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Heterogeneity and Clonality among Isolates of *Mycobacterium kansasii*: Implications for Epidemiological and Pathogenicity Studies

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The reservoir and transmission route of *Mycobacterium kansasii* are largely unknown. In addition, culturing of *M. kansasii* from human sources is not proof of disease because it may represent colonization rather than infection. Unfortunately, investigation of the epidemiology and pathogenicity of *M. kansasii* is complicated by evidence of heterogeneity within the species. A comprehensive study by detailed genotypic analysis of a large collection of *M. kansasii* isolates ($n = 276$) from various geographical sources within Europe was conducted. Five defined subtypes of *M. kansasii* were identified; of these subtypes, type I represents the most common isolate from humans. Although phylogenetic analysis confirmed its relationship to the other *M. kansasii* types, significant sequence divergence was found at the 16S-23S intergenic spacer. Analysis of the chromosomal polymorphism of type I demonstrated a marked clonal structure for this particular organism. Because *M. kansasii* is becoming a significant pathogen among immunodeficient hosts, future epidemiological and pathogenicity studies should take into consideration both the heterogeneity within the species and the apparent clonality of the most prevalent *M. kansasii* isolates infecting humans.

Mycobacterium kansasii remains a significant cause of human disease, and it is one of the most frequent nontuberculous mycobacterial pathogens isolated from clinical specimens. Annual rates of infection in the general population have been in the range of 0.5 to 1 per 100,000; however, significant geographical variability is observed. While in certain areas of the world (Australia, Japan, and southern California, and Virginia in the United States) *M. kansasii* constitutes a rare isolate (7, 12, 20, 42), regions such as Louisiana in the United States and North Moravia in the Czech Republic report annual rates as high as 2.4 and 17.6 per 100,000 population, respectively (18, 43). Since the start of the AIDS epidemic, an increase in *M. kansasii* disease has been observed (15). In the United States, the rates of disseminated disease among human immunodeficiency virus (HIV)-infected subjects reach 138 per 100,000 (43). Among the HIV-infected population in Switzerland the annual incidence is estimated to be greater than 300 times that found in the general population (≈330/100,000; data from the Swiss HIV Cohort study [22a]).

The natural reservoir of *M. kansasii* is still largely unknown. Several investigators postulated that water is the natural habitat (13, 17) because this microorganism has been recovered occasionally from tap water, showerheads, and drinking-water distribution systems (2, 9, 19, 23, 25, 31, 37, 46). However, *M. kansasii* has very rarely been isolated from rivers or lakes (5, 27). Exceptionally, it has been recovered from animals (cattle, swine) or soil (4, 6, 44, 45). Importantly, a definitive epidemiological link between those natural reservoirs and human disease is yet to be established. In addition, culturing of *M. kansasii* from human sources is not proof of disease: as many as one-third of isolates have been reported to represent colonization of the respiratory tract rather than infection (1). Human-to-human transmission has not been documented.

Investigation of these fundamental questions—reservoir, transmission routes, and pathogenicity—is complicated by evidence of heterogeneity within the *M. kansasii* species (33, 38, 47). While standard laboratory identifications, which rest on photochromogenicity and a limited number of biochemical reactions, have created the impression that *M. kansasii* is a homogeneous group of organisms, extensive biochemical testing and the use of commercial DNA probes and other molecular analyses point to the existence of defined subtypes within the *M. kansasii* species (30, 40, 41).

We aimed to establish a more precise characterization of *M. kansasii* isolates by using several genotypic markers to identify subtypes and their phylogenetic relationships and by using a number of molecular tools for the analysis of strain relatedness. The final goal was to set the basis for future studies on the epidemiology and pathogenicity of *M. kansasii.*

MATERIALS AND METHODS

Mycobacterial strains. Isolates of *M. kansasii* ($n = 276$) from Switzerland ($n = 103$), Spain ($n = 51$), Germany ($n = 113$), and Italy ($n = 9$), type strains ATCC 12478 and NCTC 10268, and two laboratory strains of *M. gastri* were used in this study. With the exception of 113 environmental isolates, all strains were recovered from human sources. The Spanish collection represented all available isolates for the years 1993 to 1995 from a defined geographical location (Baix Llobregat, Barcelona, Spain). The 113 environmental *M. kansasii* isolates were selected among a total of 2,032 mycobacterial strains isolated from 619 water samples from different regions of Germany. One of the environmental *M. kansasii* strains was isolated from river water; the remaining strains were isolated from domestic tap water. Human isolates were recovered by standard isolation

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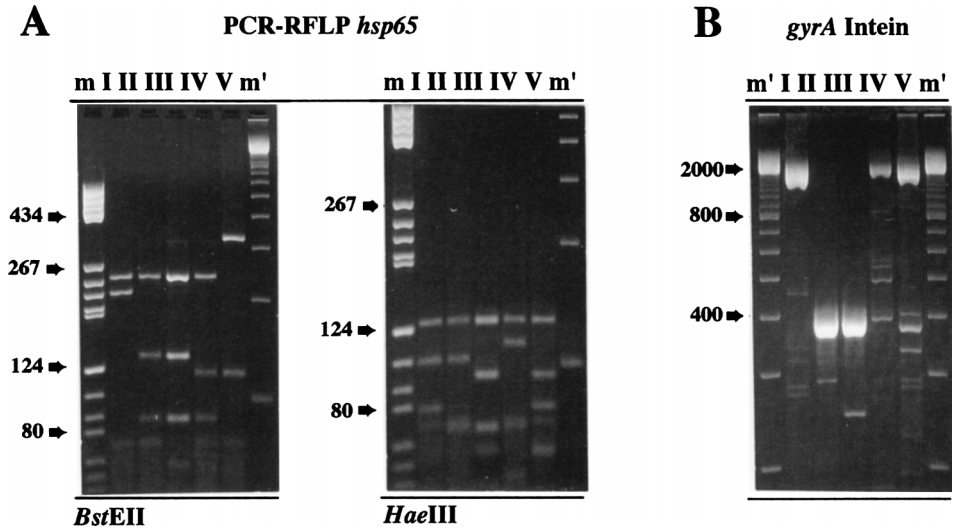

FIG. 1. Genotypic characterization of *M. kansasii*. (A) PCR-RFLP analysis of the *hsp65* gene after digestion with *Bst*EII and *Hae*III. Lanes I to V, five different patterns among the 276 *M. kansasii* isolates tested. (B) *gyrA* intein. The presence of the element is indicated by an amplification product of 1,665 bp (types I, IV, and V), and its absence is indicated by a PCR fragment of 392 bp (types II and III). Lanes m and m′ reference markers (in base pairs).

methods, whereas specially adapted methods were used to recover the environmental isolates (27). Identification was performed by conventional phenotypic methods, including slow growth, photochromogenicity, nitrate reduction, Tween hydrolysis, catalase production, and urease (28). In addition, thin-layer chromatography was performed for the characterization of environmental isolates (identification of alpha-, methoxy-, and ketomycolates).

Genotypic characterization. PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the *hsp65* gene was performed for all isolates as described previously (39). Briefly, a 439-bp segment amplified by using primers conserved throughout the genus *Mycobacterium* (Tb11, 5′-ACCAACGATGGT GTGTCCAT; Tb12, 5′-CTTGTCGAACCGCATACCCT) was digested with *Bst*EII and *Hae*III (Boehringer GmbH, Mannheim, Germany). Restriction fragments were separated by electrophoresis on a 3% Metaphor agarose gel (FMC Bioproducts, Rockland, Maine) and were visualized by staining with ethidium bromide.

The AccuProbe (conventional old formulation; Gen-Probe Inc., San Diego, Calif.) hybridization test was performed with all isolates according to the recommendations of the manufacturer (Gen-Probe Inc). Results were expressed as relative light units (RLUs). Samples producing signals greater than or equal to 30,000 RLUs were considered positive (22).

Identification of intein elements in *gyrA* was performed for all human isolates and a representative number of environmental strains by PCR analysis of genomic DNA with primers H49 (5′-AGGTTGTGCGGCGGGATATTGGT) and H50 (5′-TTCCGCCCGGACCGCAGCCACG) (10). Cycling conditions included 30 cycles of denaturation at 95°C and annealing-extension at 70°C. The presence or absence of the *gyrA* intein was determined by amplification of 1,665- or 392-bp PCR products, respectively (10).

Phylogenetic analysis. Several representative strains from each PCR-RFLP group (type I, $n = 12$; type II, $n = 10$; type III, $n = 3$; types IV and V, $n = 1$ each) were investigated by partial 16S rRNA gene sequencing and analysis of the 16S-23S rRNA spacer. Determination of the first 500 nucleotides of the 16S rRNA gene was performed by using primer 285 (5′-GAGAGTTTGATCCTGG CTCAG) and primer 264 (5′-TGCACACAGGCCACAAGGGA), and the amplification products were sequenced by using primer 244 (5′-CCCACTGCTGC CTCCCGTAG) as described previously (21). The 16S-23S rRNA gene intergenic region was amplified by using conserved primers 248 (16S rRNA gene; 5′-GTGTGGGTTTCCTTCCTTGG) and 42 (23S rRNA gene; 5′-CCACAC GGGTTAACCTCGC). Sequencing of the complete spacer region was performed with primers 293 (the 3′ part of the 16S rRNA gene; 5′-GAAGTCGTA ACAAGGTAGCC) and 41 (the 5′ part of the 23S rRNA gene; 5′-TCCCAC GTCCTTCATCGGCTC) by PCR-mediated *Taq* cycle sequencing with an ABI 373 sequencer. The complete spacer sequences ranging from 270 to 292 bp were aligned with selected intergenic spacer sequences by considering the secondary structures. For phylogenetic analysis, regions of alignment uncertainty were omitted. Pairwise distances of more than 250 remaining nucleotide positions were calculated by weighting nucleotide differences and insertions-deletions equally (hamming distances) (8). The phylogenetic tree was constructed by using the neighborliness method (36) as described previously (32).

Strain relatedness analysis. Analysis of chromosomal DNA restriction patterns by pulsed-field gel electrophoresis (PFGE) was performed as described previously (3) for all strains of PCR-RFLP type I and representative strains of all other types. In brief, subcultures of *M. kansasii* isolates were grown in Middlebrook 7H9 broth for 3 days and were incubated for the last 24 h with cycloserine and ampicillin. Pellets were incorporated into agarose plugs and lysed with lysozyme, sodium dodecyl sulfate, and proteinase K. Genomic DNA was digested with *Spe*I and *Dra*I (Boehringer Mannheim). Electrophoresis was done with a CHEF-DR III system (Bio-Rad) at 14°C for 22 h at 6 V/cm. The pulse time was ramped from 1 to 35 s for 22 h and then from 0.5 to 15 s for 6 h after *Spe*I digestion and from 4 to 44 s after *Dra*I digestion. Restriction fragments were visualized by staining with ethidium bromide, photographed, and scanned for analysis by using GelCompare package (Windows version 3.10; Applied Math, Kortrijk, Belgium). The results were presented as a dendrogram.

Two additional approaches for analysis of strain relatedness were based on the investigation of the major polymorphic tandem repeat (MPTR), originally described in *M. tuberculosis*, *M. gordonae*, and *M. kansasii* (14). Southern blot analysis was performed by using a probe from the *M. tuberculosis* MPTR. A PCR-based technique with primers MPTR-I (5′-CGCCGGTGCCGACGT TGCCC) and MPTR-F (5′-CTCTTCAATGCCGGCAGCTT) (14) at low and intermediate annealing temperatures (37 and 55°C, respectively) allowed for the generation of complex patterns for the purpose of strain typing.

RESULTS

Genotypic characterization. *hsp65* PCR-RFLP analysis generated five different patterns among the 276 *M. kansasii* strains tested (Fig. 1A and Table 1). One hundred eleven clinical isolates (60 isolates from Switzerland and all 51 isolates from a defined geographical region in Spain) and the 2 reference strains presented the type I PCR-RFLP pattern. These isolates were also defined by a positive *M. kansasii* AccuProbe hybridization test result and the presence of *gyrA* intein. Of the remaining 165 isolates, most ($n = 68$) presented the PCR-RFLP type II pattern and were AccuProbe and *gyrA* intein negative. Isolates with PCR-RFLP patterns III ($n = 34$), IV ($n = 60$), and V ($n = 3$) appeared to be rarely isolated from human sources. Subtypes III and V could hybridize with AccuProbe, with borderline readings ranging from 25,600 to 61,737 RLUs, in contrast to RLU values of >200,000 for type I strains. Type IV and V isolates carried a *gyrA* intein (Fig. 1B). The PCR-RFLP patterns *M. gastri* isolates were indistinguishable from those of *M. kansasii* type II isolates.

Phylogenetic analysis. The discriminatory power of partial 16S rRNA gene sequence analysis was limited, with nucleotide differences between the two main types, types I and II, being present at around nucleotides 35 and 50 (Fig. 2A). In contrast, the results obtained with the 16S-23S intergenic spacer con-

TABLE 1. Genotypic characterization of *M. kansasii*

Strain and type	No. of isolates		Bands (bp) by PCR-RFLP analysis of *hsp65*		AccuProbe result	*gyrA* intein result
	Human	Environmental	*Bst*II	*Hae*III		
M. kansasii						
I	111	0	245, 220	140, 105, 80	+	+
II	50	18	245, 145, 85	140, 105, (70)[a]	−	−
III	2	32	245, 145, 85	140, 100, 70	Borderline	−
IV	0	60	245, 115, 85	140, 115, 70	−	+
V	0	3	325, 125	140, 100, 80	Borderline	+
M. gastri			245, 145, 85	140, 105, 70	−	Indeterminate

[a] Not present in all isolates.

firmed the *hsp65* PCR-RFLP data indicating the existence of well-defined types within *M. kansasii* (Fig. 2B). Nucleotide differences at the 16S-23S intergenic region between the type I and other *M. kansasii* types ranged between 13 and 38 bp (5 to 15%), with the highest homology being to *M. kansasii* type II. As expected, *M. gastri* was found to be closely related to *M. kansasii*, in particular to type IV. It is also noteworthy that other mycobacteria—in particular, *M. szulgai*—exhibited close homology with the various *M. kansasii* types (Fig. 3A), which determines the relative positions suggested by the phylogenetic tree (Fig. 3B). Minor differences in the spacer region were found among *M. kansasii* type II isolates; four (one strain representative of each type II variant; strains E-234, E-235, E-251, and E-263) are included in the phylogenetic tree.

Strain relatedness analysis. PFGE of the *M. kansasii* chromosomal DNA digested with *Dra*I generated 10 to 15 fragments ranging in size from 48.5 to 700 kb (Fig. 4). The degree of polymorphism within *M. kansasii* type I was limited, consistent with a clonal structure for this particular organism (24). This was particularly apparent when evaluating *M. kansasii* strains isolated over 3 years from patients living in a defined geographical location in Spain: all strains were included within five patterns representing minor differences in macrorestriction fragment polymorphisms. Furthermore, 11 of 51 (21.5%) type I clinical isolates from Spain presented a PFGE pattern identical to those of both type strains (ATCC 12478 and NCTC 10268) and one of four *M. kansasii* type I isolates from Switzerland. In contrast, significant polymorphism was found among the limited number of type II isolates investigated by PFGE (Fig. 4). Because only representative strains of types III and V were investigated by PFGE, no conclusion as to the diversity within those groups can be inferred. The marked degree of clonality for type I strains was further confirmed by analysis of the MPTR region by Southern blotting and by PCR-based techniques (data not shown).

DISCUSSION

Two separate issues became apparent in this study: the heterogeneity of what is denominated *M. kansasii* and the clonal structure of the most prevalent subtype found in humans, *M. kansasii* type I. These two aspects have relevance for the char-

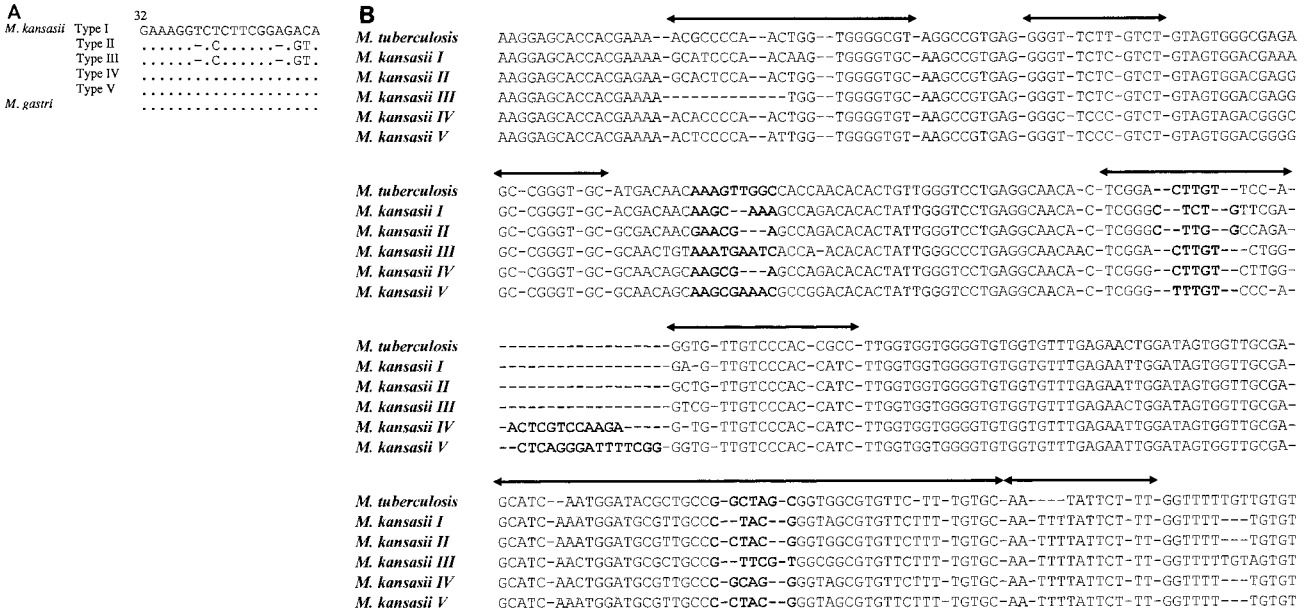

FIG. 2. Sequence analysis. (A) Alignment of partial 16S rRNA gene sequence data from the five types of *M. kansasii* and *M. gastri*. The display is limited to the region of polymorphism. Nucleotides different from those of *M. kansasii* type I are shown. Dashes indicate deletions. Dots indicate identity. (B) Alignment of the 16S-23S rRNA gene spacer region for the five types of *M. kansasii* and *M. tuberculosis*. Strain E-235 was chosen as a representative of *M. kansasii* type II. Arrows indicate helical structures. Positions that were not considered for phylogenetic analysis are printed in boldface.

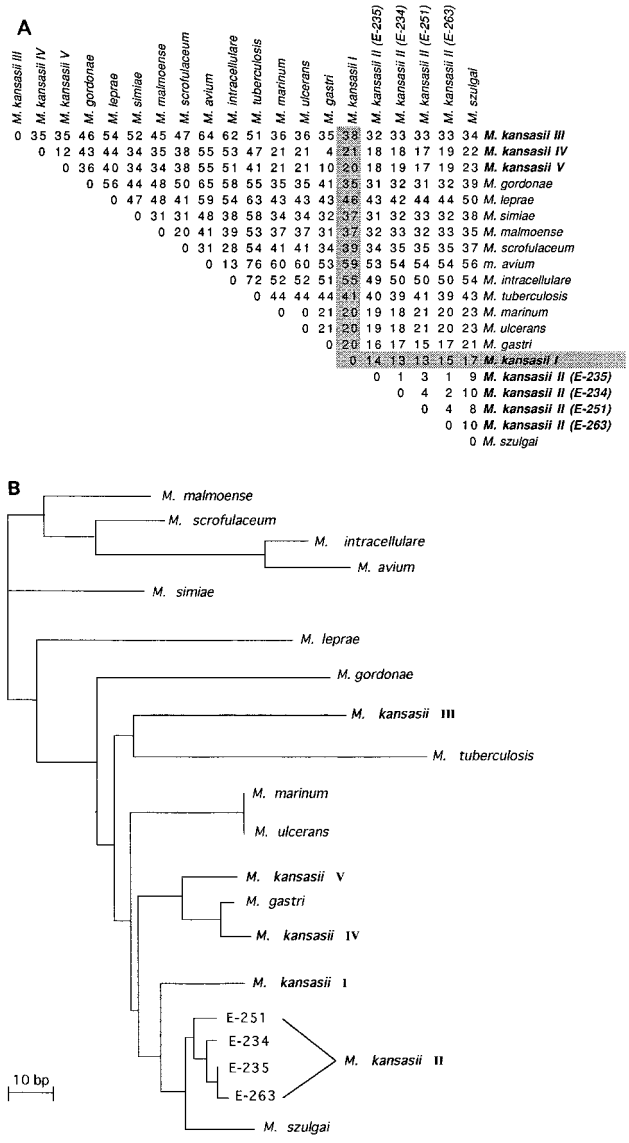

FIG. 3. Phylogenetic analysis. Hamming distances (A) and phylogenetic tree (B) derived from intergenic sequences of the 16S-23S rRNA gene showing the relationships of *M. kansasii* types to other representative mycobacteria. Hamming distances represent nucleotide differences among species for the approximately 250-bp spacer region investigated (identical organisms yield a value of 0). E-234, E-235, E-251, and E-263 represent minor variants of *M. kansasii* type II.

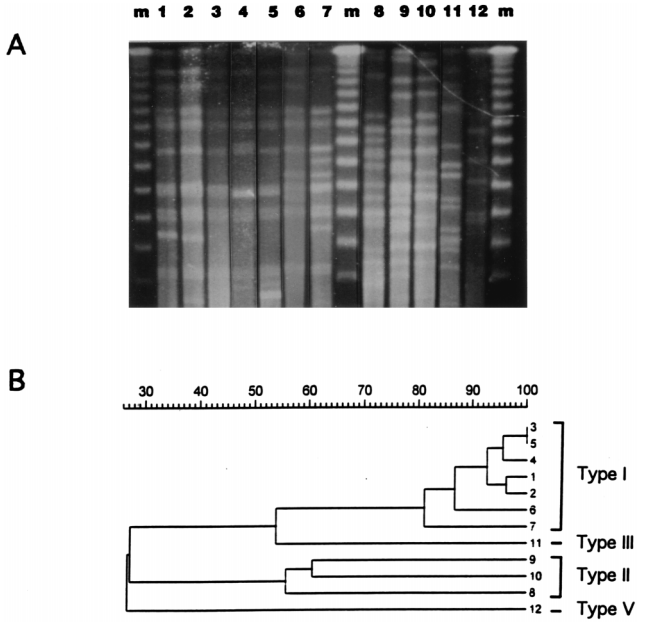

FIG. 4. Analysis of strain relatedness. (A) *Dra*I macrorestriction patterns by PFGE of representative isolates of reference strain ATCC 12478 (lane 1), *M. kansasii* type I (lanes 2 to 7), type II (lanes 8 to 10), type III (lane 11), and type V (lane 12). (B) The dendrogram is based on computer-assisted comparison of PFGE patterns. m, bacteriophage lambda ladder marker (range, 48.5 to 970 kbp).

acterization of the species (and eventually for its taxonomy) and for future analysis of questions on the pathogenicity and epidemiology of this organism(s).

Detailed genotypic analysis of *M. kansasii* isolates allowed us to establish the existence of five well-defined types within this complex. Thus, our data extend and confirm prior reports of the heterogeneity within *M. kansasii* (14, 30, 33, 40, 41, 48). PCR-RFLP analysis of the *hsp65* gene readily distinguishes the different types, which we have numbered to coincide with the recent classification by Picardeau et al. (30). Of the five types, two, types I and II, constitute frequent clinical isolates. Type I, generally identified by a positive AccuProbe test, is the most frequent *M. kansasii* isolate from human sources worldwide (22, 33, 41). The environmental habitat of *M. kansasii* type I is unknown; it was not present among the 113 environmental *M. kansasii* strains isolated in Germany; however, it has recently been isolated from water (30). In contrast, type II is frequently isolated from both humans and the environment, and it is characterized by negative hybridization to the AccuProbe. The variability in the geographical distribution of type II is underscored by different isolation rates among laboratories (22, 33, 41). In the present study, type II represented one-third of clinical isolates from Switzerland, but was absent from the *M. kansasii* collection from Barcelona, Spain. Types III, IV, and V were rarely isolated from humans, but were present in environmental samples, most frequently from tap water. A comprehensive study (exposure, skin testing, and characterization of isolates) of patients, their contacts, and the environment in a defined geographical region would solve many of these questions.

Studies that use a precise description of the genotype of *M. kansasii* isolates will also be necessary to establish the role of environmental reservoirs in relation to human disease and to determine their respective abilities to colonize humans and to cause pulmonary infection or disseminated disease. In this context, it is interesting to analyze data from a recent study from Italy (41). There, it was reported that type I *M. kansasii* was responsible for pulmonary disease among nonimmunocompromised patients, while a majority of HIV-associated *M. kansasii* infections in that region were caused by AccuProbe-negative organisms (likely to represent type II). A possible interpretation would be that HIV-infected patients become infected from a different source than nonimmunosuppressed hosts and that the organism infecting HIV-infected individuals may act as an opportunistic agent with a low intrinsic pathogenicity. A comprehensive analysis of the epidemiology and pathogenicity of *M. kansasii* isolates will require a precise definition of the infecting type.

The degree of heterogeneity within *M. kansasii* isolates raises the issue of definition of species. In the laboratory, *M.*

kansasii is characterized by a limited number of biochemical tests and photochromogenicity (28). However, analysis of ribosomal DNA sequences (in particular, the intergenic spacer) indicates a moderate degree of divergence between the various *M. kansasii*, which in some cases positions some types closer to *M. gastri* and other mycobacteria than to *M. kansasii* type I. The findings with the *gyrA* intein (29) also deserve comment. In homogeneous taxa, such as *M. tuberculosis*, *M. leprae*, *M. malmoense*, and *M. xenopi*, the presence or absence of the *gyrA* intein is a characteristic of all strains within a species. In contrast, in poorly defined taxa, such as *M. flavescens*, *M. gordonae*, and *M. kansasii*, this characteristic fails to separate organisms along taxonomic lines (34). Whether the results of the overall analysis of genotypic data are enough to question the integrity of the species is the subject of debate (33). However, should major differences in pathogenicity and epidemiology among the various types emerge, it would constitute a reason to reassess the taxonomy of *M. kansasii.*

For the purpose of investigating the degree of strain relatedness and the issue of transmissibility in a population, as we have previously done for other mycobacteria (3, 11), a 3-year collection of clinical isolates of *M. kansasii* from a defined geographical area in Spain (all of which corresponded to type I isolates) was analyzed. PFGE and other means of establishing strain relatedness identified minimal genetic polymorphism within the collection. When the analysis was extended to include strains from Switzerland and two reference strains (ATCC 12478 and NCTC 10268), a remarkable degree of conservation of the chromosomal structure at the macromolecular level was confirmed. While this phenomenon was recognized in previous studies (14, 16, 30, 33, 48), it has generated a minimal discussion about its significance. The apparent tight clonal structure of *M. kansasii* type I may result from the insufficient discriminatory powers of the techniques used, a paucity of repetitive mobile elements, or limited recombination within the species. However, it may reflect close adaptation to a host and a nonrandom association of virulence properties which effectively restrict divergence (26). Thus, the question emerges whether *M. kansasii* type I actually represents a strict human pathogen and raises the issue of human-to-human transmission. On the other hand, the clonal structure of type I and the lack of discrimination achieved with current molecular tools will limit the ability of investigators to study *M. kansasii* type I transmission events, in contrast to the significant contribution of these techniques to the understanding of the epidemiology of tuberculosis (35).

A more precise definition of the various types (subspecies or species?) among *M. kansasii* isolates will help provide an understanding of the key aspects of their biology. Because these organisms are becoming significant pathogens among immunodeficient hosts, future studies should take into consideration both the heterogeneity of the species and the apparent clonality of the most prevalent *M. kansasii* isolates infecting humans.

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