecular weight marker genome (molecular [in kilobases] are indicated on the left); lane 1, *M. xenopi*; lane 2, *M. branderi*; lanes 3 to 5, *M. celatum* type 1 strains 4, 12, and 13, respectively; lane 6, *M. celatum* type 3 strain 15; lanes 7 and 8, *M. celatum* type 2 strains 9 and 11, respectively.

RESULTS

PRA of the *hsp-65* gene. The *M. celatum* type 1 strains and the type strain of *M. celatum* type 3 produced identical restriction patterns with the two enzymes used; the *Bst*EII pattern had bands at 245 and 220 bp, and the *Hae*III pattern had bands at 140 and 90 bp. The *Bst*EII patterns of *M. celatum* type 2 strains contained 245-, 140-, and 80-bp fragments, and the *Hae*III patterns of these strains contained 140- and 105-bp fragments. *M. branderi* and *M. xenopi* produced specific patterns. *M. branderi* produced the *M. celatum* type 1 and 3 pattern with *Bst*EII and the *M. celatum* type 2 pattern with *Hae*III, whereas *M. xenopi* produced dissimilar patterns with both enzymes (Fig. 1).

Description of insertion sequence IS*1407*. Screening of a partial library of the *M. celatum* type 1 genome with an IS*1395* probe revealed a recombinant plasmid designated pMP002, which was purified and shown by restriction analysis to contain a 3.5-kb insert. A nucleotide sequence analysis of subclones of this plasmid showed that the element is 1,325 bp long and contains at its extremities a 15-bp inverted repeat with one mismatch. The element contains a 1,245-bp open reading frame that encodes a putative 415-residue transposase preceded by a sequence which could serve as a ribosome binding site (data not shown). The putative transposase exhibits sequence homology to the *Staphylococcus aureus* IS*256* family.

RFLP. Analysis of a Southern blot of *Pvu*II-digested DNAs of 18 nonrelated strains, including *M. celatum* type 1 and 2 strains and the type strain of *M. celatum* type 3, showed that

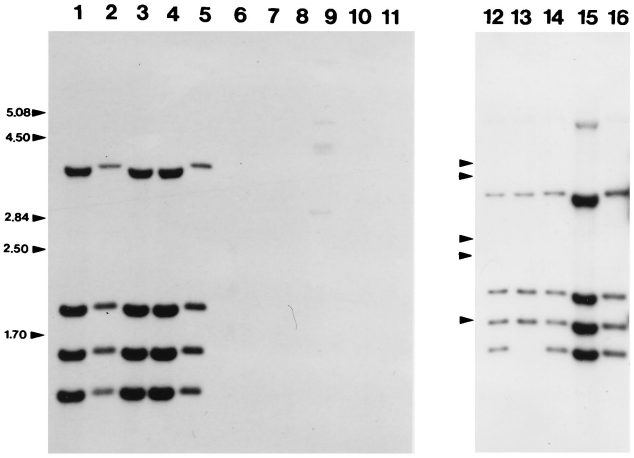

FIG. 2. Southern blot analysis of *Pvu*II-digested DNAs of *M. celatum* strains probed with IS*1395*. Lanes 1 to 5, *M. celatum* type 1 strains 1 to 5, respectively; lanes 6 to 11, *M. celatum* type 2 strains 6 to 11, respectively; lanes 12 to 15, *M. celatum* type 1 strains 12 to 14 and 16, respectively; lane 16, *M. celatum* type 3 strains 15. The molecular weight marker was bacteriophage λ DNA restricted with *Pst*I; the sizes of the fragments (in kilobases) are indicated on the left.

three or four copies of IS*1407* were present in the *M. celatum* type 1 and 3 genomes. Nevertheless, the DNA fingerprints observed were not polymorphic. Identical patterns were obtained for most type 1 and type 3 strains; the only exceptions were two isolates that harbored one copy less. The element was not detected in any *M. celatum* type 2 strain (Fig. 2).

PFGE. *M. celatum* type 1, 2, and 3 genomes restricted with low-frequency cleavage enzymes *Dra*I and *Ase*I produced about 15 restriction fragments in the size range from 50 to 600 kb. All of the patterns generated with *Xba*I were complex (with more than 30 large restriction fragments [LRF]) and contained numerous fragments (Fig. 3).

The LRF patterns showed that the level genome conservation in type 1 was high. The only difference found in the nonrelated type 1 strains was a 300-kb *Xba*I fragment (Fig. 3, lanes 2, 5, and 10). Moreover, the type 3 reference strain pattern was identical to the type 1 pattern. Unlike the type 1 and 3 patterns, the patterns for the *M. celatum* type 2 strains were polymorphic, which allowed differentiation of these organisms from each other. However, for each enzyme used, the strains tested shared about 10 LRF (Fig. 3).

The PFGE analysis of unrestricted DNA revealed extrachromosomal elements in *M. celatum* type 2 strains and the type 3 reference strain but not in *M. celatum* type 1 strains. Two of the *M. celatum* type 2 strains tested, strains 10 and 11, harbored a 140-kb plasmid, strain 8 harbored a 180-kb plasmid, and strain 6 harbored a ca. 20-kb element (Table 1 and Fig. 4). A 50-kb extrachromosomal element was detected in the *M. celatum* type 3 reference strain (Fig. 4). The PFGE migration of total DNA in the absence and presence of the intercalating agent ethidium bromide (data not shown) and the results obtained when different electrophoretic conditions were used suggested that these elements behaved like large linear plasmids similar to the *M. xenopi* extrachromosomal elements detected recently (Fig. 4) (10, 15).

DISCUSSION

The molecular epidemiology of *M. celatum* needs to be clarified, especially because of the association of this organism with disseminated infections in immunosuppressed patients. *M. celatum* was described for strains which had indistinguishable biochemical features but differed in their 16S rRNA sequences (5, 7). 16S rRNA sequencing revealed a 7-base difference between type 1 and type 3 and a 17-base difference between type 2 and type 3 (5). Previous studies have established that a cutoff value based on the number of different nucleotides in the 16S rRNA gene cannot be defined for delineating species (11, 24). For example, in the genus *Mycobacterium*, *Mycobacterium szulgai* and *Mycobacterium malmoense* differ by only a single nucleotide in the 1,384-nucleotide seg-

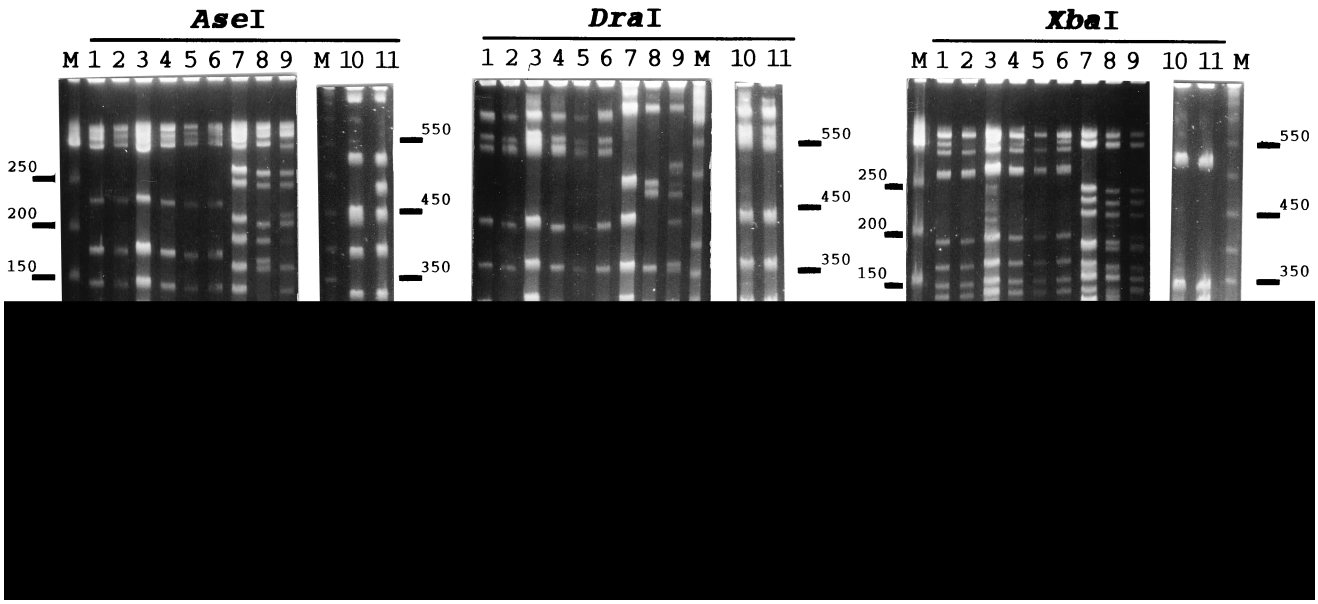

FIG. 3. PFGE separation of *Ase*I-, *Dra*I-, and *Xba*I-digested genomic DNAs from *M. celatum* strains. Lanes 1 to 6, *M. celatum* type 1 strains 18, 3, 4, and 12 to 14, respectively; lanes 7 to 9, *M. celatum* type 2 strains 9 to 11, respectively; lane 10, *M. celatum* type 1 strain 1; lane 11, *M. celatum* type 3 strain 15; lanes M, molecular weight marker consisting of DNA concatemers of 50 kb of the bacteriophage λ genome (the sizes [in kilobases] are indicated beside the gels).

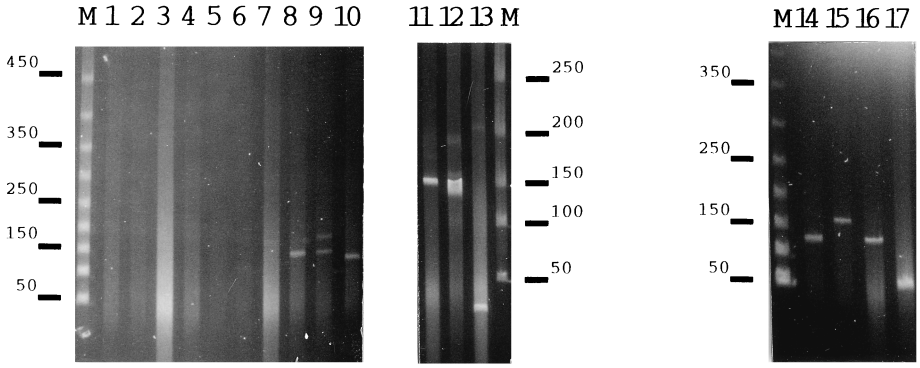

FIG. 4. PFGE of nondigested DNA: zero-integrated-field electrophoresis with the 8- to 200-kb ROM card (lanes 11 to 13) and with the 8- to 500-kb ROM card (lanes 1 to 10 and 14 to 17). Lanes 1 to 5, *M. celatum* type 1 strains 1 to 5, respectively; lanes 6 and 7, *M. celatum* type 2 strains 7 and 9, respectively; lanes 8 and 9, *M. celatum* type 2 strains 10 and 11, respectively; lanes 10 and 11, *M. xenopi*; lane 12, *M. celatum* type 2 strain 11; lane 13, *M. celatum* type 2 strain 6; lane 14, *M. xenopi*; lane 15, *M. celatum* type 2 strain 8; lane 16, *M. celatum* type 2 strain 10; lane 17, *M. celatum* type 3 strain 15; lanes M, molecular weight marker consisting of DNA concatemers of 50 kb of the bacteriophage λ genome (the sizes [in kilobases] are indicated beside the gels).

ment examined, whereas some *M. avium* serotypes differ by one to seven nucleotides in the 782-nucleotide segment examined (3, 24). On the other hand, DNA-DNA hybridization studies and RFLP analyses established that *M. malmoense* and *M. szulgai* are distinct species and that the serotypes referred to above belong to a single species. Therefore, the 16S rRNA sequence variations observed in *M. celatum* types cannot be considered sufficient to assign the types to separate species, as Butler et al. (7) concluded previously.

We used three molecular methods to try to improve differentiation of the known *M. celatum* types and to distinguish strain variations within the types. A PRA of the *hsp-65* gene showed excellent reproducibility, but could only be used to separate types 1 and 3 from type 2. The patterns obtained by using *Bst*EII and *Hae*III confirmed the differentiation of types 1 and 2 described previously on the basis of results obtained with restriction enzymes *Bst*NI and *Xho*I (7). The number of restriction fragments indicate that these two *M. celatum* PRA types differ by a single restriction site for each enzyme. These results are indirect evidence that there is a high degree of conservation of the *hsp-65* gene in these organisms. In addition to the conserved *hsp-65* and 16S rRNA genes, the superoxide dismutase (SOD) gene has also been used for taxonomic studies (6, 27). An analysis of partial SOD sequences did not reveal significant differences between *M. celatum* types 1 and 3, yet there were significant differences between *M. celatum* types 3 and 2 (4).

It is possible, therefore, that the prevalence of *M. celatum* type 3 in our study was underestimated. It is interesting that all of the *M. celatum* strains identified outside the United States (i.e., the isolates identified in the United Kingdom, Italy, and France) were not type 2 strains (5, 19). *M. celatum* type 3, as well as most of the *M. celatum* type 2 strains, harbored extrachromosomal elements ranging in size from about 20 to 180 kb; none of these elements were detected in *M. celatum* type 1 strains (Fig. 4). However, the reliability of the presence of plasmids in *M. celatum* types must be confirmed by studying additional strains. These extrachromosomal elements are currently being investigated.

To determine strain differences within types, PFGE was used, and the results confirmed that *M. celatum* strains belong to two distinct genomic groups. The type 1 strains and the type strain of *M. celatum* type 3 belong to a compact cluster whose members produce a single PFGE pattern. Minor pattern differences were observed for some strains, which may have been due to DNA rearrangements. This homogeneity is in contrast to the great diversity described for other mycobacterial species, especially *M. avium* strains (2, 12, 15, 22, 25, 26). However, a lack of genomic polymorphism has been observed previously in the PFGE patterns of epidemiologically unrelated strains of *Mycobacterium genavense*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *silvaticum* (8, 21). In contrast, PFGE provides valuable insight into the differentiation of *M. celatum* type 2 strains. Each of the strains tested produced a strain-specific pattern, but the patterns shared several LRF for each endonuclease tested. This finding is compatible with a high level of genetic relatedness among the strains.

In this study we identified a new insertion element, IS*1407*, that is found only in *M. celatum* types 1 and 3. Insertion sequences have several characteristics that make them very useful for phylogenetic and epidemiological studies. A previous study showed that there was cross-hybridization of IS*1395*, an insertion sequence found recently in *M. xenopi*, with an element present in four copies in the genome of a *M. celatum* type 1 strain (15). In the present study the latter element was identified as IS*1407*, which belongs to the IS*256* family. This finding expands the members of this family found in mycobacterial species, including IS*1395* in *M. xenopi*, IS*1081* in *Mycobacterium bovis*, IS*1245* and IS*1311* in *M. avium*, and IS*6120* in *Mycobacterium smegmatis*. The highest level of similarity between other sequences was the level of similarity with *M. xenopi*, a result which confirms the genetic relatedness of these mycobacterial species.

Unlike the other insertion elements belonging to the IS*256* family described previously in mycobacteria, IS*1407* is not present in all types of its host species, as it could not be detected in *M. celatum* type 2. Considering the high levels of homology between *M. celatum* types determined by 16S rRNA and SOD gene sequence analyses, we suggest that the *M. celatum* types may have originated from a common ancestor. IS*1407* could have appeared in the *M. celatum* type 1 (and type 3) genomes by horizontal transfer after the divergence of types 1 and 2 or may have been lost by type 2 because of mutations that affected the function of the transposase, thereby causing a loss of the element.

The IS*1407* RFLP patterns were identical for all *M. celatum* type 1 strains and the type strain of *M. celatum* type 3 (three or four copies at the same positions), although the isolates tested were from different countries (United States, United Kingdom, and France). Thus, IS*1407* is not suitable as an epidemiological

marker for strain differentiation. Moreover, an analysis of the flanking sequences of IS*1407* revealed similarities with the flanking sequences of two related elements, *M. bovis* IS*1081* and *M. xenopi* IS*1395* (9, 15) (data not shown). The absence of position polymorphism could be due to insertion of IS*1407* at specific identical positions in the chromosome.

DNA-DNA hybridization, the reference technique used for species definition, could be used to conclusively determine the taxonomic hierarchy of the three *M. celatum* types. However, all of the results described above are consistent with the hypothesis that types 1 and 3 are closely related taxa and type 2 represents a separate species. A proposal for a new species name for type 2 is not presented here due to the recommendation of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics (23) which requires defining phenotypic properties for species characterization.

In this study we demonstrated that *M. celatum* types 1 and 3 are very homogeneous. The results obtained for the type strain of *M. celatum* type 3 and the results obtained for the *M. celatum* type 1 strains were identical, as determined with the molecular markers used. Only the presence of an extrachromosomal element distinguished types 1 and 3. The molecular markers used in our study especially highlighted the close phylogenetic relationship of *M. celatum* with *M. xenopi* and *M. branderi*, which is consistent with the similar phenotypic properties of these species. The *hsp-65* gene is highly conserved in *M. celatum* and *M. branderi*; in particular, the PRA profiles of *M. branderi* are either identical to the PRA profiles of *M. celatum* types 1 and 3 or identical to the PRA profiles of *M. celatum* type 2, depending on the enzyme used. The three species harbor the related insertion sequences IS*1407*, IS*1395*, and IS*1408* (GenBank accession no. U62766), respectively, all of which belong to the IS*256* family and exhibit high levels of homology based on nucleotide and amino acid sequences.

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