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Characterization of Mycobacteria from a Major Brazilian Outbreak Suggests that Revision of the Taxonomic Status of Members of the *Mycobacterium chelonae-M. abscessus* Group Is Needed[▽]

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An outbreak of postsurgical infections caused by rapidly growing mycobacteria has been ongoing in Brazil since 2004. The degrees of similarity of the *rpoB* and *hsp65* sequences from the clinical isolates and the corresponding sequences from both the *Mycobacterium massiliense* and the *M. bolletii* type strains were above the accepted limit for interspecies variability, leading to conflicting identification results. Therefore, an extensive characterization of members of the *M. chelonae-M. abscessus* group was carried out. The *M. abscessus*, *M. chelonae*, *M. immunogenum*, *M. massiliense*, and *M. bolletii* type strains and a subset of clinical isolates were analyzed by biochemical tests, high-performance liquid chromatography, drug susceptibility testing, PCR-restriction enzyme analysis of *hsp65* (PRA-*hsp65*), *rpoB*, and *hsp65* gene sequencing and analysis of phylogenetic trees, DNA-DNA hybridization (DDH), and restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (RFLP-16S rRNA). The clinical isolates and the *M. abscessus*, *M. massiliense*, and *M. bolletii* type strains could not be separated by phenotypic tests and were grouped in the phylogenetic trees obtained. The results of DDH also confirmed the >70% relatedness of the clinical isolates and the *M. abscessus*, *M. massiliense*, and *M. bolletii* type strains; and indistinguishable RFLP-16S rRNA patterns were obtained. On the contrary, the separation of clinical isolates and the *M. abscessus*, *M. massiliense*, and *M. bolletii* type strains from *M. chelonae* and *M. immunogenum* was supported by the results of PRA-*hsp65*, DDH, and RFLP-16S rRNA and by the *rpoB* and *hsp65* phylogenetic trees. Taken together, these results led to the proposition that *M. abscessus*, *M. massiliense*, and *M. bolletii* represent a single species, that of *M. abscessus*. Two subspecies are also proposed, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*, and these two subspecies can be distinguished by two different PRA-*hsp65* patterns, which differ by a single HaeIII band, and by differences in their *rpoB* (3.4%) and *hsp*65 (1.3%) sequences.

Since 2004, a series of localized skin and soft tissue infections caused by rapidly growing mycobacteria have occurred in Brazil in patients who have undergone invasive procedures, such as laparoscopic, arthroscopic, plastic surgery, or cosmetic interventions (12, 16, 23, 37). In 4 years, more than 2,000 cases were officially reported to Brazilian federal authorities, who consider this problem an epidemiological emergency (5). Almost all isolates studied so far have belonged to the *Mycobacterium chelonae-M. abscessus* group (39). The majority of them were identified as members of two recently described emerging pathogens, *Mycobacterium massiliense* (3) and *Mycobacterium bolletii* (1), both of which belong to the *Mycobacterium chelonae-M. abscessus* group.

All five members of the *Mycobacterium chelonae-M. abscessus* group, *M. chelonae*, *M. abscessus* (21), *Mycobacterium immunogenum* (40), *M. massiliense* (3), and *M. bolletii* (1), are nearly indistinguishable phenotypically. Common features include growth in less than 7 days, the absence of pigmentation, better growth at 30°C than at 35°C, a positive 3-day arylsulfatase test result, a negative nitrate reductase test result, and a negative iron uptake test result (41). Two biochemical tests, sodium chloride tolerance and the utilization of citrate, are useful in distinguishing the five members (1, 3, 40, 41).

Antimicrobial susceptibility can also be used to differentiate the members of the *M. chelonae-M. abscessus* group. *M. abscessus* is generally susceptible to cefoxitin (MIC < 16 μg/ml) and *M. chelonae* is resistant (MIC > 128 μg/ml). Otherwise, *M. abscessus* is resistant to tobramycin (MIC > 16 μg/ml) and *M. chelonae* is susceptible (MIC < 4 μg/ml) (9). *M. immunogenum* is resistant to both drugs (40). *M. massiliense* was initially reported to be susceptible to doxycycline (3), but clinical isolates with intermediate susceptibility and also resistance to this drug have also been described (30, 37). *M. bolletii* was described to be a highly resistant species, and clarithromycin was among the drugs to which it was resistant (1). However, the results displayed by these tests can vary between strains, as has been shown for *M. massiliense* (3, 30). Lipid analysis by high-

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performance liquid chromatography (HPLC) produces very similar mycolic acid patterns for *M. chelonae*, *M. abscessus*, and *M. immunogenum* and thus has limited discriminatory power (10). The HPLC profiles of *M. massiliense* and *M. bolletii* have not yet been reported.

Genotypic characterization of the group has shown that the 16S rRNA sequences of *M. chelonae* and *M. abscessus* differ by only 4 bp, while they have identical hypervariable region A sequences (9). Both *M. massiliense* and *M. bolletii* show 100% 16S rRNA sequence similarity to the 16S rRNA sequence of *M. abscessus* (1, 3). Finally, the *M. immunogenum* 16S rRNA sequence differs from the corresponding sequences of *M. abscessus* and *M. chelonae* by 8 bp and 10 bp, respectively.

PCR-restriction enzyme analysis (PRA) of a 441-bp fragment of the 65-kDa heat shock protein-encoding gene, *hsp65* (PRA-*hsp65*), has thus far yielded five different patterns within the members of the *M. chelonae-M. abscessus* group: *M. chelonae* has a single pattern (pattern 1), while *M. abscessus* and *M. immunogenum* have two patterns each (patterns 1 and 2) (13, 29, 40). *M. massiliense* and *M. bolletii* have identical PRA-*hsp65* patterns, and these in turn, are identical to pattern 2 of *M. abscessus* (1, 3). The *M. abscessus* PRA-*hsp65* pattern 2 was also found in 152 isolates from the Brazilian outbreak mentioned above (data not shown).

The sequence of the *rpoB* gene has been demonstrated to be a powerful tool for the delineation of bacterial species (4) and was applied to the identification of rapidly growing mycobacteria, including members of the *M. chelonae-M. abscessus* group (2). The degree of interspecies variation found in the *rpoB* sequence is variable. Nevertheless, a 3% cutoff value can generally be applied to the mycobacteria, but some exceptions to that rule have been described. Analysis of interspecies variations in the *rpoB* gene sequence showed that the members of the *Mycobacterium avium* complex have a range of 4.5 to 5.7% sequence divergence (7) compared to the *rpoB* sequence of the closely related species *M. marinum* and *M. ulcerans*, whose *rpoB* sequences showed 0.4% divergence from each other (4). On the other hand, *M. mucogenicum* and *M. abscessus* have shown wider internal heterogeneity in the *rpoB* gene sequence, with the latter having up to 4.3% internal species variation (2).

The papers describing *M. massiliense* and *M. bolletii* have indicated that a specific sequence of the *rpoB* gene is a main characteristic in these species (1, 3). Consequently, sequence analysis of the *rpoB* gene as well as of the *hsp65* gene, which we used in a previous study, led to the identification of several isolates from surgical patients as *M. massiliense* and other isolates from mesotherapy patients as *M. bolletii* because of the highest degrees of similarity of their *rpoB* and *hsp65* gene sequences (37). Several unclear findings, however, emerged from that analysis. High degrees of similarity of the *rpoB* sequences of surgical isolates to the *rpoB* sequences of both *M. massiliense* (99.72%) and *M. bolletii* (98.45%) were detected. Moreover, the *rpoB* sequences of isolates from patients undergoing mesotherapy showed 100% similarity to the *rpoB* sequence of *M. bolletii* and 98.54% similarity to that of *M. massiliense*. According to the 3% cutoff that has been proposed (2), all isolates could therefore be identified either as *M. massiliense* or as *M. bolletii* on the basis of their *rpoB* gene sequences.

Similar results were observed with the *hsp65* sequences. The *hsp65* sequences of surgical isolates showed 100% and 99.25% similarities to the *hsp65* sequences of *M. massiliense* and *M. bolletii*, respectively. Again, the *hsp65* sequences of isolates from patients who had undergone mesotherapy displayed 100% similarity to the *hsp65* sequence of *M. bolletii* and 99.24% similarity to the *hsp65* sequence of *M. massiliense*. For *hsp65*, the value of 97% similarity is suggested by McNabb et al. (25) as the limit for the separation of species.

These inconclusive results prompted us to undertake an extensive phenotypic and genotypic characterization of the isolates from the Brazilian outbreaks. We applied a wide set of procedures, including DNA-DNA hybridization (DDH), which is so far considered the "gold standard" for the delineation of bacterial species (4, 31). Selected isolates from the outbreaks were compared to each other and also to reference strains representative of the five members of the *M. chelonae-M. abscessus* group. We showed that the DNA-DNA relatedness clearly identifies *M. massiliense* and *M. bolletii*, together with our clinical isolates, as *M. abscessus*, indicating that all of these isolates make up a single genomic species. Finally, taking into account the internal variability detected in the sequence of *M. abscessus*, we propose the description of two subspecies within this species.

MATERIALS AND METHODS

Mycobacterial strains. Six clinical isolates and five reference strains were included in this study. Three isolates from the state of Para in Brazil were previously identified as *M. massiliense* (two were from the laparoscopic surgery outbreak [referred to as isolates B5 and B31 in this report] and one was from an abscess that formed after an intramuscular injection [referred to as isolate B67 in this report]), and three isolates from the state of Para were previously identified as *M. bolletii* and were from patients who had undergone mesotherapy (37). The latter three isolates are referred to as B60, B61, and B66 in this report. The type strains of the five species *M. abscessus*, *M. chelonae*, *M. immunogenum*, *M. massiliense*, and *M. bolletii* were included as reference strains. The *M. fortuitum* type strain (ATCC 6841) was also used as an outgroup and a control in some experiments. Bacteria were cultivated in Mueller-Hinton liquid or solid medium; Middlebrook 7H9 broth supplemented with albumin, dextrose, and catalase; or Middlebrook 7H10 solid medium supplemented with oleic acid, albumin, dextrose, and catalase. Frozen stocks were prepared.

Phenotypic identification. (i) Biochemical tests. The isolates were evaluated for a panel of biochemical and cultural features by standard procedures (18). The tests included analysis of the growth rate; growth at 25°C and 45°C; pigment production; colony morphology; growth on MacConkey agar; biochemical tests (nitrate reduction; Tween hydrolysis; tellurite reduction; and niacin, catalase, beta-glucosidase, arylsulfatase, and urease tests); and tolerance to thiophenecarboxylic acid, tiacetazone, *p*-nitrobenzoic acid, isoniazid, hydroxylamine, oleate, NaCl, and sodium citrate.

(ii) HPLC. HPLC of cell wall mycolic acids, which may be a valid aid in the identification of mycobacterial species, was carried out as reported before (12).

(iii) Susceptibility testing. MICs were determined by the microdilution method, according to the recommendations of the CLSI (formerly NCCLS) (27), with commercially available microplates (Sensititer RGMYCO; Trek Diagnostic Systems Inc., Cleveland, OH).

Genotypic identification. (i) DNA isolation. Total genomic DNA was purified from liquid bacterial cultures as described previously (36).

(ii) PRA-*hsp65*. The band patterns produced by PRA-*hsp65* were obtained as described by Telenti et al. (33) and were compared to those in the PRASITE database (http://app.chuv.ch/prasite/index.html).

(iii) DNA sequencing and phylogenetic analysis. The *hsp65* and *rpoB* gene sequences were obtained and analyzed as described by Viana-Niero et al. (37). The corresponding sequences from the type strains included in this study were also retrieved from the GenBank database for comparative purposes. The alignment of the *rpoB* and *hsp65* gene sequences was performed with ClustalX (version 2.0) software (22). Two separate phylogenetic trees were constructed for both genes by using *M. tuberculosis* strain H37Rv (ATCC 27294) as the outgroup. The evolutionary history was generated by the neighbor-joining method. The evolutionary distances were computed by use of the Kimura two-parameter

TABLE 1. Comparison of phenotypic and biochemical characteristics of isolates and strains

Phenotypic or biochemical test	Result for the following isolates and strains[a]:										
	1	2	3	4	5	6	7	8	9	10	11
Niacin accumulation	−	−	−	−	−	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−	−	−	−	−	−
68°C catalase	−	−	−	−	−	−	+/−	+/−	−	+	+
PNB[b] tolerance	+	+	+	+	+	+	+	+	+	−	+
Catalase over 45 mm	+	+	+	+	+	+	+	−	+	+	+
Pigmentation	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent	Absent
Beta-glucosidase	−	−	−	+/−	−	+/−	−	−	+	−	+
Tween 80 hydrolysis	−	−	−	−	−	−	−	−	−	−	−
Growth rate	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid	Rapid
Growth at 25°C	+	+	+	+	+	+	+	+	+	+	+
Growth at 45°C	−	−	−	−	−	−	−	−	−	−	−
MacConkey growth	+	+	+	+	+	+	+	+	+	−	−
Tellurite reduction	+	+	+	+	+	+	+	+	+	−	+
3-day arylsulfatase	+	+	+	+	−	+	+	+	+	+	+
Colony morphology	SM[c]	SM	SM	SM	SM	SM	SM	SM	RG[d]	SM	SM
Urease	+	+	+	+	+	+	+	+	+	−	−
TCH[e] tolerance	+	+	+	+	+	+	+	+	+	+	+
Tiacetazone tolerance	+	+	+	+	+	+	+	+	+	+	−
Hydroxylamine tolerance	+	+	+	+	+	+	+	+	−	−	−
Isoniazid tolerance	+	+	+	+	+	+	+	+	+	+	+
Oleic acid tolerance	+	+	+	+	+	+	+	+	+	+	−
NaCl 5% tolerance	+	+	+	+	−	+	+	+	+	−	−
Sodium citrate	−	−	−	−	−	−	−	−	−	+	−

[a] Brazilian isolates, as follows: 1, B67; 2, B31; 3, B5; 4, B60; 5, B61; 6, B66. 7, *M. bolletii* CIP 108541; 8, *M. massiliense* CIP 108297; 9, *M. abscessus* ATCC 19977; 10, *M. chelonae* ATCC 35752; 11, *M. immunogenum* ATCC 700505.
[b] PNB, *p*-aminobenzoic acid.
[c] SM, smooth.
[d] RG, rough.
[e] TCH, thiophene-carboxylic acid.

model and bootstrap analysis with 1,000 replications. Phylogenetic analysis was performed with MEGA (version 4) software (32).

DNA-DNA similarity. The total genomic relationships of DNAs were investigated for the six clinical isolates and the corresponding type strains of *M. abscessus*, *M. chelonae*, *M. immunogenum*, *M. massiliense*, and *M. bolletii*. *M. fortuitum*^{T} DNA was also analyzed so that DNA from a non-closely related fast-growing mycobacterium was included. DDH experiments were performed on membrane filters by a dot blot-based procedure, as described previously (17). Genomic DNAs from *M. massiliense* CIP 108297^T and *M. bolletii* CIP 108541^T were radioactively labeled in vitro and were used as probes to hybridize with 500 ng of each unlabeled DNA. Unlabeled DNAs were dot blotted and bound to nylon membranes (Hybond-N+; Amersham). The relative binding ratios (expressed as percentages) were calculated from the counts of bound homologous DNA, as measured with a Typhon Trio apparatus (GE Healthcare). The amount of DNA fixed in each dot was calculated by measuring the amount of radioactivity when the 16S rRNA gene was used as a second probe (17).

RFLP of 16S rRNA gene. RFLP analysis of the 16S rRNA gene was also performed. This procedure allowed the identification of the number of *rrn* operons per genome carried by the mycobacteria (15). A previously described experimental procedure (14) was used, with the exception that ECL buffer (Amersham) was used for both the prehybridization and the hybridization steps.

RESULTS

Phenotypic identification. The biochemical and culture tests turned out to be poorly discriminative (Table 1). Most of the clinical isolates presented almost overlapping results with the closely related rapid growers (*M. abscessus*, *M. chelonae*, *M. immunogenum*). The few discrepancies within the strains belonging to the species *M. massiliense* and *M. bolletii* may well be imputable to biological variability. The results of HPLC were also poorly informative. All the species investigated here were, in fact, characterized by a single pattern, with only minor differences being detected among the species (Fig. 1).

Although the majority of the test results confirmed the susceptibility patterns available in the literature for the reference strains (3, 9, 30, 40), several clear differences emerged (Table 2). The reference strain of *M. massiliense*, as well as two of three *M. massiliense* clinical strains (strains B67 and B31), turned out to be susceptible to amikacin (MIC range, 8 to 16 μg/ml for all except for one strain, strain B5; Table 1) while in the species nova description, a MIC of 48 μg/ml has been reported (3). In contrast, there was a discrepancy in the MICs of the tetracyclines, with all of our strains, including the *M. massiliense* reference strain, which has been reported to have a MIC of 4 μg/ml (3), being resistant (MIC $>$ 32 μg/ml). The results obtained with the commercial microplate, which included minocycline, were, in fact, confirmed by the doxycycline test, which was performed by the same technique with the prepared antibiotic. Even more striking were the discrepancies concerning *M. bolletii*, whose species nova description (1) emphasized its resistance to clarithromycin (MIC $>$ 256 μg/ml). In contrast, all of our strains, including the *M. bolletii* reference strain, were susceptible (MIC range, $<$0.12 to 1 μg/ml).

Genotypic identification. Initially, the two methods that are the more frequently applied to the identification of mycobacterial species were used, i.e., PRA-*hsp65* gene and DNA sequencing of the two selected genome targets, namely, the *rpoB* and *hsp65* genes.

On the basis of the previously defined PRA-*hsp65* patterns (13, 37), the PRA-*hsp65* type 2 pattern of *M. abscessus* was shared by all six of our clinical isolates and by the *M. massiliense* and *M. bolletii* type strains (data not shown). The other type strains showed the expected patterns, according to the

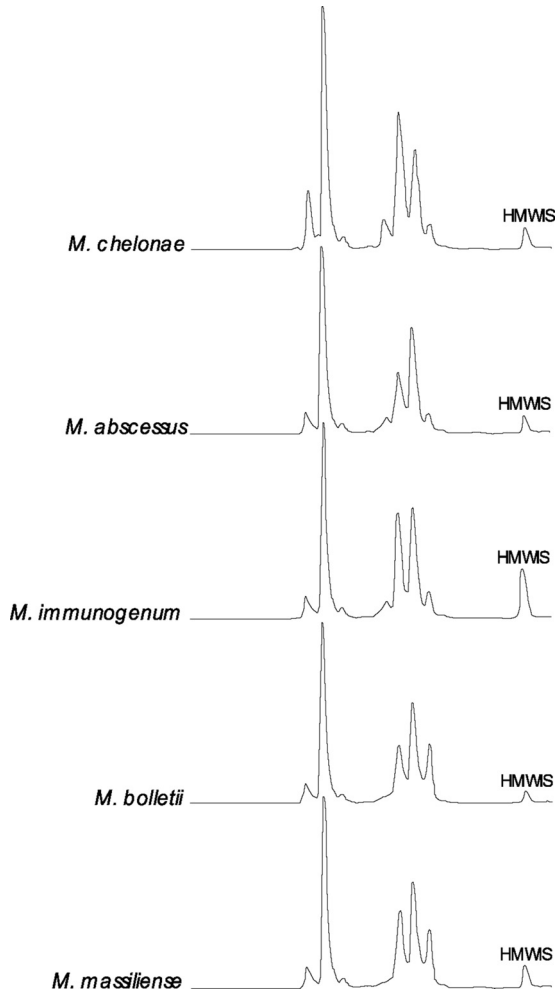

FIG. 1. Comparison of representative HPLC mycolic acid patterns of members of the *M. chelonae-M. abscessus* group. HMWIS, high-molecular-weight internal standard.

information in the PRASITE database (http://app.chuv.ch/prasite/index.html).

***rpoB* and *hsp65* sequencing.** The sequences of region V of the *rpoB* gene were compared. The *rpoB* gene sequences of the Brazilian isolates were found to be almost identical to those of the *M. massiliense*^{T} and *M. bolletii*^{T} strains from GenBank (greater than 98.5%) and were found to be closely related to the *rpoB* sequence of *M. abscessus*^{T} (96.5% and 95.6%, respectively).

The *rpoB* sequences were translated into amino acid sequences for further comparison. Most of the nucleotide polymorphisms detected represented silent substitutions, with no amino acid change in the corresponding protein sequences being detected. The exceptions were the E/D, A/Q, A/E, and T/L substitutions in the *M. abscessus*^{T} *rpoB* sequence and the V/I and N/D substitutions in the *M. chelonae*^{T} *rpoB* sequence (data not shown). In both cases, substitutions appeared in a nonconserved region from the RNA polymerase RPB10 interaction site, according to the Conserved Domain Database at NCBI (24). Comparisons of the sequences from the *hsp65* gene indicated results similar to those corresponding to *rpoB* gene sequences, with even higher percentages of similarities being detected. The *hsp65* sequence (401 bp) from the *M. abscessus* type strain showed 98.5 and 98.7% similarities to the corresponding sequences from *M. massiliense* and *M. bolletii*, respectively. A higher similarity index (99.2%) between the corresponding sequences from *M. massiliense* and *M. bolletii* was observed. Again, the *hsp65* gene sequences of the Brazilian isolates were found to be almost identical to the *M. massiliense*^{T} and *M. bolletii*^{T} *hsp65* sequences (greater than 99%) and closely related to the *M. abscessus*^{T} *hsp65* sequence (98% similarity). All nucleotide polymorphisms detected represented silent substitutions.

The close relationship detected by comparison of the nucleotide sequences of both the *rpoB* and *hsp65* genes is shown in the phylogenetic trees (Fig. 2a and b, respectively).

DDH. Due to the high degree of similarity shown by our selected isolates of *M. massiliense*, *M. bolletii*, and *M. abscessus*, total DNA-DNA similarities were determined, because DDH is considered the reference method for the delimitation of bacterial species (4, 31). The results are summarized in Table 3. The levels of DDH obtained by using *M. massiliense*^{T} and *M. bolletii*^{T} as probes with DNA from *M. abscessus*^{T} and all six clinical isolates were greater than 70%, indicating that they all belong to a single species. On the contrary, the level of hybridization with DNA from other species, including *M. chelonae*^{T}

TABLE 2. Antimicrobial susceptibility results for isolates and strains included in this study

Drug	MIC (μg/ml) for the following isolates and strains[a]:										
	1	2	3	4	5	6	7	8	9	10	11
Linezolid	32	32	32	8	32	16	8	8	16	2	32
Clarithromycin	1	0.5	0.25	<0.12	0.5	<0.12	<0.12	0.25	0.25	0.25	0.25
Amikacin	16	16	32	16	16	8	8	8	16	8	16
Cefoxitin	64	>256	64	128	32	64	64	64	32	>256	>256
Ceftriaxone	>64	>64	>64	>64	>64	32	>64	>64	>64	>64	>64
Imipenem	32	64	64	64	16	8	32	32	8	16	64
Tobramycin	32	>64	>64	16	16	>64	16	16	16	2	16
Ciprofloxacin	>16	>16	>16	16	16	>16	8	>16	8	0.5	8
Gatifloxacin	>8	>8	>8	8	>8	>8	8	>8	>8	0.25	>8
Minocycline	>32	>32	>32	<0.5	>32	>32	>32	>32	>32	16	32
Amoxicillin-clavulanic acid	>64/32	>64/32	>64/32	>64/32	>64/32	>64/32	>64/32	>64/32	>64	>64	>64
Trimethoprim-Sulfamethoxazole	>8/152	>8/152	>8/152	>8/152	>8/152	>8/152	>8/152	>8/152	>152	76	>152

[a] Strains 1 to 11 correspond to the strains described in footnote *a* of Table 1.

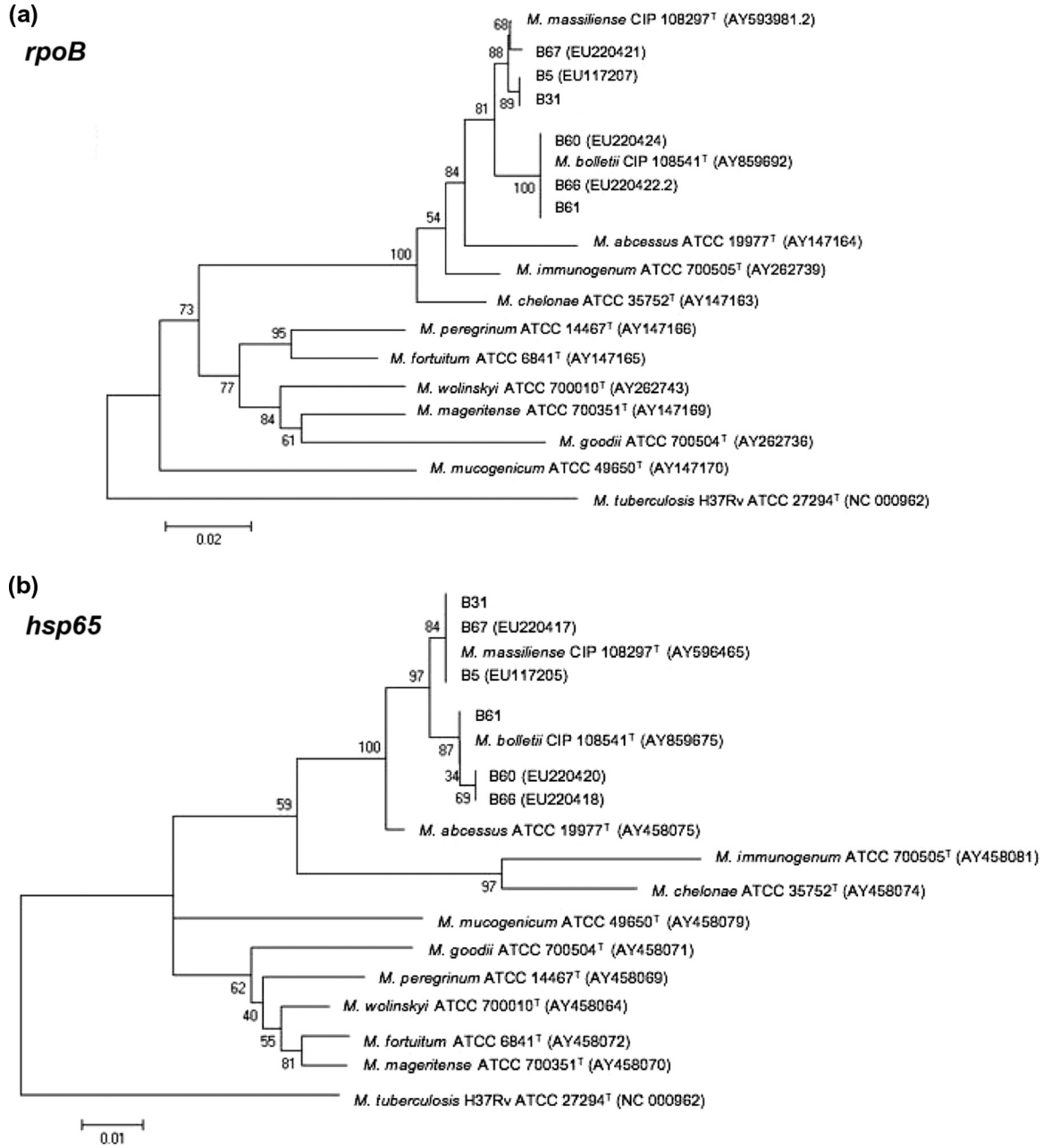

FIG. 2. Phylogenetic tree based on *rpoB* (a) and *hsp65* (b) gene sequences, showing the relationships between Brazilian clinical isolates (isolates B5, B31, B60, B61, B66, and B67) and other type strains of selected rapidly growing mycobacteria. GenBank accession numbers are shown in parentheses. *M. tuberculosis* ATCC 27294^T was included as the outgroup.

and *M. immunogenum*^{T}, was less than 44%, showing a clear differentiation at the genomic level between these closely related species.

RFLP of 16S rRNA gene. RFLP analysis with 16S rRNA as the probe was used to characterize the mycobacteria. According to the patterns obtained, all strains except *M. fortuitum* and *M. immunogenum* have a single *rrn* operon per genome (Fig. 3). The patterns for *M. fortuitum* and *M. immunogenum* are in agreement with the corresponding data published previously (15, 40).

BamHI-digested DNAs showed a single band pattern for all strains except *M. fortuitum* and *M. immunogenum*. Compared to the bands from *M. massiliense*, *M. bolletii*, and *M. abscessus*, the single band was slightly smaller for isolates B67, B31, B60, and B66 and in these isolates was in a position similar to that of the smaller single band shown by *M. chelonae* (Fig. 3a). These close patterns were distinguished by using PstI as second restriction enzyme.

The RFLP patterns of PstI-digested DNAs showed two bands that were in identical positions for all six Brazilian isolates, as well as *M. massiliense*, *M. bolletii*, and *M. abscessus* (Fig. 3b). On the contrary, PstI-digested DNA from *M. chelo-*

TABLE 3. DNA-DNA genomic pairing between *M. massiliense*^{T} and *M. bolletii*^{T} and other species, including Brazilian clinical isolates (B67, B31, B5, B60, B61, and B66)

Strain no.	Assigned species	Isolate or strain	Procedure	% DNA-DNA relatedness obtained with the probe for[a]:	
				M. massiliense	*M. bolletii*
1	*M. massiliense*	B67	Intramuscular injection	81.8 ± 11.3	83.8 ± 9.5
2	*M. massiliense*	B31	Laparoscopic surgery	91.7 ± 13.2	80.4 ± 10.5
3	*M. massiliense*	B5	Laparoscopic surgery	84.4 ± 9.5	87.5 ± 12.3
4	*M. bolletii*	B60	Mesotherapy	91.7 ± 8.2	104.3 ± 8.2
5	*M. bolletii*	B61	Mesotherapy	76.6 ± 9.4	88.6 ± 6.1
6	*M. bolletii*	B66	Mesotherapy	90.5 ± 12.4	105.7 ± 14.3
7	*M. bolletii*^{T}	CIP 108541		76.5 ± 4.8	100
8	*M. massiliense*^{T}	CIP 108297		100	73.4 ± 8.5
9	*M. abscessus*^{T}	ATCC 19977		92.02 ± 13.4	95.5 ± 12.9
10	*M. chelonae*^{T}	ATCC 35752		28.1 ± 1.2	44 ± 1.1
11	*M. immunogenum*^{T}	ATCC 700505		20.3 ± 1.1	22.4 ± 9.04
12	*M. fortuitum*^{T}	ATCC 6841		5.7 ± 2.3	4.3 ± 1.9

[a] Data are mean values ± standard deviation of two to five different experiments.

nae showed a different two-band pattern. These results showed that the Brazilian isolates, together with *M. massiliense* and *M. bolletii*, share RFLP-16S rRNA patterns with *M. abscessus*. It has been shown that the RFLP-16S rRNA patterns are species specific within rapidly growing mycobacteria (8, 14, 17, 26), and therefore, they should all be considered the species *M. abscessus*, which is clearly differentiated from *M. chelonae* and *M. immunogenum* according to their RFLP-16S rRNA patterns.

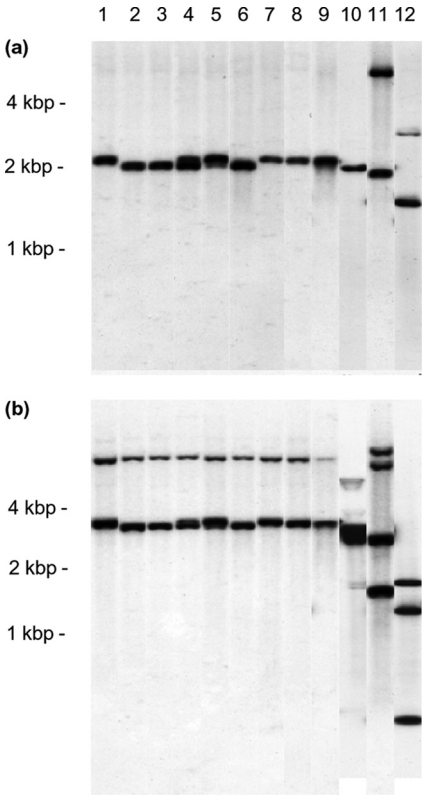

FIG. 3. RFLP-16S rRNA patterns of mycobacterial DNAs digested with the restriction enzymes BamHI (a) and PstI (b). Lanes 1 to 11, isolates 1 to 11, respectively, as described in footnote *a* of Table 1; lane 12, *M. fortuitum* ATCC 6841.

DISCUSSION

The increasing availability of gene sequences has had a tremendous influence on the taxonomy of bacteria, particularly that of the mycobacteria, with great increases in newly described species occurring every year (34). Some of the newly described species are considered emerging pathogens due to their relationships to clinical outbreaks (20, 29, 37). Besides the 16S rRNA gene, another conserved gene, *rpoB*, has widely contributed to the characterization of the species in the genus *Mycobacterium* (4). The degree of intraspecific *rpoB* sequence similarity has been estimated to range from 98.2 to 100% (4), with a few exceptions, such as the >4.3% intraspecies sequence divergence in the species *M. abscessus* (2).

Members of the *M. chelonae-M. abscessus* group are ubiquitous environmental organisms frequently associated with nosocomial outbreaks and pseudo-outbreaks (6, 20, 28, 29, 37, 38). Five related species with different associations with infection have been described within that group, making the proper identification of members of this complex important for both therapeutic management and epidemiological studies. The two species most recently incorporated into the group are *M. massiliense* and *M. bolletii*. Remarkably, the separation of these species was established mainly on the basis of their different *rpoB* gene sequences (1, 3). In the original description of these species, important differences in antimicrobial susceptibilities were also reported and were considered to support their separation.

In the recent outbreak of infections related to surgical and cosmetic procedures occurring in Brazil, the isolates were initially identified as *M. massiliense* (isolates from surgical cases) and *M. bolletii* (isolates from mesotherapy cases) (37). However, such identifications appeared to be inconclusive, with the isolates showing several characteristics that would allow them to be assigned to either one of the two species. In order to clarify their identities, a subset of six clinical isolates was extensively analyzed and compared to type strains not only of *M. massiliense* and *M. bolletii* but also of all other members of the *M. chelonae-M. abscessus* group. The analysis undertaken included both phenotypic and genotypic characterization.

M. massiliense^T, *M. bolletii*^{T}, *M. abscessus*^{T}, and the Brazilian

clinical isolates could not be clearly distinguished on the basis of their phenotypic characteristics. Biochemical tests produced almost indistinguishable patterns (Table 1). HPLC analysis, which is well known to be unable to differentiate *M. abscessus*, *M. chelonae*, and *M. immunogenum* (10, 40), turned out to be of no use with *M. bolletii* and *M. massiliense* (Fig. 1), whose profiles had not been investigated before. Even more confusing were the antimicrobial susceptibility data, especially with regard to clarithromycin. In the species nova description by Adekambi et al. (1), who used a nonapproved CLSI method, a MIC of >256 μg/ml was reported for *M. bolletii*, while the same type strain, as well as all other clinical isolates of the same species, were fully susceptible on the basis of our findings. In the only other study (19) in which the susceptibility of *M. bolletii* has been investigated so far, again, resistance to clarithromycin was detected, although the MIC range (8 to 16 μg/ml) was very different from our MIC range and that of Adekambi et al. (1).

M. abscessus^T could be separated from *M. massiliense*^{T} and *M. bolletii*^{T} by use of the PRA-*hsp*65 pattern. The first species showed the type 1 pattern of *M. abscessus*, while the latter two shared the type 2 pattern of *M. abscessus* (1, 3, 37), with the difference between the two patterns being limited to a single HaeIII band. All the Brazilian clinical isolates under study also had the type 2 pattern of *M. abscessus*.

Genetic sequencing of the *rpoB* region, as proposed by Adekambi et al. (2) for the separation of rapidly growing mycobacteria, confirmed the high degree of heterogeneity within clinical isolates of *M. abscessus*, which was also reported by the same authors. Our results showed that the similarities in the *rpoB* gene sequences place *M. abscessus*, *M. bolletii*, and *M. massiliense* with the Brazilian isolates on the same branch, in which all their degrees of identity are within the range of *M. abscessus* intraspecies variability (Fig. 2a). These results are consistent with the similarities found in a different conserved DNA target, the *hsp65* gene (Fig. 2b), whose use has also been proposed to be effective for the identification of mycobacteria (25).

The global DNA-DNA pairing values still remain the gold standard for the delineation of bacterial species (4, 9, 31, 42). Therefore, with the aim of clarifying the differences found, experiments were performed to analyze the total genomic relationships of the DNAs from the Brazilian clinical isolates and the type strains of the five species belonging to the *M. chelonae-M. abscessus* group. In the experimental design, the DNAs from *M. massiliense* and *M. bolletii* were used as probes. The DDH results revealed the clear species separation of *M. chelonae* and *M. immunogenum* from *M. massiliense* and *M. bolletii*, with the DDH values being less than 44% (Table 3). On the contrary, *M. massiliense* and *M. bolletii* could not be differentiated from each other or from *M. abscessus*, as the total DDH values were greater than 70% (Table 3). High hybridization values were also observed when the total DNAs of *M. massiliense* and *M. bolletii* were compared to the DNAs from the Brazilian clinical isolates (Table 3). According to these results, the two recently described species as well as the Brazilian clinical isolates appear to be members of the species *M. abscessus*.

M. abscessus as well as *M. chelonae*, unlike the large majority of rapidly growing mycobacteria, carry a single *rrn* operon per genome (15). RFLP analysis of the 16S rRNA genes also revealed in *M. massiliense*, *M. bolletii*, and the subset of Brazilian clinical isolates (Fig. 3) the presence of a single *rrn* operon per genome, similar to the findings for *M. abscessus*. Moreover, their RFLP patterns, which have been characterized as being species specific within rapidly growing mycobacteria (8, 15, 17, 26), were identical to the RFLP pattern of *M. abscessus* and different from the patterns of the other members of the *M. chelonae-M. abscessus* group (Fig. 3).

All results presented above indicate that the Brazilian *M. abscessus*, *M. bolletii*, and *M. massiliense* isolates belong to a single species. Since their description, "*M. massiliense*" and "*M. bolletii*" have been recognized to cause infectious diseases worldwide (11, 19, 20, 30, 35, 37). According to our results, from a public health perspective, the species responsible for all those cases should actually be assigned to the species *M. abscessus*.

The internal variability emerging from our findings and other previous data (2, 6, 28, 38) for the species *M. abscessus* suggests a new arrangement for the *M. chelonae-M. abscessus* group, in which only three species, *M. chelonae*, *M. immunogenum*, and *M. abscessus*, would be present, with *M. abscessus* being split into two subspecies, namely, *M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*. For the latter subspecies, including the isolates previously identified as "*M. massiliense*" and "*M. bolletii*," the name "massiliense" was chosen as it appeared first in the literature.

The two subspecies of *M. abscessus* can be distinguished genotypically by their PRA pattern type (the *M. abscessus* type 1 pattern for *M. abscessus* subsp. *abscessus* and the *M. abscessus* type 2 pattern for *M. abscessus* subsp. *massiliense*) and by differences in the *rpoB* and *hsp65* sequences. Thus, the 711-bp *rpoB* sequence and the 401-bp *hsp65* sequence from *M. abscessus* subsp. *abscessus* show similarities of less than 96.6% and 98.7%, respectively, with the sequences of *M. abscessus* subsp. *massiliense* (which includes the species formerly named "*M. bolletii*").

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