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Mycobacterium africanum Genotyping Using Novel Spacer Oligonucleotides in the Direct Repeat Locus

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This study involves a first evaluation of 25 novel spacer oligonucleotides in addition to the 43 routine spacers for molecular characterization of a panel of 65 isolates of tubercle bacilli from different geographic origins that were initially classified as *Mycobacterium africanum* based on phenotypic characters. The 68-spacer format defined four additional patterns, and three groups were identified. The relatively homogeneous groups A1 and A2 included strains from West Africa, and A3-1 included strains from East Africa. The presence of deletion region RD9 confirmed the reclassification of the *M. africanum* subtype II spoligopattern within group A3-1 as *Mycobacterium tuberculosis*. These isolates may represent a diverging branch of *M. tuberculosis* in Africa. The use of new spacers also suggested an undergoing evolution of *M. africanum* subtype I in West Africa. Our results showed that the strain differentiation within the *M. tuberculosis* complex is improved by using novel spacers, and extensive studies using new-generation spoligotyping may be helpful to better understand the evolution of *M. africanum*.

Many recent studies have used spoligotyping, a commonly used PCR-based method, for *Mycobacterium tuberculosis* complex identification and genotyping (13, 19). Recently, Viana-Niero et al. showed that a collection of isolates tentatively identified as *Mycobacterium africanum* could be successfully subdivided into three groups, A1 to A3, by spoligotyping, IS*6110*-restriction fragment length polymorphism analysis, and analysis by variable number of tandem DNA repeats and that a specific spoligotyping signature of *M. africanum* was the absence of spacers 8, 9, and 39 (25). This was followed by a study on isolates from Uganda, which showed that *M. africanum* subtype II differed from the classical *M. africanum* subtype I isolates by the absence of spacers 33 to 36 and 40 (16, 21).

Although spoligotyping is easier to perform than IS*6110*-restriction fragment length polymorphism analysis, its discriminatory power is not optimal when it is used in a routine 43-spacer format (14). Recently, a panel of novel spacers was used in spoligotyping to improve *M. tuberculosis* complex differentiation (23, 24). As these studies did not investigate any *M. africanum* strains, we thought it desirable to investigate the variability of a set of novel sequences in addition to the routine spoligotyping spacers with a panel of isolates of tubercle bacilli from different geographic origins that were initially classified as *M. africanum* based on phenotypic characters. The present study involves a first evaluation of the 25 novel spoligotyping spacers for *M. africanum* characterization.

MATERIALS AND METHODS

Mycobacterial strains. This study used a collection of 65 clinical isolates from Benin, Cameroon, France, Guinea-Bissau, Nigeria, Senegal, Sierra-Leone, and Uganda and from African patients residing in the United States. Twenty-nine clinical isolates were from the National Reference Laboratory for Mycobacteria, Institut Pasteur, Paris, France, and 36 were from the Clinical Mycobacteriology Laboratory, Wadsworth Center, Albany, New York. Among the latter, two were *M. africanum* American Type Culture Collection strains (ATCC 25420^T and ATCC 35711), and six were isolates that were recently reclassified as variants of *M. tuberculosis* by genomic deletion analysis (3, 17).

Genotyping. DNA was extracted by a simple thermolysis method. Spoligotyping using 43 spacers was performed as previously described by Kamerbeek et al. (13). The 68-spacer format with 25 novel spacers was used according to the scheme of van Embden et al., which numbers the spacers according to their position in the direct repeat locus (24). It should be emphasized that this representation of spacers, although genomically true, is completely different from the initial arbitrary designation of Kamerbeek et al. (13); e.g., spacer 12 in the scheme of van Embden et al. corresponds to spacer 4 in that of Kamerbeek et al. The hybridization results (positive or negative) were documented in the form of a binary code for each spacer and entered in an Excel spreadsheet file. The results were further analyzed by using the Bionumerics software (Applied Maths, St. Maarten Latem, Belgium), following the instruction manual for similarity analysis with the Jaccard index. Dendrograms were built by the unweighted pair group method of arithmetic averages method. The relative discriminatory powers of the 43- and 68-spacer spoligotyping methods were determined by using the Hunter-Gaston discriminatory index (11). A more exhaustive analysis, using a full set of deletion regions RD1 to RD11 and TbD1, was also performed on some isolates for each of the group defined by 68-spacer spoligotyping (3, 17).

RESULTS AND DISCUSSION

Characterization of *M. africanum* by 43-spacer spoligotyping. Classical spoligotyping showed a total of 41 different patterns for 65 isolates studied (results not shown). The results

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TABLE 1. Summary of spoligotypes, geographic origins, groups, and subgroups of the 65 strains studied

Strain	Origin[a]	Type	Group defined by using:		Strain	Origin[a]	Type	Group defined by using:	
			43 spacers	68 spacers				43 spacers	68 spacers
960090	SLE	Orphan 1	A2-2	A2-2	221372	X	Orphan 8	A1	A1
960091	SLE	1338	A3	A3-2	200100[d]	SEN	181a[b]	A1	A1
960092	SLE	438	A2-2	A2-2	200101[e]	X	181a[b]	A1	A1
960093	SLE	Orphan 2	A1	A1	940960	FXX	325	A1	A1
960094	SLE	Orphan 3	A1	A1	940961	FXX	181a[b]	A1	A1
960095	NGA	438	A2-2	A2-2	950468	FXX	181a[b]	A1	A1
960096	UGA	52	A3	A3-1	951936	FXX	326	A1	A1
960097	GIN	Orphan 4	A1	A1	961262	FXX	181a[b]	A1	A1
960098	NGA	331c[b]	A2-1	A2-1	961389	FXX	Orphan 9	A2-1	A2-1
960099	NGA	331a[b]	A2-1	A2-1	970133	FXX	181a[b]	A1	A1
960100	NGA	Orphan 5	A2-1	A2-1	970866	X	Orphan 10	A3	A3-2
960101	NGA	438	A2-2	A2-2	970869	CIV	319	A2-1	A2-1
960102	GIN	181a[b]	A1	A1	970872	CIV	331a[b]	A2-1	A2-1
960103	GIN	326	A1	A1	970874	CIV	Orphan 11	A2-1	A2-1
960104	GIN	50	A3	A3-1	970877	CIV	318	A1	A1
960105	GIN	181b[b]	A1	A1	980338	CMR	856	A2-3	A2-3
960106	UGA	135	A3	A3-1	980698	CMR	328	A2-1	A2-1
960107	UGA	52	A3	A3-1	980699	CMR	Orphan 12	A2-3	A2-3
960108	UGA	52	A3	A3-1	980701	CMR	328	A2-1	A2-1
960109	UGA	420	A3	A3-1	980702	CMR	Orphan 13	A2-3	A2-3
970697	X[c]	181a[b]	A1	A1	981444	CMR	320	A2-1	A2-1
973402	X	181a[b]	A1	A1	981445	CMR	861	A2-1	A2-1
990984	X	101	A2-1	A2-1	981446	CMR	858	A2-3	A2-3
991541	X	Orphan 6	A1	A1	981448	CMR	328	A2-1	A2-1
993083	X	181a[b]	A1	A1	981449	CMR	329	A2-1	A2-1
220064	X	181a[b]	A1	A1	981538	BEN	Orphan 14	A2-2	A2-2
221703	X	187a[b]	A1	A1	14003001	SEN	799	A1	A1
990315	X	187b[b]	A1	A1	14003009	SEN	Orphan 15	A2-1	A2-1
210271	X	Orphan 7	A2-2	A2-2	14003011	SEN	323	A1	A1
210390	X	187b[b]	A1	A1	14003028	CMR	Orphan 16	A2-3	A2-3
211535	X	331b[b]	A2-1	A2-1	14003041	CMR	Orphan 17	A3	A3-2
220097	X	331b[b]	A2-1	A2-1	14003048	SEN	Orphan 18	A3	A3-2
220476	X	1153	A3	A3-2					

[a] BEN, Benin; CIV, Ivory Coast; CMR, Cameroon; FXX, metropolitan France; GIN, Guinea-Bissau; NGA, Nigeria; SEN, Senegal; SLE, Sierra Leone; UGA, Uganda.
[b] Strain for which a different pattern was observed when spoligotyping with 68 spacers was used.
[c] X, strain isolated from African patients residing in the United States.
[d] Strain 200100 was *M. africanum* type strain ATCC 25420^T.
[e] Strain 200101 was *M. africanum* type strain ATCC 35711.

obtained corroborated the typical absence of spacers 8, 9, and 39 in 57 subtype I isolates (25). The six subtype II pattern strains (strains 960096, 960104, 960106, 960107, 960108, and 960109 in Table 1) showed a positive hybridization with these three spacers, which corroborated their classification as *M. tuberculosis* (3, 17). Two other isolates were also atypical, since one showed positive hybridization signals with spacers 8 and 9 but not 39 (isolate 960091), whereas the other (isolate 220476) was positive for spacer 8 but not for spacers 9 and 39.

The spoligotyping patterns were compared to those found in the international spoligotyping database at the Institut Pasteur de Guadeloupe (7). Its recent version (SpolDB4), which is under development, contained patterns from 23,200 clinical isolates, split into 1,340 shared types (STs) and 2,000 unique types, at the time of the comparison. Eighteen unique patterns and 23 STs (two or more isolates with the same pattern in the SpolDB4 database) were observed. Among the 23 STs, seven clusters containing 2 to 12 isolates were defined: 1 cluster of 12 strains (ST 181), 1 of 5 strains (ST 331), 4 of 3 strains (STs 52, 187, 328, and 438) and 1 of 2 strains (ST 326). Detailed analysis of the patterns (p), and not the strains, led to the identification of three main groups (Table 1), designated A1 (p-12), A2 (p-20), and A3 (p-9). It should be emphasized that the classification of strains within various groups in the present study does not follow the scheme of Viana-Niero et al. (25).

Groups A1 and A2 were relatively homogeneous, whereas A3 was more heterogeneous and included isolates that could not be grouped as either A1 or A2. The group A1 signature appeared to be the absence of spacers 7 to 9 and 39 (Table 2). Group A2 could be divided into three subgroups: A2-1 (p-11), whose characteristic was the absence of spacers 8 to 12, 21 to 24, and 37 to 39; subgroup A2-2 (p-4), with the absence of spacers 8 to 12 and 37 to 39; and subgroup A2-3 (p-5), which showed no hybridization with spacers 8 to 12, 25 to 33, 35, and 37 to 39 (Table 2). A3 arbitrarily contained all of the isolates that were not grouped as either A1 or A2. A tentative profile for some of the A3 strains was the absence of spacers 33 to 36 with or without spacer 40 (subtype II [16]). The absence of spacer 40 in strains from Africa has been reported (20).

Characterization of *M. africanum* by new-generation spoligotyping. As summarized in Tables 1 and 2 and Fig. 1, a total of 45 different patterns were obtained for the 65 isolates by

68-spacer spoligotyping. Group A1 (p 14) was characterized by the absence of spacers 15 to 19 and 53 to 57 (Table 2). Group A2 (p 22) was divided into three different subgroups (Table 2): A2-1 (p 13), characterized by the absence of spacers 18 to 22, 31 to 34, and 51 to 60; A2-2 (p 4), characterized by the absence of spacers 18 to 22 and 51 to 60; and A2-3 (p 5), characterized by the absence of spacers 18 to 22, 25, 35 to 43, and 51 to 60. This method also highlighted a new subgroup (p 4) with a potential signature characterized by the absence of spacers 5 to 11, 16 to 17, 43 to 50, and 54 to 62 within the previously defined group A3. It contained the six subtype II pattern strains that did not show a typical *M. africanum* signature in the 43-spacer format (missing spacers 8, 9, and 39) and was designated A3-1. Nonetheless, five other patterns did not fit any of the above-described groups and were arbitrarily designated A3-2 (Table 1; Fig. 1).

Regarding the geographic variability (Table 1), groups A1 and A2 included strains from West Africa (Benin, Cameroon, African patients in France, Guinea-Bissau, Ivory Coast, Nigeria, Senegal, and Sierra Leone), whereas A3-1 included strains that were mostly from East Africa (five out of six strains).

The addition of novel spacers enhanced the discriminatory power of the spoligotyping. It increased the number of patterns identified from 41 to 45, with a Hunter-Gaston discriminatory index of 0.97, instead of 0.95 for the 43-spacer format. Indeed, the 20 clinical isolates that clustered in three STs numbered 181, 187, and 331 in the classical 43-bit representation, were further subdivided by using the 68-bit representation system. The seven subtypes thus obtained are shown in Table 2. Interestingly, ST 181, which defines the majority of *M. africanum* isolates described so far (25), was characterized by an increased deletion step (absence of a block of five spacers). The same phenomenon is also apparent for shared types 187 and 331 (Table 2). These results corroborate and further extend those of van der Zanden et al. showing that the strain differentiation of *M. bovis*, *M. microti*, and *M. canettii* within the *M. tuberculosis* complex was improved by using novel spacers (23), and they suggest that extensive studies using new-generation spoligotyping may be helpful to better understand the evolution of *M. africanum*.

PCR-deletion region analysis. Out of 20 variable regions described for the *M. tuberculosis* complex (2, 3, 8), RD9 and RD10 are particularly useful to differentiate *M. africanum*, since it lacks RD9 (and sometimes RD10), regions that are present in *M. tuberculosis* (3, 10, 17). Our results showed that RD9 was present only among the 6 subtype II strains (A3-1 subgroup) and not in the remaining 59 strains. In contrast, the TbD1 region was systematically present in all *M. africanum* groups studied with the exception of six isolates in subgroup A3-1, suggesting that these isolates are "modern" strains of *M. tuberculosis* (3). This finding shows that the latter isolates are clearly different from other *M. africanum* strains seen in this and previous studies (12, 25) and is consistent with their reclassification as *M. tuberculosis* (17). Further analysis showed that RD11 was absent in all strains belonging to group A1 but was present in strains in groups A2 and A3, whereas RD10 was variable. The distribution of the RDs in conjunction with the current knowledge of *M. tuberculosis* phylogeny suggests a possible way in which *M. tuberculosis* evolution may have proceeded (3, 10, 15). It seems likely that *M. bovis* is the final

TABLE 2. Rules describing specific spoligotyping signatures for strains within each of the groups or subgroups defined by 68-spacer spoligotyping

ST	Example of spoligotype	Group[a]	Nonhybridizing spacers[b] according to reference:	
			24	23
181a		A1	15–19, 53–57	**7–9, 39**, 52–53, 58–61
181b		A1	5–9, 15–19, 53–57	**7–9, 39**, 45–49, 52–53, 58–61
187a		A1	15–19, 43, 53–57	**7–9, 33, 39**, 52–53, 58–61
187b		A1	15–19, 43, 53–60	**7–9, 33, 39**, 52–53, 58–64
331a		A2-1	18–22, 31–34, 51–60	**8–12, 21–24, 37–39**, 58–64
331b		A2-1	16, 18–22, 31–34, 51–60	**8–12, 21–24, 37–39**, 52, 58–64
331c		A2-1	18–22, 31–34, 48, 51–60	**8–12, 21–24, 37–39**, 55, 58–64
438		A2-2	18–22, 51–60	**8–12, 37–39**, 58–64
858		A2-3	18–22, 25, 35–43, 45–46, 51–60	**8–12, 25–33, 35, 37–39**, 54, 58–64
52		A3-1	5–11, 16–17, 43–50, 54–62	**33–36, 40**, 45–65

[a] A3-2 did not show a specific signature, and hence no deletion pattern could be defined.
[b] Numbers in boldface indicate the spoligotyping signatures based on the classical 43-spacer format.

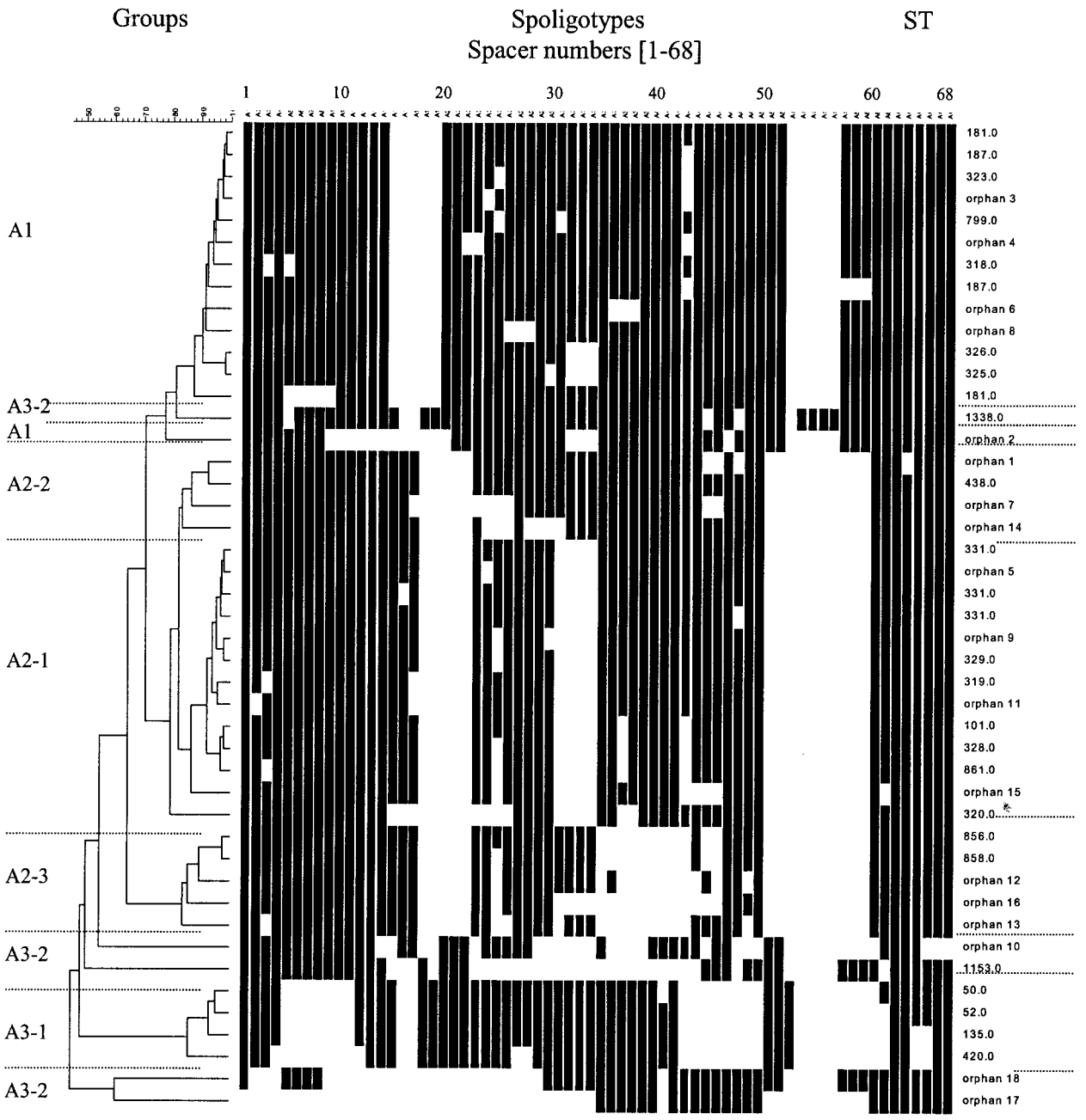

FIG. 1. Dendrogram and schematic representation of spoligotype patterns obtained by using the new 68-spacer format. The degree of similarity of the spoligotypes was calculated with the 1-Jaccard index, and the relationship between patterns was assessed by the unweighted pair group method of arithmetic averages. For descriptions of the various groups and subgroups and the STs listed, see the text.

member of a separate lineage represented by *M. africanum*, *M. microti*, *M. caprae*, and *M. bovis* and that *M. africanum* may be an offspring of *M. tuberculosis* which has lost RD9. This evolutionary hypothesis regarding *M. africanum* applies to *M. africanum* subtype I but not to *M. africanum* subtype II, which may represent a diverging branch of the *M. tuberculosis* lineage.

Phylogeographical considerations. The *M. tuberculosis* complex currently encompasses *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, *M. bovis* subsp. *caprae*, *M. canettii*, *M. microti*, and a recently described species, *M. pinnipedii* (4), with characteristic animal and/or human epidemiologies (4, 18). *M. tuberculosis* and *M. bovis* are most commonly isolated in clinical laboratories and may be easily distinguished by biochemical tests, phenotypic characters, and several genetic markers (5). However, the taxonomical status of *M. africanum* isolates, which were shown to introduce substantial phenotypic heterogeneity and genetic diversity within *M. bovis* and *M. tubercu-*

losis clusters, remained controversial (6). A distinction between West African strains (from Senegal, Mauritania, and Cameroon), which are more likely to have *M. bovis*-like phenotypic characteristics (*M. africanum* type I), and East African strains (from Burundi and Rwanda), which are more likely to have *M. tuberculosis*-like characteristics (*M. africanum* type II), was previously made by David et al. (6). Our results with the 68-spacer spoligotyping format showed that the patterns for these strains lacking spacers 33 to 36 and 40, did resemble those previously labeled as *M. tuberculosis* (24). Nonetheless, spoligotyping alone may not always allow us to gain sufficient information to establish a likely evolutionary history of *M. tuberculosis*; e.g., generation of identical spoligotype patterns among different strain families may also arise due to IS*6110*-mediated deletion polymorphism (26). For a precise phylogeographical attribution of the various *M. africanum* clades, larger studies using 68-spacer format spoligotyping in conjunction with other markers, such as mycobacterial interspersed repetitive units (22), RDs, and single-nucleotide polymorphisms (1, 9), may be rewarding.

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