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# Multilocus Sequence Analysis and *rpoB* Sequencing of *Mycobacterium abscessus* (Sensu Lato) Strains[▽]

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**Mycobacterium abscessus*, *Mycobacterium bolletii*, and *Mycobacterium massiliense* (*Mycobacterium abscessus* sensu lato) are closely related species that currently are identified by the sequencing of the *rpoB* gene. However, recent studies show that *rpoB* sequencing alone is insufficient to discriminate between these species, and some authors have questioned their current taxonomic classification. We studied here a large collection of *M. abscessus* (sensu lato) strains by partial *rpoB* sequencing (752 bp) and multilocus sequence analysis (MLSA). The final MLSA scheme developed was based on the partial sequences of eight housekeeping genes: *argH*, *cya*, *glpK*, *gnd*, *murC*, *pgm*, *pta*, and *purH*. The strains studied included the three type strains (*M. abscessus* CIP 104536$^T$, *M. massiliense* CIP 108297$^T$, and *M. bolletii* CIP 108541$^T$) and 120 isolates recovered between 1997 and 2007 in France, Germany, Switzerland, and Brazil. The *rpoB* phylogenetic tree confirmed the existence of three main clusters, each comprising the type strain of one species. However, divergence values between the *M. massiliense* and *M. bolletii* clusters all were below 3% and between the *M. abscessus* and *M. massiliense* clusters were from 2.66 to 3.59%. The tree produced using the concatenated MLSA gene sequences (4,071 bp) also showed three main clusters, each comprising the type strain of one species. The *M. abscessus* cluster had a bootstrap value of 100% and was mostly compact. Bootstrap values for the *M. massiliense* and *M. bolletii* branches were much lower (71 and 61%, respectively), with the *M. massiliense* cluster having a fuzzy aspect. Mean (range) divergence values were 2.17% (1.13 to 2.58%) between the *M. abscessus* and *M. massiliense* clusters, 2.37% (1.5 to 2.85%) between the *M. abscessus* and *M. bolletii* clusters, and 2.28% (0.86 to 2.68%) between the *M. massiliense* and *M. bolletii* clusters. Adding the *rpoB* sequence to the MLSA-concatenated sequence (total sequence, 4,823 bp) had little effect on the clustering of strains. We found 10/120 (8.3%) isolates for which the concatenated MLSA gene sequence and *rpoB* sequence were discordant (e.g., *M. massiliense* MLSA sequence and *M. abscessus rpoB* sequence), suggesting the intergroup lateral transfers of *rpoB*. In conclusion, our study strongly supports the recent proposal that *M. abscessus*, *M. massiliense*, and *M. bolletii* should constitute a single species. Our findings also indicate that there has been a horizontal transfer of *rpoB* sequences between these subgroups, precluding the use of *rpoB* sequencing alone for the accurate identification of the two proposed *M. abscessus* subspecies.**

*Mycobacterium abscessus* is a rapidly growing mycobacterium (RGM) that causes a wide spectrum of disease in humans, including chronic lung disease, skin and soft-tissue disease, and disseminated disease (12, 14, 53). *M. abscessus* lung disease mostly develops in subjects with underlying lung disorders (e.g., cystic fibrosis [CF] or prior mycobacterial infection) or Lady Windermere syndrome (10, 19, 29, 35, 40, 41, 45, 46). *M. abscessus* is also a leading cause of sporadic and epidemic cases of skin and soft-tissue RGM infections after surgery or following the use of contaminated syringes and needles (21, 22, 39). Several large outbreaks of skin and soft-tissue infection have been reported following the injection of adrenal cortex extract, mesotherapy, tattooing, and piercing (6, 8, 15, 24, 52, 58).

The *Mycobacterium massiliense* and *Mycobacterium bolletii* species were characterized in the early 2000s, and both are closely related to *M. abscessus* (100% identity of 16S rRNA sequences) (2, 5). Therefore, *M. abscessus* (now *M. abscessus*

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sensu lato) is comprised of three species: *M. abscessus* sensu stricto (for simplicity, *M. abscessus* sensu stricto here will be referred to as *M. abscessus*), *M. massiliense*, and *M. bolletii*. Although information about the pathogenic effects of *M. massiliense* and *M. bolletii* in humans still is scarce, several recent studies report that they cause a spectrum of diseases similar to those associated with *M. abscessus*. However, there may be some differences between the three species. Zelazny et al. have reported that *M. massiliense* is more frequently present in the respiratory tract of younger patients with preexisting lung disease than *M. abscessus* (59). Differences also have been reported in the susceptibility patterns of the three species (13, 14, 54, 55). For example, *M. massiliense* was reported to be susceptible to doxycycline, whereas *M. abscessus* and *M. bolletii* are not (5), although this finding has not been confirmed by others (32, 52).

*M. massiliense* and *M. bolletii* were characterized as new species distinct from *M. abscessus* on the basis of their *rpoB* sequences (>3% sequence divergence) (2, 5). Partial *rpoB* sequencing is now the gold standard for the molecular identification of the three species (3, 4, 16, 38, 48). However, recent studies have highlighted the inaccuracy of single-target sequencing, including *rpoB* sequencing, for distinguishing between *M. abscessus*, *M. massiliense*, and *M. bolletii* (18, 31, 36, 59). Zelazny et al. found that the partial sequencing of *rpoB*, *hsp65*, and *secA* led to inconsistent results in 7 of 42 clinical isolates; most of these seven isolates had an *M. abscessus rpoB* sequence and *M. massiliense hsp65* and *secA* sequences, and they clustered with the *M. massiliense* type strain in repetitive sequence-based PCR (rep-PCR) and pulsed-field gel electrophoresis (PFGE) (59). We reported similar data in a study of a panel of 59 clinical isolates by the partial sequencing of *rpoB*, *hsp65*, and *sodA* (36). Target genes yielded discordant results in 15 isolates, which had interspecific composite patterns (e.g., isolates with a *rpoB* sequence 100% identical to the *M. abscessus* type sequence and an *hsp65* sequence 100% identical to the *M. massiliense* type sequence). The identification of these isolates was substantially improved by the partial sequencing of five housekeeping gene sequences, indicating the value of a multilocus sequencing approach (26, 36).

A panel of *M. abscessus*, *M. massiliense*, and *M. bolletii* strains recently has been studied by biochemical tests, high-performance liquid chromatography (HPLC), drug susceptibility testing, PCR restriction enzyme analysis of the *hsp65* gene (PRA-*hsp65*), *rpoB* and *hsp65* gene sequencing, the restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene, and DNA-DNA hybridization (34). The clinical isolates studied and the type strains could not be separated, and DNA-DNA hybridization showed more than 70% interstrain relatedness. The authors thus proposed a revision of the taxonomic status of *M. abscessus*, *M. massiliense*, and *M. bolletii*, whereby the three species are in fact a single species (*M. abscessus*) and two subspecies (*M. abscessus* subsp. *abscessus* and *M. abscessus* subsp. *massiliense*) (34).

Multilocus sequence analysis (MLSA) is a phylogenetic analysis of multiple internal fragments of genes that are ubiquitous to the studied taxon, present as a single copy within the genome, and are not subject to selective pressure (27). MLSA defines an isolate by the sequences obtained from the internal fragments of several housekeeping genes. The usual approach in bacterial taxonomy is to concatenate the sequences of several (typically six to eight) housekeeping genes. The concatenated sequences then are used to assess clustering patterns among large numbers of strains within a genus or part of a genus (17, 23, 27, 28). This approach has been used successfully to delineate microbial species (i.e., well-resolved clusters) within various taxonomic groups, including groups of highly recombinant bacteria, like *Neisseria* spp. (37). Conversely, MLSA also allows the assignment of unknown strains to species clusters, and this can be performed via the internet, opening the way to electronic taxonomy (11).

In the present study, we developed an MLSA scheme and applied it to a large collection of *M. abscessus* sensu lato strains. The data obtained with this approach were compared to those obtained by *rpoB* sequencing to (i) help clarify the taxonomic status of *M. abscessus*, *M. massiliense*, and *M. bolletii* and (ii) evaluate the accuracy of *rpoB* as a molecular identification target.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** We studied 120 isolates of *M. abscessus* sensu lato recovered from France ($n = 97$), Germany ($n = 6$), Switzerland ($n = 7$), and Brazil ($n = 10$) between 1997 and 2007. One hundred nineteen of the isolates were from clinical samples, and one was from the environment (isolated from a sewer in Brazil). The sample origin was known for 90 of the clinical isolates: 79 (87.8%) were from respiratory samples (57 CF subjects, 22 non-CF subjects) and 11 (12.2%) were from other samples (9 from skin and soft tissue, 1 from pericardium, and 1 from a hip prosthesis). All of the clinical isolates were from unrelated cases. One clinical isolate was from a nonsporadic case, having also been recovered during a recent outbreak of *M. massiliense* skin and soft-tissue disease after a laparoscopic procedure in Brazil (15, 33). The type strains *M. abscessus* CIP 104536$^T$ (ATCC 19977$^T$), *M. massiliense* (CIP 108297$^T$), and *M. bolletii* (CIP 108541$^T$) also were included in the strain collection. Bacterial strains were stored at −70°C using cryopreservation beads and were grown on sheep blood agar at 37°C for 4 days prior to use.

***rpoB* sequencing and *rpoB*-based identification.** Mycobacterial DNA was extracted using Tris-EDTA, lysozyme, and proteinase K as described previously (36). A 940-bp fragment of the *rpoB* gene was amplified by PCR using AmpliTaq gold polymerase (Applied Biosystems, Courtaboeuf, France) with the primers MYCOF1 and MYCOR2 (Table 1). Dideoxy sequencing was carried out on both strands using a BigDye Terminator cycle sequencing kit (Applied Biosystems) with the same primers. Sequencing products were purified by gel filtration (Bio-gel P100; Bio-Rad, Marnes-la-Coquette, France) and were run on a 3700 DNA analyzer (Applied Biosystems). The *rpoB* sequences trimmed to 752 bp (3) were compared to the corresponding *rpoB* sequences from the reference type strains (http://www.ncbi.nlm.nih.gov/).

**PCR amplification and sequencing of 10 housekeeping genes in addition to *rpoB*.** Fragments from 10 housekeeping genes were amplified using the sets of primers shown in Table 1: *argH* (argininosuccinate lyase), *cya* (adenylate cyclase), *gdhA* (glutamate dehydrogenase), *glpK* (glycerol kinase), *gnd* (6-phosphogluconate deshydrogenase), *murC* (UDP *N*-acetylmuramate-L-Ala ligase), *pgm* (phosphoglucomutase), *pknA* (serine/threonine protein kinase), *pta* (phosphate acetyltransferase), and *purH* (phoshoribosylaminoimiazolcarboxylase ATPase subunit). The genes *gdhA*, *glpK*, *murC*, *pknA*, *pta*, and *purH* have been widely used in multilocus sequence typing (MLST) schemes developed for Gram-positive bacteria (e.g., *Streptococcus* spp., *Enterococcus* spp., *Staphylococcus* spp., and *Bacillus* spp.) (7, 37, 49). The gene products for *argH*, *cya*, *gnd*, and *pgm* have been used previously in the multilocus enzyme electrophoresis (MLEE) analysis of mycobacteria (20, 56, 57, 60). Amplification was performed using 25 μl of ReddyMix PCR master mix (Thermo Fisher Scientific Inc.) and 1 μl of each primer (10 pmol). The dideoxy sequencing of the amplified gene fragments was carried out on both strands with the Big Dye Terminator cycle sequencing kit (Applied Biosystems), using the same primers as those for amplification. Sequencing products were purified and analyzed with an ABI 3700 DNA analyzer as described above. The sequences were aligned and trimmed to defined start and end positions using BioEdit version 7.0.5.3 (25). Gene sequences from the three type strains were used as reference species sequences. Reference *M. abscessus* sequences of the 10 housekeeping genes were obtained from the whole-

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TABLE 1. Primers used for PCR and sequencing

| Gene | Primer name | Primer sequence | Amplified fragment (bp) | $T_m$ (°C) | Reference or source |
|---|---|---|---|---|---|
| *argH* | ARGHF | 5′-GACGAGGGCGACAGCTTC-3′ | 629 | 60 | 36 |
| | ARGHSR1 | 5′-GTGCGCGAGCAGATGATG-3′ | | 58 | |
| *cya* | ACF | 5′-GTGAAGCGGGCCAAGAAG-3′ | 647 | 58 | 36 |
| | ACSR1 | 5′-AACTGGGAGGCCAGGAGC-3′ | | 60 | |
| *gdhA* | GDHAF | 5′-GTCAGTGCCCCGATCGCT-3′ | 582 | 60 | This study |
| | GDHASR1 | 5′-GGCTCTCGGAGTACGTCGA-3′ | | 60 | |
| *glpK* | GLPKSF1 | 5′-AATCTCACCGGCGGTGTC-3′ | 609 | 58 | 36 |
| | GLPKSFR2 | 5′-GGACAGACCCACGATGGC-3′ | | 60 | |
| *gnd* | GNDF | 5′-GTGACGTCGGAGTGGTTGG-3′ | 634 | 62 | 36 |
| | GNDSR1 | 5′-CTTCGCCTCAGGTCAGCTC-3′ | | 62 | |
| *murC* | MURCSF1 | 5′-CGGACGAAAGCGACGGCT-3′ | 607 | 60 | 36 |
| | MURCSR2 | 5′-CCAAAACCCTGCTGAGCC-3′ | | 58 | |
| *pgm* | PGMSF1 | 5′-CCATTTGAACCCGACCGG-3′ | 596 | 60 | This study |
| | PGMSR2 | 5′-GTGCCAACGAGATCCTGCG-3′ | | 66 | |
| *pknA* | PKNAF | 5′-CAGGTGGACCTCGGACATG-3′ | 493 | 62 | This study |
| | PKNASR1 | 5′-AACCAGGCGCCCACCATC-3′ | | 60 | |
| *pta* | PTASF1 | 5′-GATCGGGCGTCATGCCCT-3′ | 720 | 60 | This study |
| | PTASR2 | 5′-ACGAGGCACTGCTCTCCC-3′ | | 66 | |
| *purH* | PURHSF1 | 5′-CGGAGGCTTCACCCTGGA-3′ | 634 | 64 | This study |
| | PURHSR2 | 5′-CAGGCCACCGCTGATCTG-3′ | | 60 | |
| *rpoB* | MYCOF1 | 5′-TCCGATGAGGTGCTGGCAGA-3′ | 940 | 68 | This study |
| | MYCOR2 | 5′-ACTTGATGGTCAACAGCTCC-3′ | | 68 | |

genome sequence of *M. abscessus* CIP 104536$^T$ (http://www.ncbi.nlm.nih.gov) (accession number NC_010397) (43). We recently determined the sequences of the *argH*, *cya*, *glpK*, *gnd*, and *murC* genes in the reference strains *M. bolletii* CIP 108541$^T$ and *M. massiliense* CIP 108297$^T$ (36).

**Phylogenetic analyses.** Unrooted individual gene trees and trees obtained using concatenated sequences were generated using the neighbor-joining method with 1,000 bootstrap replications. Trees were drawn to scale, with branch lengths representing the inferred evolutionary distances. The evolutionary distances were computed using the maximum composite likelihood method (51), and the data units were the number of base substitutions per site. Codon positions were in frame, and there was a total of 4,071 bp in the final data set. The neighbor-joining method and MEGA 4 software were used for the phylogenetic analysis of the sequence data (50).

**Nucleotide sequence accession numbers.** Sequences of the *pgm*, *pta*, and *purH* gene fragments from the reference strains *M. bolletii* CIP 108541$^T$ and *M. massiliense* CIP 108297$^T$ were determined in this study and submitted to the National Center for Biotechnology Information (NCBI) website (accession numbers HM371394, HM371395, and HM371396 for *M. bolletii* and HM371391, HM371392, and HM371393, for *M. massiliense*).

## RESULTS

**Selection of eight housekeeping genes eligible for MLSA.** Ten sets of primers (Table 1) were designed to amplify and sequence internal fragments of 10 housekeeping genes on a first panel, including 10 clinical isolates and the three *M. abscessus* sensu lato type strains. Fragments of the expected size were amplified from 100% of the strains for eight genes: *argH*, *cya*, *glpK*, *gnd*, *murC*, *pgm*, *pta*, and *purH*. The *gdhA* and *pknA* genes were eliminated from the subsequent MLSA study: in several strains, the amplification of *gdhA* did not yield fragments of the expected size, and the sequencing of the amplification products showed phage sequences; for *pknA*, we repeatedly obtained nonspecific amplification products even with the use of different primer pairs.

The eight selected housekeeping genes (*argH*, *cya*, *glpK*, *gnd*, *murC*, *pgm*, *pta*, and *purH*) are scattered throughout the genome of *M. abscessus* CIP 104536$^T$ and are at least 76 kb away from each other (42).

**Polymorphism of the genes included in the MLSA scheme.** Internal fragments from the eight housekeeping genes selected for MLSA were amplified, and their nucleotide sequences were determined for the 120 isolates and the three type strains. Fragment sizes varied from 480 to 549 bp, with G+C contents of between 62.4 and 68.2% (Table 2). Polymorphic sites for each gene fragment were least frequent in *pta* ($n = 24$) and most frequent in *argH* ($n = 43$). The *glpK* and *murC* fragments had the lowest number of alleles ($n = 16$), and the *cya* and *pgm* fragments had the highest number of alleles ($n = 25$). All differences between alleles were due to point mutations; neither deletions nor insertions were observed. The ratio between homologous and nonhomologous substitutions was between 0.0029 (*cya*) and 0.1971 (*pgm*) with a mean of 0.0769; thus, most of the mutations were silent and did not generate nucleotide substitutions (Table 2). The maximum sequence divergence between the 123 strains was greatest for *argH* (5.42%) and lowest for *pgm* (2.02%); divergence exceeded 3% for four genes: *argH*, *cya*, *gnd*, and *murC*.

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TABLE 2. Genes studied[c]

| Gene(s) | Size of analyzed fragment (bp) | %G+C | No. of polymorphic sites | No. of alleles | dN/dS | Maximum % of nt divergence[a] | % of nt divergence between type strain sequences[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | MabsT versus MmasT | MabsT versus MbolT | MbolT versus MmasT |
| *argH* | 480 | 66.8 | 40 | 19 | 0.1529 | 5.47 | 4.58 | 3.54 | 2.92 |
| *cya* | 510 | 68.2 | 31 | 25 | 0.0029 | 3.62 | 1.96 | 1.96 | 2.75 |
| *glpK* | 534 | 62.7 | 25 | 16 | 0.0380 | 2.48 | 1.69 | 1.31 | 0.94 |
| *gnd* | 480 | 65.2 | 33 | 20 | 0.0307 | 4.73 | 2.29 | 4.58 | 3.54 |
| *murC* | 537 | 68.6 | 39 | 16 | 0.1259 | 4.44 | 2.05 | 3.54 | 3.35 |
| *pgm* | 495 | 62.2 | 29 | 25 | 0.1900 | 2.02 | 1.41 | 1.41 | 0.81 |
| *pta* | 486 | 66.1 | 20 | 20 | 0.0110 | 2.74 | 1.85 | 1.65 | 2.26 |
| *purH* | 549 | 65.6 | 31 | 21 | 0.0502 | 2.98 | 1.82 | 2.00 | 0.18 |
| All MLSA genes | 4,071 | 65.7 | 248 | 79 | ND | 2.85 | 2.19 | 2.43 | 2.01 |
| *rpoB* | 752 | 65.6 | 43 | 22 | 0.0827 | 4.79 | 3.32 | 4.12 | 1.46 |

[a] Maximum nucleotide divergence found between the 123 strains studied.
[b] *M. abscessus* CIP 104536$^T$, *M. massiliense* CIP 108297$^T$, and *M. bolletii* CIP 108541$^T$.
[c] ND, not done; dN/dS, ratio of the rate of nonsynonymous substitutions (dN) to the rate of synonymous substitutions (dS); MabsT, *M. abscessus* type strain sequence; MbolT, *M. bolletii* type strain sequence; MmasT, *M. massiliense* type strain sequence.

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***rpoB* tree and polymorphism of *rpoB* sequences.** The tree built from the 123 *rpoB* sequences showed three distinct clusters, each comprising the type strain of one species (Fig. 1). According to Adékambi et al. (2), these three clusters could be equated with the species clusters *M. abscessus* (59 isolates), *M. bolletii* (24 isolates), and *M. massiliense* (37 isolates). The mean sequence divergence within each cluster was extremely low, ranging from 0.36 to 0.41%. The mean divergence between the *M. abscessus* and *M. bolletii* clusters was 4.38%, with all values exceeding 3% (extremes were 3.59 to 4.65%), divergence between the *M. abscessus* and *M. massiliense* clusters was 3.40%, with some values below 3% (extremes were 2.66 to 3.59%), and that between *M. massiliense* and *M. bolletii* was only 1.68%, and all values were below 3% (extremes were 1.33 to 2.13%). The divergence values between type strain sequences were consistent with these differences: 4.12% between *M. abscessus* CIP 104536$^T$ and *M. massiliense* CIP 108297$^T$, 3.32% between *M. abscessus* CIP 104536$^T$ and *M. bolletii* CIP 108541$^T$, and 1.46% between *M. massiliense* CIP 108297$^T$ and *M. bolletii* CIP 108541$^T$ (Table 2).

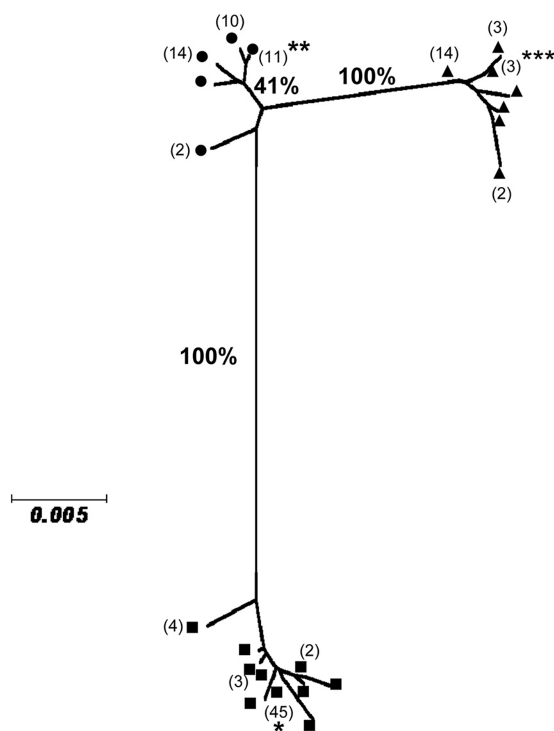


FIG. 1. Tree constructed from partial *rpoB* gene sequences. The tree for all studied strains ($n$ = 123) was generated using the neighbor-joining method. Bootstrap support values (%) are indicated for each node. Species assignment of clinical isolates are according to the criteria in Adékambi et al. (2): ■, *M. abscessus*; ●, *M. massiliense*; ▲, *M. bolletii*; numbers in parentheses are the numbers of isolates if there are two or more. Type strains: *, *M. abscessus* CIP 104536$^T$; **, *M. massiliense* CIP 108297$^T$; ***, *M. bolletii* CIP 108541$^T$.

**Single-gene trees obtained with each of the MLSA genes.** A single-gene tree was built from the sequences for each of the eight genes included in the final MLSA scheme. The trees built using *argH*, *cya*, *gnd*, *murC*, *pta*, and *purH* sequences showed three main branches, each carrying the type strain of one species except for the *purH* tree (*M. massiliense* and *M. bolletii* type strain sequences on the same branch) (Fig. 2). Bootstrap values for the *argH* and *murC* genes were >80%. However, there was no tree in which each of the three clusters was made up of strains assigned to a single species by *rpoB* sequencing (Fig. 2). The resolution obtained with the *glpK* and *pgm* sequences was very poor, and this was consistent with the very low polymorphism in these genes (as described above).

The divergence between type strain sequences was highest with *argH* and *gnd* and lowest with *glpK* and *pgm*. With the *argH* type sequences, divergence was 4.58% between *M. abscessus* and *M. massiliense* and 3.54% between *M. abscessus* and *M. bolletii* (3.54%), but it was only 2.92% between *M. massiliense* and *M. bolletii*. With the *gnd* type sequences, divergences were 4.58% between *M. abscessus* and *M. bolletii*, 3.54% between *M. massiliense* and *M. bolletii*, and only 2.29% between *M. abscessus* and *M. massiliense* (Table 2).

**Trees obtained with concatenated sequences of the eight MLSA genes.** Figure 3A shows the tree obtained for the 123 strains by concatenating the sequences of the eight housekeeping gene fragments (4,071bp). This tree shows three principle clusters, each containing the sequence of only one of the three type strains; it contains, as expected, the *M. abscessus*, *M.*

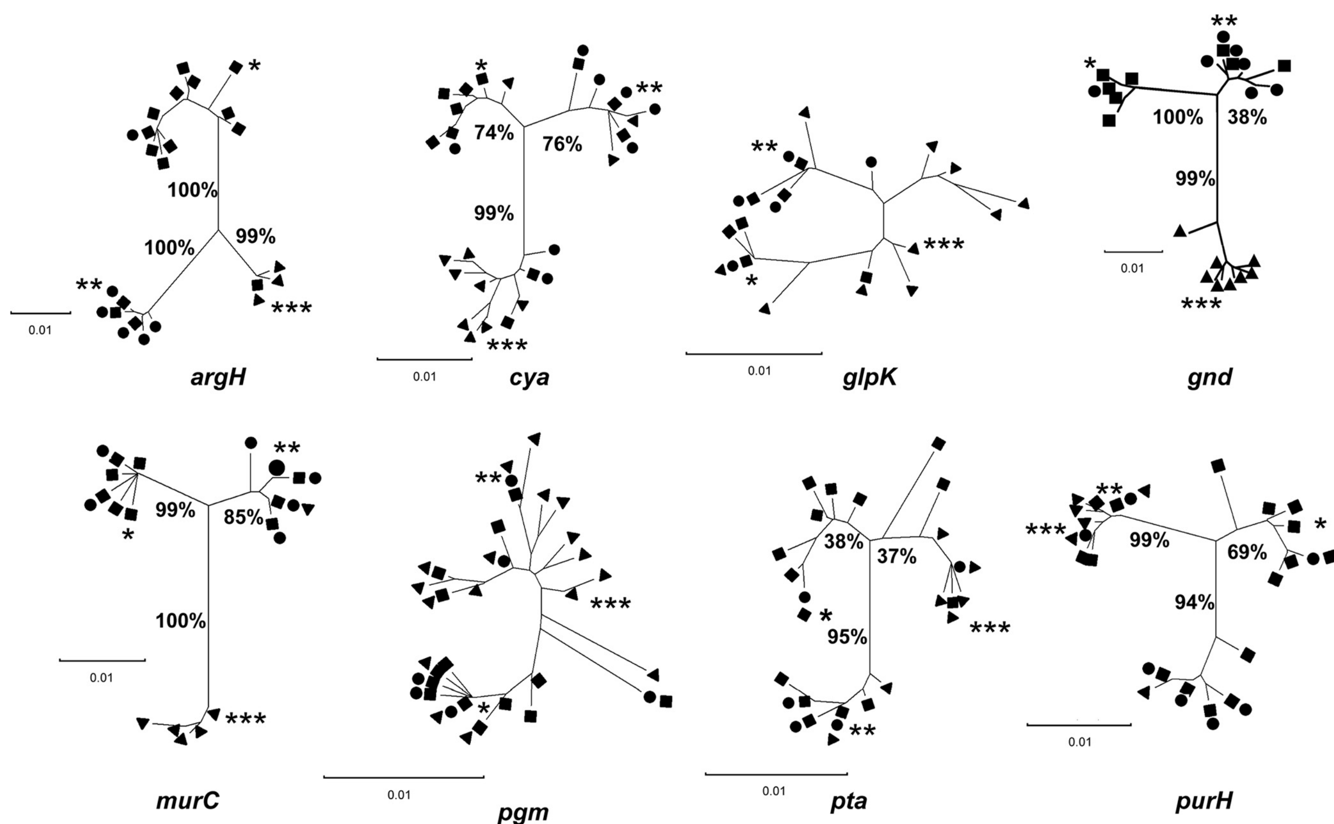


FIG. 2. Trees constructed from the sequences of the eight individual genes included in the final MLSA scheme. The trees for all studied strains ($n$ = 123) were generated using the neighbor-joining method. Bootstrap support values (%) at each of the nodes are indicated only for trees showing well-defined clusters. Species assignment of clinical isolates according to the criteria of Adékambi et al. (3): ■, *M. abscessus*; ●, *M. massiliense*; ▲, *M. bolletii*. Type strains: *, *M. abscessus* CIP 104536$^T$; **, *M. massiliense* CIP 108297$^T$; ***, *M. bolletii* CIP 108541$^T$.

*massiliense*, and *M. bolletii* clusters. Apart from a few exceptions, the *M. abscessus* cluster is compact, with a bootstrap value of 100%. The respective bootstrap values for the *M. massiliense* and *M. bolletii* branches are much lower at 71 and 65%, respectively. The *M. massiliense* cluster is more dispersed, with some sequences situated close to the other clusters. One of these sequences is from the type strain CIP 108297$^T$, which is not closely related to most of the other sequences in the cluster. The *M. bolletii* cluster is more compact, but some of its sequences are close to the main branch point.

The mean nucleotide divergence values were 0.45% (range, 0 to 1.65%) within the *M. abscessus* cluster, 0.72% (0 to 1.25%) within the *M. massiliense* cluster, and 0.64% (0 to 2.06%) within the *M. bolletii* cluster. The mean divergence values between clusters were 2.17% (range, 1.13 to 2.58%) between the *M. abscessus* and *M. massiliense* clusters, 2.37% (1.5 to 2.85%) between the *M. abscessus* and *M. bolletii* clusters, and 2.28% (0.86 to 2.68%) between the *M. massiliense* and *M. bolletii* clusters. Divergence values for the MLSA-concatenated sequences from type strains were 2.19% between *M. abscessus* and *M. massiliense*, 2.43% between *M. abscessus* and *M. bolletii*, and 2.01% between *M. massiliense* and *M. bolletii*.

**Effect of adding the *rpoB* sequence to the final MLSA scheme.** We studied the effect of adding the *rpoB* sequence to the concatenated sequences of the eight MLSA genes. The tree obtained (Fig. 3B, MLSA + *rpoB* tree) was very similar to the tree constructed from the MLSA gene sequences only (MLSA tree). However, the bootstrap value for the *M. massiliense* branch was even lower (24 versus 71%). With a few exceptions (see isolates 12 and 63), the distribution of isolates into the three groups was very similar in the two trees (Fig. 3A and B).

**Discrepancies between MLSA data and *rpoB*-based identification.** We compared the MLSA and *rpoB* sequences from each of the 120 isolates using the type strain sequences for reference. The results of this analysis were consistent for 110 isolates and inconsistent for 10 isolates (Table 3). The most frequent inconsistency ($n$ = 7; isolates 18, 23, 46, 97, 103, 107, and 134) was an MSLA sequence with 99.14 to 99.75% identity to the *M. massiliense* type strain (versus ≤98.03% identity to the *M. abscessus* and *M. bolletii* type strains) and an *rpoB* sequence similar to that of the *M. abscessus* type strain. Other inconsistencies were an MLSA sequence similar to that of the *M. bolletii* type strain, an *rpoB* sequence similar to that of the *M. abscessus* type strain (isolate 144), an MLSA sequence similar to that of the *M. abscessus* type strain, and an *rpoB* sequence similar to that of the *M. massiliense* type strain (isolates 71 and 121). Four of the isolates with an MLSA sequence similar to that of *M. massiliense* and an *rpoB* sequence similar to that of *M. abscessus* clustered together on the *M. massiliense*

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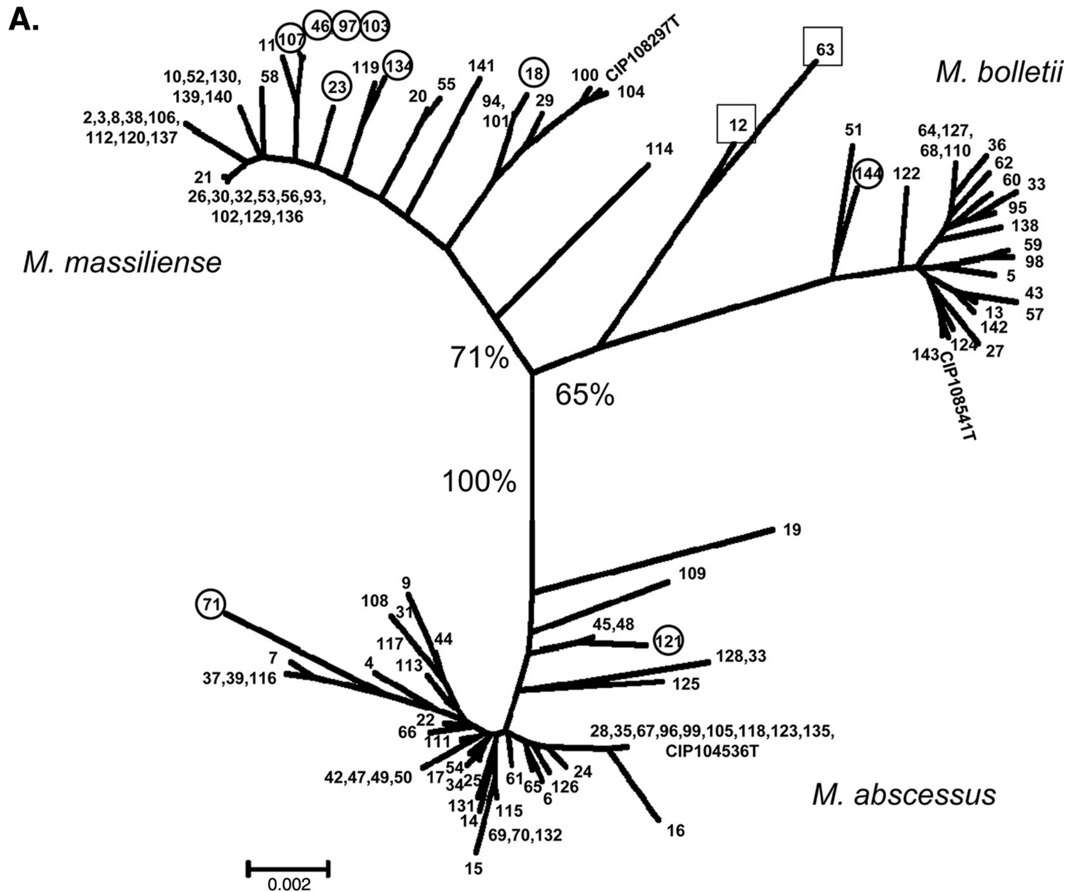


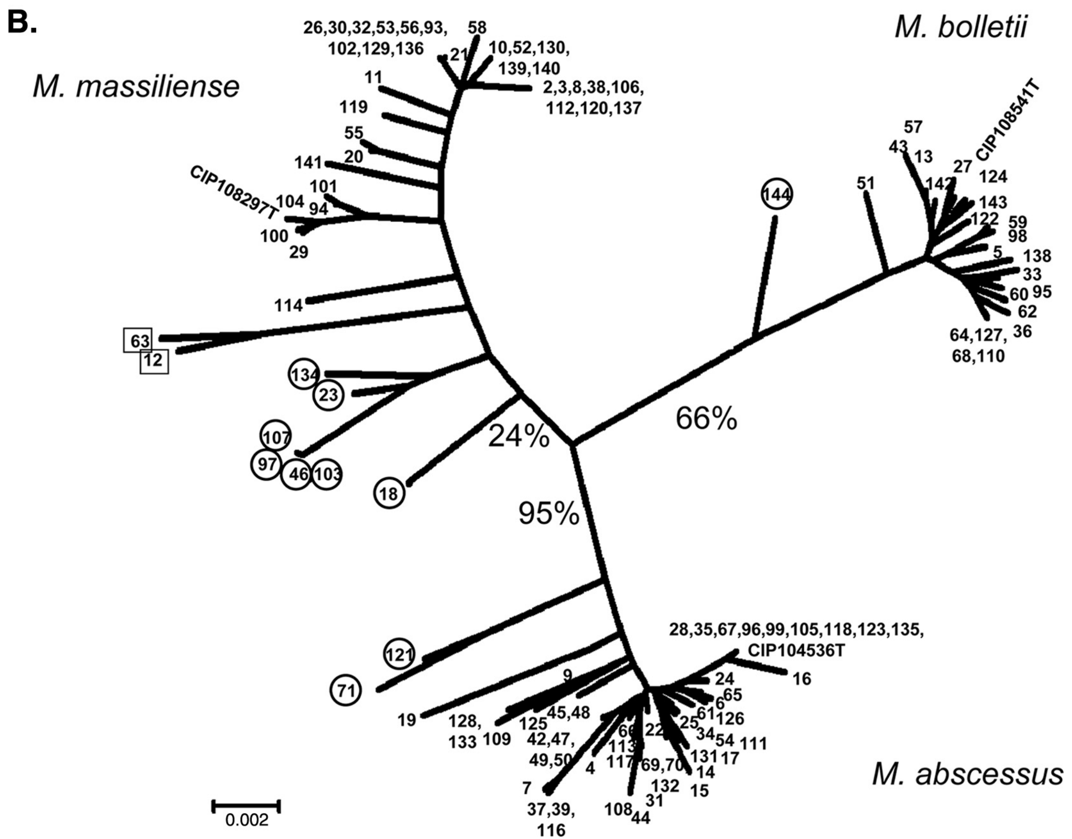


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TABLE 3. Isolates with discordant concatenated MLSA sequence and *rpoB* sequence[c]

| Isolate no. | % of nt identity with type strain sequence | | | | | |
|---|---|---|---|---|---|---|
| | MLSA sequence[a] | | | *rpoB* sequence | | |
| | MabsT | MbolT | MmasT | MabsT | MbolT | MmasT |
| 18 | 98.03 | 97.99 | **99.75** | **100** | 95.88 | 96.68 |
| 23 | 97.89 | 97.62 | **99.14** | **100** | 95.88 | 96.68 |
| 46[b] | 97.69 | 97.86 | **99.19** | **99.34** | 96.28 | 97.34 |
| 97[b] | 97.69 | 97.86 | **99.19** | **99.34** | 96.28 | 97.34 |
| 103[b] | 97.69 | 97.86 | **99.19** | **99.34** | 96.28 | 97.34 |
| 107[b] | 97.67 | 97.84 | **99.16** | **99.34** | 96.28 | 97.34 |
| 134 | 97.84 | 97.57 | **99.34** | **99.73** | 96.14 | 96.81 |
| 144 | 97.96 | **99.31** | 98.01 | **99.87** | 96.01 | 96.68 |
| 71 | **98.85** | 97.89 | 98.18 | 96.68 | 98.54 | **99.73** |
| 121 | **99.04** | 97.35 | 97.94 | 96.68 | 98.54 | **99.73** |

[a] Concatenated MLSA gene sequences (4,071 bp).
[b] Also see Fig. 3.
[c] MabsT, *M. abscessus* type strain sequence; MbolT, *M. bolletii* type strain sequence; MmasT, *M. massiliense* type strain sequence. Boldface numbers indicate discordant species identification by either MLSA or *rpoB* analysis for a given isolate.

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branch of the MLSA tree (Fig. 3, isolates 46, 97, 103, and 107). These four isolates shared identical *rpoB* sequences.

## DISCUSSION

Both the molecular identification and the taxonomy of *M. abscessus*, *M. massiliense*, and *M. bolletii* currently are based upon the partial sequencing of *rpoB* (2, 3, 16). However, several studies recently have questioned the effectiveness of sequencing only the *rpoB* gene for the molecular identification of these species (36, 59). More recently, doubts have been raised about the taxonomic classification of *M. massiliense* and *M. bolletii* (34). For the first time for this group of species, we used an MLSA scheme with the aim of resolving these issues. We compared the results obtained from using this scheme to those obtained from the partial sequencing of *rpoB*.

The study of our collection of isolates with the chosen MLSA scheme (eight housekeeping gene sequences, 4,071 bp) clearly shows the existence of three principal groups, with each containing the type strain of one of the three species (*M. abscessus* CIP 104536$^T$, *M. massiliense* CIP 108297$^T$, and *M. bolletii* CIP 108541$^T$). At first sight, these groups can be termed *M. abscessus*, *M. massiliense*, and *M. bolletii*. However, although the *M. abscessus* branch is robust, the *M. bolletii* and *M. massiliense* branches have relatively low bootstrap values (65 and 71%, respectively) and a diffuse appearance, particularly in the *M. massiliense* branch. Furthermore, the current *M. massiliense* type strain (CIP 108297$^T$) does not cluster closely with the more tightly grouped isolates in the *M. massiliense* branch.

Sequence analysis of the fragment of the *rpoB* gene, described by Adékambi as the gold standard for the molecular diagnosis of RGM infection (3), also was very informative. Our study is the first to use this approach with a large and diverse collection of isolates. We found that the isolates can indeed be divided into three distinct groups, each clustered around one of the three type strains. However, the divergence between the groups casts doubts about the extent to which these groups are different species according to the criteria of Adékambi (i.e., >3% *rpoB* sequence divergence between two RGM species) (2, 5). This was most marked for the *M. massiliense* and *M. bolletii* groups and for the *M. massiliense* and *M. bolletii* type strains. The most different *rpoB* sequences in the *M. massiliense* and *M. bolletii* groups strains diverged by only 2.13%, and the divergence between the *rpoB* sequences of the *M. massiliense* and *M. bolletii* type strains (not reported by Adékambi et al. [2, 5]) was only 1.46%. Therefore, according to the criteria of Adékambi et al., *M. massiliense* and *M. bolletii* do not constitute different species (3).

The divergence of *rpoB* between *M. massiliense*/*M. bolletii* and *M. abscessus* similarly raises doubts about whether *M. massiliense* and *M. bolletii* are species distinct from *M. abscessus*. The divergence of the *rpoB* sequences between the *M. abscessus* and *M. massiliense* type strains and between the *M. abscessus* and *M. bolletii* type strains were more than 3% (4.12 and 3.32%, respectively). However, in our collection, the divergence threshold of 3% was exceeded between the *M. abscessus* and *M. bolletii* groups (values from 3.59 to 4.65%) but not between the *M. abscessus* and *M. massiliense* groups (values from 2.66 to 3.59%). As *M. massiliense* and *M. bolletii* appear not to be entirely separate species, we compared the *M. massiliense*/*M. bolletii* group to the *M. abscessus* group: the divergence values were between 2.66 and 4.79%. Therefore, the combined *M. massiliense*/*M. bolletii* group and the *M. abscessus* group are not entirely separate species. These findings indicate the need for the complete revision of the current taxonomic classification of *M. abscessus* sensu lato; as recently proposed

FIG. 3. Trees constructed from concatenated sequences. (A) Concatenated MLSA sequences. (B) Concatenated MLSA + *rpoB* sequences. The trees for all studied strains ($n = 123$) were generated by using the neighbor-joining method. Bootstrap support values (%) are indicated for each node. Each isolate is indicated by its number in our collection. CIP type strains also are indicated. Boxes indicate isolates with discordant *rpoB*-based identification (see Table 3 for further details). Note that isolates 12 and 63 are located on the *M. bolletii* branch of the MLSA tree (A) and on the *M. massiliense* branch of the MLSA + *rpoB* tree (B).

by Leao et al. (34), *M. abscessus*, *M. massiliense*, and *M. bolletii* should comprise a single species (*M. abscessus*).

The other objective of our study was to evaluate the use of *rpoB* gene sequencing for the molecular identification of isolates and discrimination between the different groups of *M. abscessus* sensu lato. Several recent studies have questioned the reliability of approaches based solely on *rpoB* sequencing (31, 34, 47, 48). This is because of the possible horizontal transfer of the *rpoB* gene between the different groups of *M. abscessus* sensu lato, especially from the *M. abscessus* group to the *M. massiliense* group. Our comparative analysis of the data for *rpoB* and MLSA confirmed that such horizontal transfer has occurred: almost 10% of the isolates studied had discordant MLSA and *rpoB* sequences. The most frequent situation was isolates having an *rpoB* sequence belonging to the *M. abscessus* group and an MLSA sequence belonging to the *M. massiliense* group. Thus, *rpoB* sequencing can identify the revised *M. abscessus* species (formerly *M. abscessus* sensu lato) but cannot discriminate 100% between *M. abscessus*, *M. massiliense*, and *M. bolletii*. We recently have shown that other potential targets for the molecular identification of RGM strains, such as *hsp65* and *sodA*, also can be horizontally transferred within the *M. abscessus* sensu lato groups (36). We also found evidence of lateral transfer events involving the recently proposed target *secA* (unpublished data). If it were useful to distinguish between the *M. abscessus*, *M. massiliense*, and *M. bolletii* groups, because of clinical or epidemiological features specific to the infectious agent, for example, it would be valuable to identify other targets or combinations of targets to overcome the problem of horizontal gene transfer between these groups. Our team currently is addressing this issue.

This study provides information relevant to the phylogeny of *M. abscessus* sensu lato. We show the existence of three groups, *M. abscessus*, *M. bolletii*, and *M. massiliense*. Of these, the *M. massiliense* group seems to have emerged most recently. Our findings also reveal substantial horizontal gene transfer between the three groups, with a particularly marked flow of *rpoB* from *M. abscessus* to *M. massiliense*. Some transfer from *M. abscessus* to *M. massiliense* may be recent, as a small subgroup of isolates in the *M. massiliense* group (obtained from respiratory samples in France and Brazil) had an *rpoB* sequence that was almost 100% identical to the *M. abscessus* CIP 104536$^T$ sequence. Although the three groups are not entirely separate species, our data suggest that each group evolves in its own way, maintaining a certain cohesion, and that genetic material is exchanged between the groups, most likely via phages (30, 43, 44). The fact that they exchange genetic material indicates that they share similar biotopes, but their distinctness indicates a degree of specialization of each group. However, almost all of the isolates that we tested were from clinical samples; few *M. abscessus* sensu lato isolates from the environment are available (see below). We therefore cannot totally exclude sampling bias toward human pathogenic strains.

The analysis of the *M. abscessus* CIP 104536$^T$ genome by our group has improved our understanding of the microorganism's natural lifestyle. Genes in the *M. abscessus* CIP 104536$^T$ genome also are found in bacteria living in soil or aquatic environments, probably in close contact with plants. However, *M. abscessus* also contains a large number of genes known to be involved in intracellular parasitism, suggesting that it may have evolved to escape free-living amoebas (1, 5, 9); this would explain why *M. abscessus* is rarely isolated from soil or water, although there is general agreement that it lives in such environments. We have launched an extensive investigation of the presence of RGM in the water treatment systems in the Paris region, a region where respiratory infections involving *M. abscessus* are particularly prevalent in at-risk populations. Our MLSA approach will be valuable for comparing the population structures of *M. abscessus* sensu lato isolates obtained in this survey to those of clinical strains.

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