JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2004, p. 5493–5501 Vol. 42, No. 12
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.12.5493–5501.2004

Amoebal Coculture of "*Mycobacterium massiliense*" sp. nov. from the Sputum of a Patient with Hemoptoic Pneumonia

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Received 15 April 2004/Returned for modification 21 July 2004/Accepted 10 August 2004

A nonphotochromogenic, rapidly growing *Mycobacterium* strain was isolated in pure culture from the sputum and the bronchoalveolar fluid of a patient with hemoptoic pneumonia by using axenic media and an amoebal coculture system. Both isolates grew in less than 7 days at 24 to 37°C with an optimal growth temperature of 30°C. The isolates exhibited biochemical and antimicrobial susceptibility profiles overlapping those of *Mycobacterium abscessus*, *Mycobacterium chelonae*, and *Mycobacterium immunogenum*, indicating that they belonged to *M. chelonae-M. abscessus* group. They differed from *M. abscessus* in β-galactosidase, β-*N*-acetyl-β-glucosaminidase, and β-glucuronidase activities and by the lack of nitrate reductase and indole production activities, as well as in their in vitro susceptibilities to minocycline and doxycycline. These isolates and *M. abscessus* differed from *M. chelonae* and *M. immunogenum* by exhibiting gelatinase and tryptophane desaminase activities. Their 16S rRNA genes had complete sequence identity with that of *M. abscessus* and >99.6% similarity with those of *M. chelonae* and *M. immunogenum*. Further molecular investigations showed that partial *hsp65* and *sodA* gene sequences differed from that of *M. abscessus* by five and three positions over 441 bp, respectively. Partial *rpoB* and *recA* gene sequence analyses showed 96 and 98% similarities with *M. abscessus*, respectively. Similarly, 16S-23S rRNA internal transcribed spacer sequence of the isolates differed from that of *M. abscessus* by a A→G substitution at position 60 and a C insertion at position 102. Phenotypic and genotypic features of these two isolates indicated that they were representative of a new mycobacterial species within the *M. chelonae-M. abscessus* group. Phylogenetic analysis suggested that these isolates were perhaps recently derived from *M. abscessus*. We propose the name of "*Mycobacterium massiliense*" for this new species. The type strain has been deposited in the Collection Institut Pasteur as CIP 108297^T and in Culture Collection of the University of Göteborg, Göteborg, Sweden, as CCUG 48898^T.

During the last few years, the number of nontuberculous mycobacteria (NTM) reported in various clinical situations has greatly increased because of opportunistic infections in immunocompromised patients and improved culture and identification techniques (18). The 16S rRNA gene sequence analysis of NTM led to the description of 40 new species since 1992 and contributed to the description of new clinical forms (19, 35, 43, 44). Particularly, mycobacteria of the *Mycobacterium chelonae-Mycobacterium abscessus* group (*M. chelonae*, *M. abscessus*, and *Mycobacterium immunogenum*) emerged as opportunistic pathogens (45, 47–49, 51), causing hypersensitivity pneumonitis in automobile production workers and chronic lung disease in elderly women with bronchiectasis and in young adults with cystic fibrosis (4, 9, 16, 36, 49). Precise species identification in this group of mycobacteria remains difficult. Only two biochemical tests, i.e., sodium chloride tolerance and utilization of citrate, allow species identification of these organisms (37, 49), but these tests require 4 weeks to complete (8). Identification of species of the *M. chelonae-M. abscessus* group by using high-performance liquid chromatography of mycolic acids has been limited by profile similarities (49). The 16S rRNA gene sequence of *M. abscessus* differs from the respective sequences of *M. chelonae* and *M. immunogenum* by only four and eight positions, respectively, over 1,483 bp (25, 49). We therefore developed partial PCR sequencing of *rpoB* gene for improved identification of NTM (1), and we also demonstrated its usefulness for taxonomy (2). When applying this tool to a collection of 23 *M. chelonae-M. abscessus* group clinical isolates, we found two isolates from the same patient that exhibited 96.0% similarity only with the sequence of *M. abscessus* type strain, although they shared 100% similarity of the 16S rRNA sequence and similar standard phenotypic profile. We further characterized these isolates by extensive phenotypic and molecular methods and found that they were representative of a hitherto-undescribed rapidly growing *Mycobacterium* (RGM) species. The name "*Mycobacterium massiliense*" sp. nov. is proposed for this new species.

CASE REPORT

A 50-year-old woman presented with an 8-year history of bronchiectasis and hemoptysis. She had no history of past tuberculosis but received substitutive corticosteroid treatment for an Addison's disease for 2 years. No bacteriological investigations had been performed during the course of her illness. The patient had had close contacts with horses, dogs, and cats and presented with a fever of 38°C, subcutaneous nodules of

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TABLE 1. Comparison of biochemical characteristics of "*M. massiliense*" and closely related mycobacterial species[a]

Characteristic	Presence (+) or absence (−) of various characteristics in:			
	"*M. massiliense*"	*M. abscessus*	*M. chelonae*	*M. immunogenum*
Growth at 24°C	+	+	+	+
Growth at 30°C	+	+	+	+
Growth at 37°C	+	+	+	+
Growth at 42°C	−	−	−	−
Growth at 45°C	−	−	−	−
Pigmentation	−	−	−	−
Arylsulfatase (3 days)	+	+	+	+
Catalase	+	+	+	+
Semiquantitative catalase	+	+	+	+
5% NaCl tolerance (30°C)	+	+	−	−
MacConkey (without crystal violet)	+	+	+	+
Degradation of *p*-aminosalicylate	−	−	−	−
Iron uptake	−	−	−	−
Nitrate reductase	−	+	+	−
Pyrazinamidase	+	+	+	±
Pyrrolidonyl arylamidase	+	+	+	+
Phosphatase alkaline	+	+	+	+
β-Glucuronidase	−	+	−	−
β-Galactosidase	+	−	−	+
α-Glucosidase	+	+	+	+
N-Acetyl-β-glucosaminidase	+	−	−	−
Esculin	+	+	+	+
Urease	−	−	−	−
Gelatinase	+	+	−	−
Arginine dihydrolase	−	−	−	−
Lysine decarboxylase	−	−	−	−
Ornithine decarboxylase	−	−	−	−
Citrate	−	−	+	−
H_2O production	−	−	−	−
Tryptophane desaminase	+	+	−	−
Indol production	−	+	−	−
Acetoin production	+	+	+	+
Glucose	−	−	−	−
Ribose	−	−	−	−
Xylose	−	−	−	−
Mannitol	−	−	−	−
Maltose	−	−	−	−
Lactose	−	−	−	−
Saccharose	−	−	−	−
Glycogen	−	−	−	−
Inositol	−	−	−	−
Sorbitol	−	−	−	−
Rhamnose	−	−	−	−
Meliobiose	−	−	−	−
Amygdaline	−	−	−	−
Arabinose	−	−	−	−

[a] Results were reproducible over triplicate testing. Colony morphology was rough/smooth for all four species.

both forearms, impaired respiratory function, and micronodular lesions in the upper lobe of the right lung. Blood examination was unremarkable apart from a biological inflammatory syndrome. An NTM was isolated from the sputum sample and from a bronchoalveolar lavage obtained during bronchoscopy 2 months later by using amoebal coculture according to our laboratory protocol (26) and conventional medium inoculation. Direct Ziehl-Neelsen staining showed acid-fast bacilli. Histological examination of the forearm nodules disclosed swelling and nongranulomatous, nonspecific inflammatory infiltrate with lymphocytic cells predominantly present around the blood vessels. Ziehl-Neelsen and periodic acid-Schiff (PAS) stainings were negative, and the culture remained sterile. After isolation and identification of a *M. chelonae-M. abscessus* group strain in respiratory samples, the patient was treated with oral clarithromycin (1 g/day) for 1 month and oral minocycline (100 mg/day) for an additional 6 months. Clinical improvement was noted at the 6-month follow-up examination with disappearance of the cutaneous nodules and marked improvement of the respiratory function.

MATERIALS AND METHODS

Identification and characterization of the isolates. Sputum and bronchoalveolar samples were decontaminated by using the *N*-acetyl-L-cysteine sodium hydroxide (NALC-NaOH) protocol (21, 23). Briefly, an equal volume of digestant (3% NaOH, 1.45% sodium citrate, 0.5% NALC [Merck, Darmstadt, Germany]) was added to up to 10 ml of specimen. After being vortexed, the mixture was shaken for 30 min and neutralized by adding sterile phosphate buffer (bio-

TABLE 2. Antimicrobial susceptibility test results for "*M. massiliense*" and closely related mycobacterial species[a]

Antimicrobial	MIC (μg/ml) for:			
	"*M. massiliense*"	*M. abscessus*	*M. chelonae*	*M. immunogenum*
Clarithromycin	0.064	1.5	0.125	0.75
Minocycline	4	>256	16	>256
Doxycycline	0.75	>256	>256	>256
Cefoxitin (disc, 30 μg)	>32	>32	>32	>32
Colistin (disc, 50 μg)	>2	>2	>2	>2
Rifamplin	>32	>32	>32	>32
Ciprofloxacin	>32	>32	0.5	>32
Sparfloxacin	>32	>32	8	>32
Ofloxacin	>32	>32	2	>32
Amikacin	48	32	48	>256
Imipenem	>32	>32	>32	>32
Penicillin	>32	>32	>32	>32
Amoxicillin	>256	>256	>256	>256
Ceftriaxone	>256	>256	>256	>256
Cefotaxime	>256	>256	>256	>256
Vancomycin	>256	>256	>256	>256
Teicoplanin	>256	>256	>256	>256
Metronidazole	>256	>256	>256	>256
Tobramycin (disc, 10 μg)	>8	>8	>8	>8
Trimethoprim-sulfamethoxazole (disc, 1.25 μg + 23.75 μg)	>8 and >152	>8 and >152	>8 and >152	>8 and >152
Acid pipemidic (disc, 20 μg)	>16	>16	>16	>16
Isoniazid	>10	>10	NT[b]	NT
Streptomycin	>4	>4	NT	NT
Ethambutol	>2	>2	NT	NT

[a] Results were reproducible over triplicate testing.
[b] NT, not tested.

Mérieux, La Balme les Grottes, France) to a final volume of 50 ml and then centrifuged at 3,000 rpm for 10 min. The supernatant was discarded, and the sediment was resuspended in 2 ml of phosphate buffer. Half of the sediment was frozen at −80°C, and the other part was used for acid-fast staining and inoculation into BACTEC 9000MB broth according to the manufacturer's instructions (BD Biosciences, Sparks, Md.). Mycobacterial isolates were subcultured on Middlebrook 7H10 agar, Lowenstein-Jensen (LJ) (egg-based) slants (bioMérieux), and 5% sheep-blood agar (bioMérieux). Solid and liquid cultures were inspected twice weekly for colonies and turbidity, respectively.

Amoebal coculture. Respiratory specimens were cocultivated with amoebae according to our laboratory protocol for respiratory specimen processing (26). An *Acanthamoeba polyphaga* strain, Linc-AP1, kindly provided by T. J. Rowbotham (Public Health Laboratory, Leeds, United Kingdom), was grown at 28°C for 4 days in 150-cm^3 culture flasks (Corning) with 30 ml of peptone-yeast extract-glucose broth to reach an average of 5×10^5 amoebae/ml (14, 15, 26). Amoebae were then harvested by centrifugation at 2,000 rpm for 10 min, and the pellet was resuspended twice in 30 ml of Page's modified Neff's amoeba saline (PAS) (14, 26). Then, 1 ml of a suspension of 5×10^5 *A. polyphaga*/ml was distributed into each well of a 12-well Costar microplate (Corning), and the decontaminated sputum sample was inoculated onto amoebal microplates. The microplates were then centrifuged at 4,000 rpm for 30 min, followed by incubation at 33°C under a humidified 5% CO_2 atmosphere. The amoebal cocultures were subcultured onto fresh amoebae on day 4 and then examined daily for the occurrence of amoebal lysis. The presence of intra-amoebal bacteria was detected, after gentle shaking and cytocentrifugation at 800 rpm for 10 min as described by Gimenez (12), by Ziehl-Neelsen and Gram stainings.

Electron microscopy. Inoculated *A. polyphaga* were washed overnight in monophosphate buffer (pH 12) and fixed for 1 h at 4°C at room temperature with 1% osmium tetroxide. Dehydration was performed by successive washes in increasing acetone concentrations (25 to 100%). After the preparations were incubated for 1 h in a suspension of acetone-Epon and overnight in Epon, they were embedded in araldite (Fluka, St. Quentin Fallavier, France). Thin sections were cut from blocks by using an Ultracut microtome (Reichert-Leica, Marseille, France), deposited on copper grids coated with Formvar (Sigma-Aldrich, Taufkirchen, Germany), and stained for 10 min with a solution of methanol-uranyl acetate and lead nitrate with sodium citrate in water (14). Grids were examined with a Morgani 268D electron microscope (Philips, Eindhoven, The Netherlands).

Phenotypic analysis. We observed the colony morphology, pigmentation, and the ability of the isolates to grow at various temperatures (24, 30, 37, 42, and 45°C) on 5% sheep blood agar, Middlebrook 7H10 agar, and LJ slants. The following biochemical and physiological values were determined: the activities of arylsulfatase and catalase, the extent of iron uptake, the degradation of *p*-aminosalicylic acid, and growth on MacConkey agar without crystal violet and on LJ slants in the presence of 5% sodium chloride (21, 30). Additional biochemical tests were performed by inoculation of API Coryne and API 20E (bioMérieux) strips (42) according to the instructions of the manufacturer, with the exception that incubation time was increased to 3 days at 30°C under a highly humidified atmosphere. Every test was done three times in order to ensure the reproducibility of results.

Susceptibility testing. Suspensions were prepared by emulsifying colonies into 5 ml of sterile water to achieve a density equal to a 1.0 McFarland turbidity standard by visual examination. Suspensions were mixed vigorously on a vortex mixer for 15 to 20 s and then used to inoculate the entire surface of a 5% sheep blood agar plate. The MICs of rifampin, ciprofloxacin, sparfloxacin, ofloxacin, minocycline, clarithromycin, doxycycline, amikacin, penicillin, imipenem, amoxicillin, ceftriaxone, cefotaxime, metronidazole, teicoplanin, and vancomycin were determined by incubation with the respective E-test (AB Biodisk, Solna, Sweden) at 30°C on 5% sheep blood agar for 3 days. For each drug, the MIC was recorded as the point of intersection between the zone edge and the E-test strip. The strains were classified as either susceptible, intermediate, or resistant to a particular antibiotic according to breakpoints recommended by the National Committee for Clinical Laboratory Standards (29) Additional disk diffusion method on 5% sheep blood agar for 3 days at 30°C was used to determine the susceptibility to trimethoprim-sulfamethoxazole (1.25 μg/23.75 μg), tobramycin (10 μg), pipemidic acid (30 μg), and cefoxitin (30 μg). Using a disk diffusion method for certain antimicrobials, the zone sizes for resistance used were those of Grange and Stanford (13). Growth on LJ slants in the presence of isoniazid (0.1, 1, and 10 μg/ml), streptomycin (4 μg/ml), ethambutol (2 μg/ml), and *para*-aminosalicylic acid (0.5 μg/ml) was also evaluated. Each susceptibility test was done in triplicate in three separate days in order to ensure the reproducibility of results.

Measurement of G+C content of DNA. After culture on 5% sheep blood agar, DNA extraction, purification, degradation, and G+C content determinations by HPLC were done as described by Mesbah et al. (27), except that a Waters 625 LC system with a Waters 486 Tenable Absorbance Detector and a Waters 746 Data

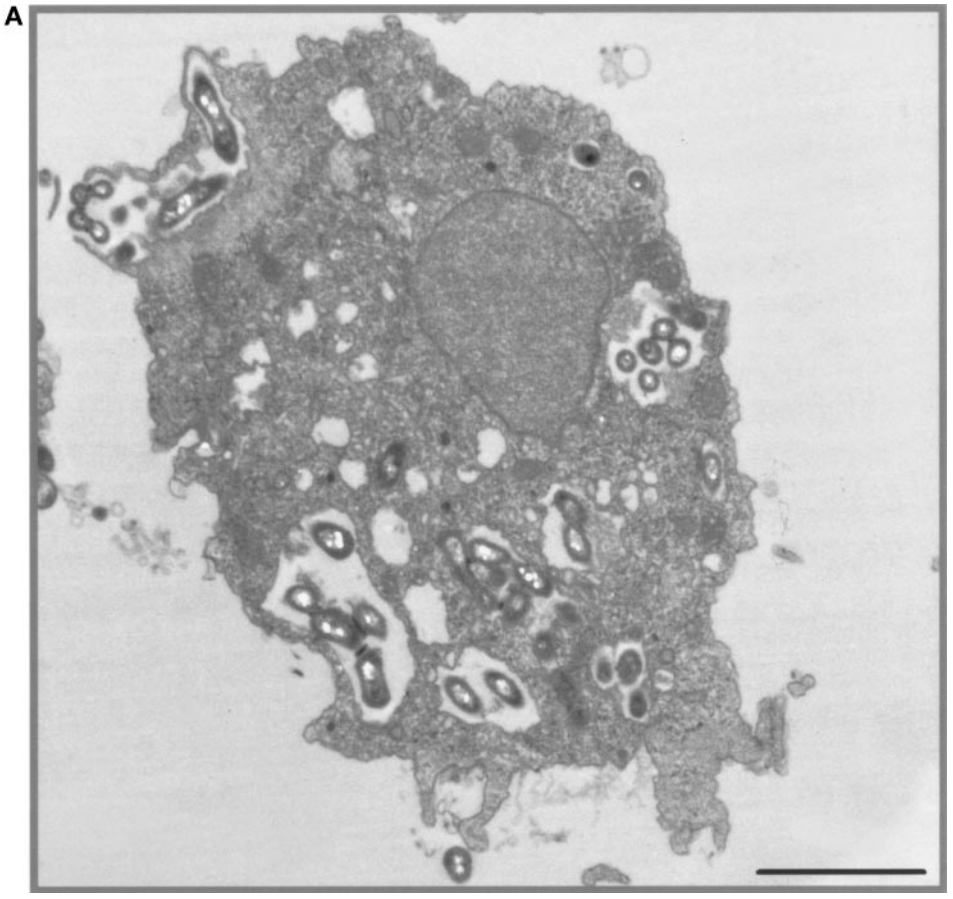

FIG. 1. (A) Intravacuolar location of "*M. massiliense*" within *A. polyphaga*, as shown by electron microscopy. Magnification, ×39,200. Bar, 2 μm. (B) Trilamellar structure of the cell wall of "*M. massiliense*," as seen by electron microscopy. The electron micrograph shows the rod element. Magnification, ×30,800. Bar, 0.2 μm.

Module (Millipore, Saint Quentin en Yvelines, France) were used. Triplicate determinations were made.

Sequence and phylogenetic analyses. DNA was extracted from colonies grown on 5% sheep blood agar by using the Fast-Prep device and the FastDNA kit according to the recommendations of the manufacturer (Bio 101, Inc., Carlsbad, Calif.). We performed the amplification and sequencing of the 16S rRNA (50), *hsp65* (40), *rpoB* (1), *sodA* (2), and *recA* (3) genes. The 16S-23S rRNA internal transcribed spacer (ITS) was amplified and sequenced by using the primer pair Sp1 and Sp2 (33). Products of sequencing reactions were recorded with an ABI Prism 3100 DNA sequencer according to the standard protocol of the supplier (Perkin-Elmer Applied Biosystems). The percentages of similarity between the sequences were determined by using the CLUSTAL W program supported by the PBIL website (http://npsa-pbil.ibcp.fr/cgi-bin/npsa). For phylogenetic analyses, sequences were trimmed in order to start and finish at the same nucleotide position for all of the strains under study. Multisequence alignment was performed by using the CLUSTAL X program (version 1.81) in the PHYLIP software package (41). Phylogenetic trees were obtained from DNA sequences by using the neighbor-joining method with Kimura's two-parameter distance correction model with 1,000 bootstrap replications in the MEGA version 2.1 software package (24). The sequences determined in the present study for "*M. massiliense*" were deposited in GenBank under the following access numbers: 16S rRNA, AY593980; *hsp65*, AY596465; *sodA*, AY593975; *recA*, AY593979; *rpoB*, AY593981; and 16S-23S ITS, AY593978.

RESULTS

Phenotypic characteristics and susceptibility testing. The two isolates were strictly aerobic, nonmotile, non-spore-forming, acid-fast, gram-positive rods. The colonies on 5% sheep blood agar were nonphotochromogenic and intermediate between smooth and rough. Growth occurred by between 2 and 4 days on 5% sheep blood agar, Middlebrook 7H10 agar, and LJ slants at between at 24 and 37°C; no growth was observed at 42 and 45°C. Both isolates grew at 30°C on MacConkey agar without crystal violet and LJ slants containing 5% NaCl. They exhibited pyrazinamidase, pyrrolidonyl arylamidase, phosphatase alkaline, β-galactosidase, α-glucosidase, esculine (β-glucosidase), *N*-acetyl-β-glucosaminidase, gelatinase, catalase, and 3-day arylsulfatase activities and produced acetoin. The isolates lacked nitrate reductase, β-glucuronidase, urease, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, sodium citrate utilization, and tryptophane desaminase activities and H_2S production. They did not show iron uptake and degradation of *para*-aminosalicylic acid. Also, they did not utilize glucose, ribose, xylose, mannitol, maltose, lactose, saccharose, glycogene, inositol, sorbitol, rhamnose, melobiose, amygdaline, or arabinose as the sole carbon source. Antibiotic susceptibility profiles for the two isolates included susceptibility to amikacin (MIC, 32 μg/ml), clarithromycin (MIC, 0.064 μg/ml), minocycline (MIC, 4 μg/ml),and doxycycline (MIC, 0.75 μg/ml) and resistance to cefoxitin (MIC, >2 μg/ml), rifampin (MIC, >32 μg/ml), ciprofloxacin (MIC, >32 μg/ml), sparfloxacin (MIC > 32 μg/ml), ofloxacin (MIC, >32 μg/ml),

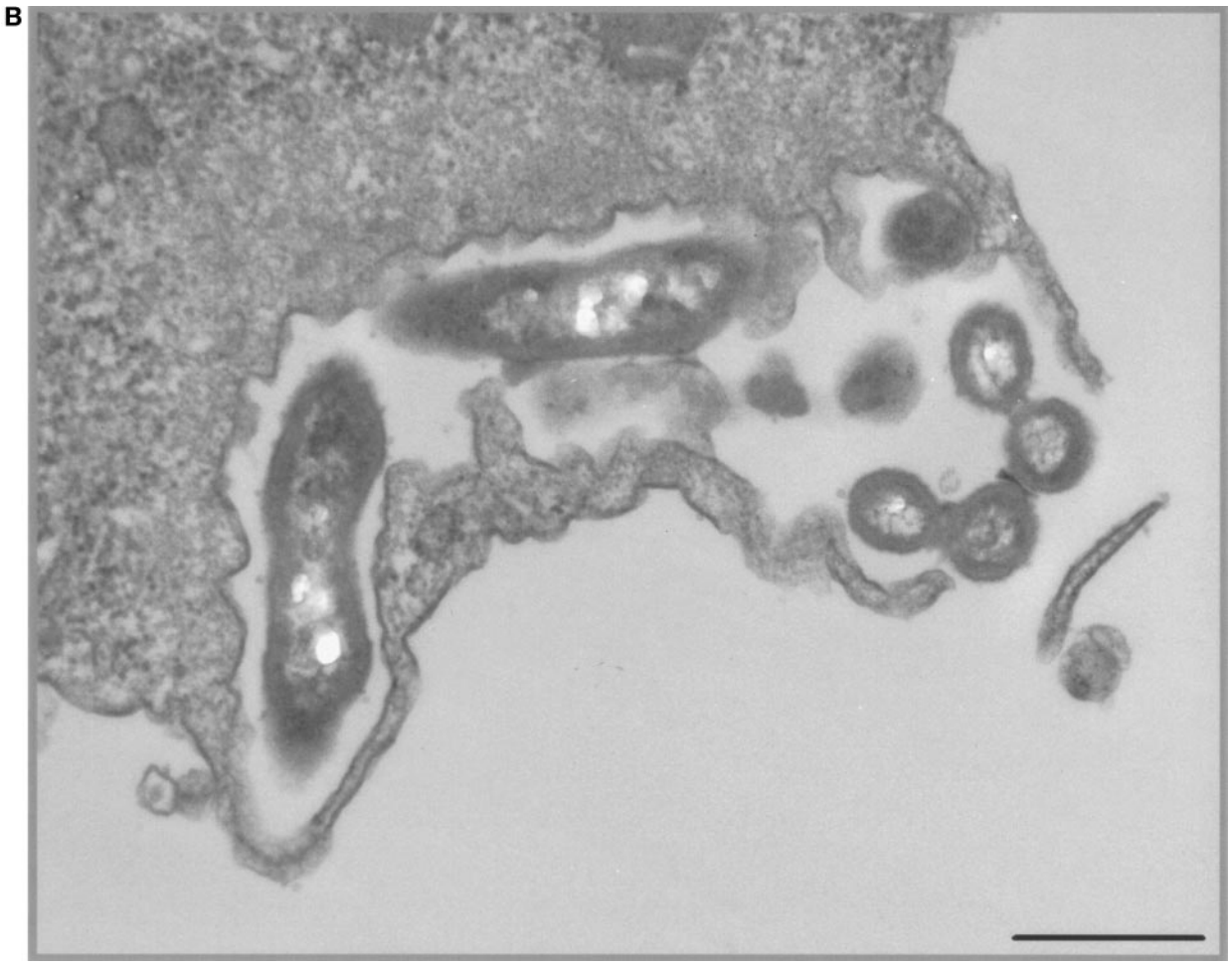

FIG. 1—*Continued.*

streptomycin (MIC, >4 μg/ml), tobramycin (MIC, >8 μg/ml), penicillin (MIC, >32 μg/ml), amoxicillin (MIC, >32 μg/ml), imipenem (MIC, >32 μg/ml), ceftriaxone (MIC, >256 μg/ml), cefotaxime (MIC, >256 μg/ml), vancomycin (MIC, >256 μg/ml), teicoplanin (MIC, >256 μg/ml), metronidazole (MIC, >256 μg/ml), trimethoprim-sulfamethoxazole (MIC, >8 μg/ml), isoniazid (MIC, >10 μg/ml), and ethambutol (MIC, >2 μg/ml). All of the phenotypic and antibiotic susceptibility tests gave identical results over three replicates. Biochemical and susceptibility characteristics that differentiated the isolates from the species of the *M. chelonae-M. abscessus* group are summarized in Tables 1 and 2.

Intra-amoebal survival and growth. No amoebal lysis was observed during the time of coculture. Acid-fast and Gimenez stainings, and transmission electron microscopy demonstrated mycobacterial internalization by *A. polyphaga* during coculture. After 3 days of coculture, electron microscopy observation disclosed the intravacuolar location with one to five mycobacteria per vacuole (Fig. 1A). They appeared as slightly curved or straight bacilli, 0.4 μm in width and 2 μm in length. Cell walls exhibited a trilamellar structure, including waxes with characteristic mycolic acid with long-branched chains (Fig. 1B).

G+C content of DNA. The G+C content of the DNA was 65 ± 3 mol%.

Genotypic analyses of the 16S rRNA, *hsp65*, *sodA*, *recA*, and *rpoB* genes and of the 16S-23S rRNA ITS. The 16S rRNA gene sequence of both isolates had complete identity with that of *M. abscessus* CIP 104536^T over 1,483 bp. The 441-bp *hsp65* gene region described by Telenti et al. (40) differed by 5, 31, and 35 nucleotides from that of *M. abscessus*, *M. chelonae*, and *M. immunogenum*, respectively. The partial 441-bp *sodA* gene region (2) differed by 3, 26, and 31 nucleotides from that of *M. abscessus*, *M. chelonae*, and *M. immunogenum*, respectively. The partial 915-bp *recA* gene sequence analysis (3) showed 98.0, 89.8, and 91.8% similarity with *M. abscessus*, *M. chelonae*, and *M. immunogenum*, respectively. The partial 723-bp *rpoB* gene sequence analysis (1) showed 96.0, 95.4, and 97.0% sequence similarity with *M. abscessus*, *M. chelonae*, and *M. immunogenum*, respectively. Based on the 16S-23S rRNA ITS (33), this new species differed from *M. abscessus* by the substitution of A→G at position 60 and the insertion of a C at position 102 of the spacer sequence (Fig. 2). Further 16S-23S rRNA ITS sequence analysis showed 19 and 32 position differences from *M. chelonae* and *M. immunogenum*, respectively,

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                          10         20         30         40         50         60
                           |          |          |          |          |          |
M. abscessus     AAGGAGCACCATTTCCCAGTCGAATGAACTAGGGAACATAAAGTAGGCATCTGTAGTGGA
M. massiliense   AAGGAGCACCATTTCCCAGTCGAATGAACTAGGGAACATAAAGTAGGCATCTGTAGTGGG
                 ************************************************************

                          70         80         90        100        110        120
                           |          |          |          |          |          |
M. abscessus     TATCTACTTGGTGAATATGTTTTGTAAATCCTGTCCACCCC-GTGGATGGGTAGTCGGCA
M. massiliense   TATCTACTTGGTGAATATGTTTTGTAAATCCTGTCCACCCCCGTGGATGGGTAGTCGGCA
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FIG. 2. Alignment of the of partial 16S-23S rRNA ITS region (120 nucleotides) of "*M. massiliense*" (GenBank accession number AY593978) and *M. abscessus* (GenBank accession number AY593976).

over 224-bp. A phylogenetic tree based on combined *rpoB* and *recA* gene partial sequences by incorporating 17 RGM species and the new taxon is shown in Fig. 3. A bootstrap value of 100% in the neighbor-joining tree supported the fork separating "*M. massiliense*" from *M. abscessus*. Parsimony and maximum-likelihood methods confirmed that "*M. massiliense*" clustered with species of the *M. chelonae-M. abscessus* group. Its lineage was clearly different from that of *M. abscessus* and quite distant from other recognized species (Fig. 3).

DISCUSSION

When we applied *rpoB* to the identification of clinical isolates belonging to the *M. chelonae-M. abscessus* group (1), we

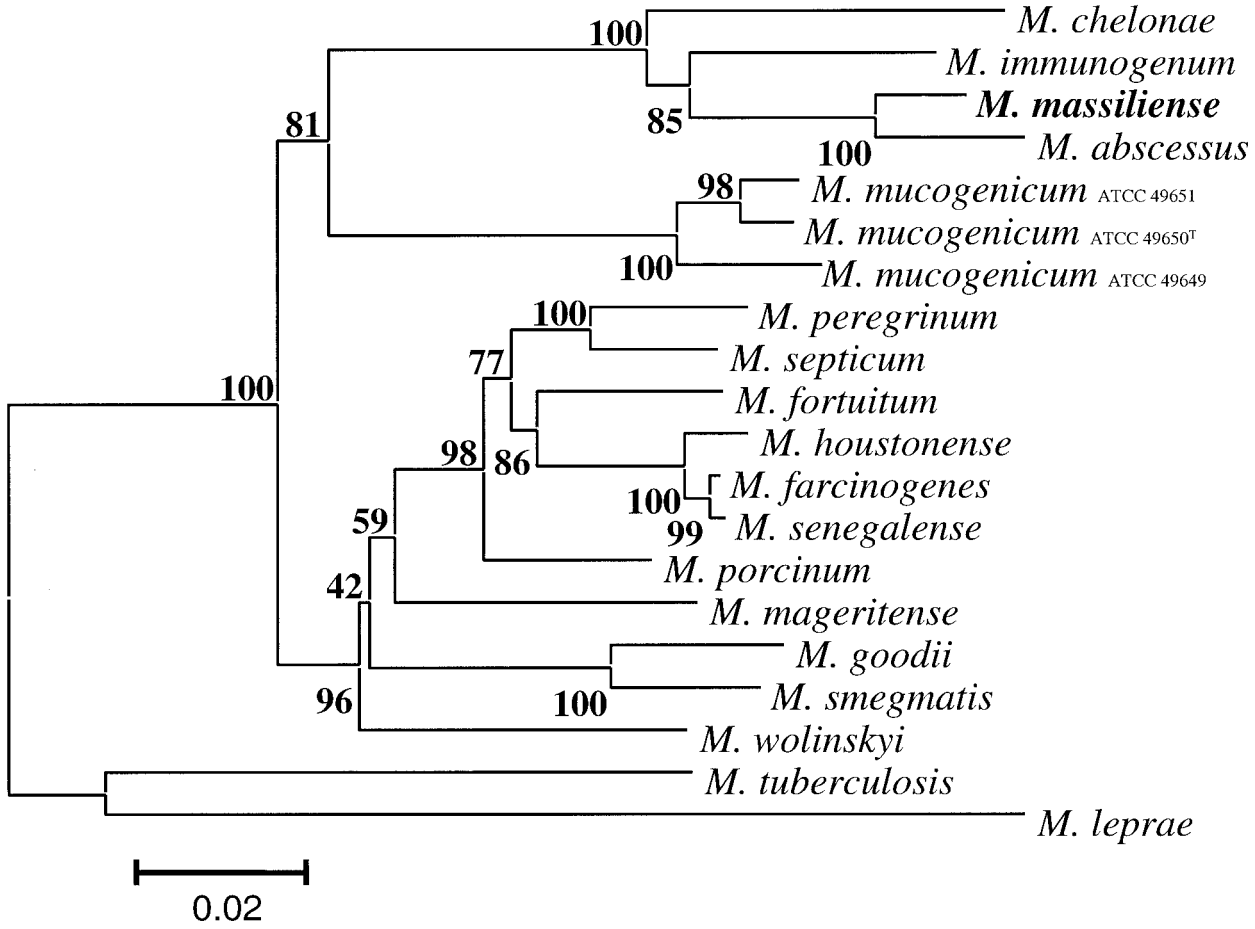

FIG. 3. Phylogenetic tree of the combined *rpoB+recA* gene sequences of "*M. massiliense*" and 17 RGM prepared by using the neighbor-joining method and Kimura's two-parameter distance correction model. The support of each branch, as determined from 1,000 bootstrap samples, is indicated by the value at each node (as a percentage). *M. tuberculosis* and *M. leprae* were used as the outgroups. The scale bar represents a 2% difference in nucleotide sequences.

found two isolates from the same patient sharing 96.0% sequence similarity only with that of *M. abscessus* type strain, suggesting that they were significantly distant from known taxons of this group. Indeed, in a previous study, we showed that an RGM belonged to the same species if it had <2% sequence divergence with one of the 20 studied species and that it belonged to a new species if it exhibited >3% sequence divergence from the corresponding type strain with the partial 723-bp *rpoB* sequence (2). We therefore further characterized these two isolates by a polyphasic approach comprising biochemical and antimicrobial susceptibility tests and genotypic analysis. One carbohydrate source and three enzymatic activities clearly separated both isolates from *M. abscessus* CIP 104536^T, since "*M. massiliense*" exhibited β-galactosidase and *N*-acetyl-β-glucosaminidase activities, whereas *M. abscessus* did not. "*M. massiliense*" had no β-glucuronidase activity and did not produce indole, in contrast to *M. abscessus*. Interestingly, "*M. massiliense*" and *M. abscessus* both differed from *M. chelonae* and *M. immunogenum* type strains by exhibiting gelatinase and tryptophane desaminase activities. "*M. massiliense*" and *M. immunogenum* were negative for the nitrate reduction, whereas *M. abscessus* and *M. chelonae* were positive. Discrepancies in the result of nitrate reduction test with that previously reported (37) may due to the fact that we used miniaturized API strip instead of conventional method (30). "*M. massiliense*" did not exhibit both iron uptake and urease activities, like the three species of the *M. chelonae-M. abscessus* group (37, 49). However, it exhibited some reactions typical of *M. abscessus*, such as the inability to utilize citrate, sorbitol, inositol, and mannitol as sole carbon sources (37) (Table 1).

Previous antibiotic susceptibility testing of a large collection of 45 *M. abscessus* isolates found them to be resistant to doxycycline (52), in contrast to the two "*M. massiliense*" isolates that were susceptible. This could be a useful marker for differentiating "*M. massiliense*" from *M. abscessus*. However, the clinical relevance of in vitro antibiotic susceptibility testing of "*M. massiliense*" is unknown; interestingly, the patient was cured after treatment with clarithromycin and minocycline, and "*M. massiliense*" was susceptible in vitro to these antibiotics. However, "*M. massiliense*" shared resistance to tobramycin (MIC, >8 μg/ml) and ciprofloxacin (MIC, >32 μg/ml) in common with *M. abscessus* (5, 39, 52).

We previously showed that 16S rRNA gene sequence did not discriminate all NTM taxa (1, 2). We proposed that *rpoB* gene sequence difference of >3% and a *recA* gene sequence difference of >2% discriminated NTM species (1–3).The 16S rRNA sequence of both isolates showed complete identity with that of the *M. abscessus* type strain. However, the 16S rRNA gene sequence poorly discriminates mycobacterial species such as *Mycobacterium gastri* and *Mycobacterium kansasii* (11); *Mycobacterium senegalense*, *Mycobacterium farcinogenes*, and "*Mycobacterium houstonense*" (formerly *M. fortuitum* third biovariant sorbitol positive) (2); and species of the *Mycobacterium tuberculosis* complex (11, 44). The two isolates exhibited 96.0% partial *rpo*B sequence similarity with that of the *M. abscessus*. Also, their partial *recA* gene sequence (3) showed 98.0% similarity with *M. abscessus*. Blackwood et al. found that in mycobacteria, the intraspecies sequence similarity of this region ranged from 98.7 to 100% (3). Furthermore, "*M. massiliense*" and *M. abscessus* were clearly differentiated by *hsp65* and *sodA* gene sequence analyses despite the low number of informative sites in those genes (five and three nucleotides, respectively) (2). On the other hand, *M. fortuitum* and *M. senegalense*, as well as *M. peregrinum* and *M. septicum*, which have the highest degree of 16S rRNA similarity (>99.6%) are separated from one another by only three nucleotides in their partial *hsp65* gene sequences (2, 31). Likewise, *M. senegalense* and "*M. houstonense*" have complete 16S rRNA gene sequence identity over 1,483 bp but differed from each other by two nucleotides only within the *sodA* partial sequence. Furthermore, based on the 16S-23S rRNA ITS, the new taxon differed from *M. abscessus* by one substitution and one insertion. One substitution in 16S-23S rRNA ITS discriminated sequevars among *M. farcinogenes* and *Mycobacterium avium* isolates (17, 28). Roth et al. found that insertion or deletion in the 16S-23S rRNA ITS discriminated different species (32), with the exception of members of the *M. tuberculosis* complex (10). Recently, we showed that the bootstrap values at the nodes of a few clusters with regard to the partial *sodA* and *hsp65*-based trees were too low to induce much confidence compared to the *recA* and *rpoB* gene-derived phylogenies (2), probably due to inadequate samples sizes (6). Furthermore, good congruence was obtained between the 16S rRNA and the combined *rpoB* and *recA* phylogenetic trees (2). We therefore combined the latter two genes to determine the evolutionary relationships of "*M. massiliense*" within the *M. chelonae-M. abcessus* group. This analysis demonstrated that "*M. massiliense*" was more closely related to *M. abscessus* than to *M. chelonae* and *M. immunogenum*.

One of the two isolates reported here was cultured by using an amoeba coculture system (14, 26) initially described for the isolation of *Legionella*-like amoebal pathogen in the sputum of a woman with persistent pneumonia (34). Several other mycobacterial species were observed in amoebae. *Mycobacterium leprae* was shown to survive in free-living amoebae, but no mycobacterial multiplication occurred (20). Further experiments showed that *Mycobacterium avium*, *Mycobacterium marinum*, *Mycobacterium ulcerans*, *Mycobacterium simiae*, and "*Mycobacterium habane*" could enter free-living amoebae; *Mycobacterium smegmatis*, *Mycobacterium fortuitum*, and *Mycobacterium phlei* were in large numbers in the amoebae, eventually inducing amoebal lysis (7, 22, 38).

In the present study, we describe a new RGM species, "*M. massiliense*," isolated in amoebic coculture from the lower respiratory tract of a 50-year-old woman with hemoptoic pneumonia. The same strain was recovered two times over a 2-month period. An NTM is generally considered to be pathogenic if it isolated (i) repeatedly from different samples during several weeks or in large amounts, (ii) in the presence of a coexisting bronchopulmonary disease, or (iii) when the clinical presentation and X-ray pattern is suggestive (pseudotuberculosis) (16, 46). The new taxon could therefore be considered pathogenic. Additional arguments in favor of a pathogenic role are the facts that the closely related species *M. abscessus* has been shown to cause pulmonary disease and that the underlying diseases included bronchiectasis, cystic fibrosis, gastroesophageal disorders, and prior granulomatous disease such as sarcoidosis or tuberculosis (9, 16, 36). A study of *M. abscessus* pulmonary disease emphasized striking similarities to *M. avium* complex lung disease of the type known as nodular bronchiec-

tasis (16). These similarities suggested a common pathogenicity or host susceptibility (16). Patients infected with *M. avium* complex and most patients with *M. abscessus* pulmonary disease had underlying nodular bronchiectasis (46). Approximately 20% of patients with *M. abscessus* infection eventually developed *M. avium* complex infection (16). In a later study, *M. abscessus* has been identified by using the microbiologic and radiographic criteria of the American Thoracic Society (16) due to the lack of molecular identification methods at that time. The use of the partial *rpoB* gene for further genetic analysis would be helpful to better assess the clinical relevance of the species or subspecies that are associated with disease since the *M. abscessus* type strain was first isolated from a patient with abscess (25).

Since these mycobacteria were isolated in Marseille, we propose that the isolates be included in the genus *Mycobacterium* as "*Mycobacterium massiliense*," a name that refers to Massilia, the old Greek and Roman name for Marseille.

ACKNOWLEDGMENTS

We thank Christian de Fontaine, Lina Barrassi, and Nicolas Aldrovandi for technical assistance and Babu V. A. K. Gundi for expert review of the manuscript.

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