Mycobacterium confluentis sp. nov.

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A new rapidly growing mycobacterium was isolated from human sputum. This organism grew at 22, 31, 37, and 41°C and possessed catalase, acid phosphatase, acetamidase, urease, nicotinamidase, pyrazinamidase, and nitrate reductase activities. It did not produce nicotinic acid, hydrolyze Tween, or have benzamidase, isonicotinamidase, succinamidase, and arylsulfatase activities. A mycolic acid analysis revealed a simple, unique pattern. The organism is susceptible to antituberculous drugs. A comparative 16S rRNA sequence analysis placed this organism within the confines of the genus *Mycobacterium*, most closely related to the thermotolerant rapidly growing species. On the basis of the pattern of enzymatic activities and metabolic properties, as well as the unique 16S rRNA sequence, we propose that our single strain represents a new species, for which we propose the name *Mycobacterium confluentis*. The type strain is strain 1389/90; a culture of this strain has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 44017.

Mycobacteria are aerobic, nonmobile bacteria that are widespread in nature and range from soil-dwelling saprophytes to pathogens of humans and animals (19, 27). A previously unrecognized mycobacterium was isolated from a human sputum specimen. Colonization of the respiratory tract was not associated with disease. In this paper we describe the results of a taxonomic study of this organism and propose that it should be named *Mycobacterium confluentis* sp. nov.

MATERIALS AND METHODS

Bacterial strain. A single isolate, strain 1389/90^T (T = type strain), was recovered from a sputum specimen obtained from a healthy male. The isolation method used was the standard method used for isolation of mycobacteria from clinical specimens (19). Colony morphology and the ability to grow at various temperatures (22, 31, 37, 41, 45, and 52°C) were determined after 4 weeks of incubation on Löwenstein-Jensen slants. The following properties were determined as described previously: production of nicotinic acid (16); acetamidase, benzamidase, urease, isonicotinamidase, nicotinamidase, pyrazinamidase, and succinamidase activities (2); nitrate reduction (3); Tween hydrolysis (26); acid phosphatase activity (8); production of esterases (7) and arylsulfatase (12); catalase activity (10); iron uptake (22); and resistance to isoniazid (0.25 µg/ml), streptomycin (4.0 µg/ml), and ethambutol (1.0 µg/ml) (18).

Lipid analyses were carried out by performing one- and two-dimensional thin-layer chromatography of whole-organism methanolysates (13). Freeze-dried bacteria (50 mg) were degraded by treatment at 75°C with 3 ml of methanol-toluene-sulfuric acid (30:15:1, vol/vol/vol) for 16 h. Long-chain components were extracted with hexane, and traces of acid were removed by passage through a short column containing ammonium hydrogen carbonate. The hexane extracts were examined by thin-layer chromatography on plates (20 by 20 cm), using a solution containing petroleum

ether (boiling point, 60 to 80°C) and diethyl ether (85:15, vol/vol) as the developing phase. For two-dimensional chromatography on plates (10 by 10 cm), petroleum ether-acetone (95:5, vol/vol) in the first direction was followed by a single development with toluene-acetone (97:3, vol/vol) in the second direction.

The 16S rRNA gene was amplified by using the polymerase chain reaction technique, and the sequence was determined as described previously (4, 5). With the sequencing strategy which we used we determined the nucleotides at 1,335 contiguous positions. The sequence which we obtained was aligned with selected 16S rRNA sequences (15, 21) as described previously (15). For the phylogenetic analysis regions of alignment uncertainty were omitted (positions 83 to 86 and 686 to 696 of the sequence, corresponding to *Escherichia coli* positions 212 to 217 and 837 to 848); this reduced the number of positions used from 1,335 to 1,318. Pairwise distances were calculated by weighing nucleotide differences and insertions-deletions equally (Hamming distance). The phylogenetic tree was constructed by using the neighborliness method (20) as described previously (15).

Nucleotide sequence accession number. The EMBL nucleotide sequence accession number for strain 1389/90^T is X63608.

RESULTS AND DISCUSSION

Cells of strain 1389/90^T grown on Löwenstein-Jensen agar were rod-shaped coccobacilli (0.5 to 0.8 by 0.7 to 1.7 µm), often polymorphic, and acid-alcohol fast. No spores, capsules, or aerial hyphae were observed.

On Löwenstein-Jensen slants the isolate grew as eugonic, smooth, and colorless colonies within 2 to 4 days when it was incubated at temperatures ranging from 22 to 41°C. Initially, a brownish pigmentation that appeared after 8 to 12 weeks of incubation and later turned to black drew our attention to this isolate. The dark pigment is neither dependent on light nor a stable property of the strain. A similar pigmentation property has been described previously for *M. thermoresistibile* (27) and for a strain which developed

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TABLE 1. Distinguishing characteristics of selected *Mycobacterium* species^a

Characteristic	Strain 1389/90 ^T	<i>M. thermoresistibile</i> ^b	<i>M. fortuitum</i> ^b	<i>M. chelonae</i> ^b	<i>M. smegmatis</i> ^b	<i>M. flavescens</i> ^b	<i>M. phlei</i> ^b
Enzymatic activities							
Production of nicotinic acid	0	0	0	0	0	0	0
Acetamidase	+	0	+	+	+	0	+
Benzamidase	0	0	0	0	+	0	0
Urease	+	+	+	+	+	+	+
Isonicotinamidase	0	0	0	0	+	0	0
Nicotinamidase	+	+	+	+	+	+	+
Pyrazinamidase	+	+	+	+	+	+	+
Succinidamidase	0	0	+	0	0	0	0
Tween hydrolysis	0	+	+/-	0	+	0	0
Nitrate reductase	+	+	+	0	+	+	+
Arylsulfatase (10 days)	0	0	+	+	0	0	0
Acid phosphatase	+	0	+	+	0	0	+
α -Esterase	(+)	(+)	0	0	0	+	+
β -Esterase	0	0	0	0	0	0	+
Catalase at 22°C	+	+	+	+	+	+	+
Catalase at 68°C	+	+	+	0	+	+	+
Iron uptake	0	0	+	0	+	+	+
Growth at:							
22°C	+	+	+	+	+	+	+
31°C	+	+	+	+	+	+	+
37°C	+	+	+	+	+	+	+
41°C	+	+	0	0	+	+	+
45°C	0	+	0	0	+	(+)	+
52°C	0	+	0	0	0	0	+
Tolerance to							
5% NaCl	0	+	+	0	+	+	+
Susceptibility to:							
Isoniazid (0.25 µg/ml)	S	R	R	R	R	S	R
Streptomycin (4.0 µg/ml)	S	S	R	R	R	S	S
Ethambutol (1.0 µg/ml)	S	S	R	R	R	S	S

^a 0, negative; +/-, variable; (+), weakly positive; +, positive; S, susceptible; R, resistant.^b Obtained from the strain collection of the National Reference Laboratory for Mycobacteria, Forschungsinstitut Borstel, Borstel, Germany.

spontaneously from a strain of *M. fortuitum* (6). Our taxonomic studies (Table 1 and Fig. 1 and 2) demonstrated that strain 1389/90^T is a species that is different from *M. fortuitum* and *M. thermoresistibile*.

A comparison of the properties of strain 1389/90^T with the properties of other species of rapidly growing mycobacteria is shown in Table 1. The pattern of enzymatic activities and metabolic properties demonstrates that strain 1389/90^T is different from previously described species (27). This strain possesses acetamidase, urease, nicotinamidase, pyrazinamidase, nitrate reductase, acid phosphatase, and catalase activities; neither arylsulfatase activity nor Tween hydrolysis was observed. Strain 1389/90^T does not tolerate 5% NaCl but grows at 41°C. Most remarkable is the susceptibility of this strain to isoniazid, streptomycin, and ethambutol, which is unusual for rapidly growing mycobacteria.

One-dimensional thin-layer chromatography of whole-organism acid methanolysates indicated that the pattern of long-chain components of strain 1389/90^T is distinct from the patterns of long-chain components of *M. flavescens*, *M. phlei*, *M. smegmatis*, and *M. thermoresistibile*. Two-dimensional thin-layer chromatography resulted in more precise separation of all of the components; representative test

strains produced multiple-spot patterns. In contrast, strain 1389/90^T produced a very simple, unique pattern (Fig. 1). Strain 1389/90^T synthesized only ketomycolates and traces of α -mycolates, whereas in the other thermotolerant rapidly growing mycobacteria methoxymycolates and mycolates with other functional groups and eicanols were also found.

Figure 3 shows the almost complete 16S rRNA sequence of strain 1389/90^T, consisting of a contiguous stretch of 1,335 nucleotide positions. This sequence is unique among the more than 30 published mycobacterial rRNA sequences (1, 9, 15, 21, 23), including sequences for *M. fortuitum*, *M. chelonae*, *M. thermoresistibile*, *M. triviale*, *M. phlei*, *M. senegalense*, *M. fallax*, *M. neoaurum*, *M. aurum*, *M. smegmatis*, and *M. flavescens*, as well as the "*M. sphagni*" sequence (unpublished data). A phylogenetic tree that showed the natural relationships among selected mycobacteria (Fig. 2) was constructed by using equally weighted (Hamming) distances (Table 2). In this scheme strain 1389/90^T was a member of a branch of rapid growers consisting of strain 1389/90^T, *M. phlei*, *M. thermoresistibile*, *M. flavescens*, *M. smegmatis*, and "*M. chromogen*." "*M. chromogen*" is a rapidly growing, saprophytic *Mycobacterium* species that was isolated from bovine feces and has not been

FIG. 1. Two-dimensional thin-layer chromatography of whole-organism acid methanolysates. (a) Strain 1389/90^T (= DSM 44017^T). (b) *M. thermoresistibile* (Forschungsinstitut Borstel). (c) *M. smegmatis* DSM 43756. (d) *M. flavescens* DSM 43219. (e) *M. phlei* DSM 43239. Spot A, α -mycolate (methyl mycolates devoid of oxygen functions other than the 3-hydroxy ester system); spot B, methoxymycolate; spot C, ketomycolate; spot D, carboxy mycolate methyl ester. Spot E, 2-eicosanol and homologs; spot F, nonhydroxylated fatty acid methyl esters.

FIG. 2. Phylogenetic tree. This phylogenetic tree was based on 16S rRNA sequences from selected mycobacteria (15, 21) and shows the position of strain 1389/90^T. The tree was constructed by using the neighborliness method and was rooted by using *Nocardia asteroides* as an outgroup. The slow-growing mycobacteria include *M. simiae*, *M. terrae*, *M. nonchromogenicum*, *M. xenopi*, *M. gordonae*, *M. asiaticum*, *M. tuberculosis*, *M. marinum*, *M. leprae*, *M. scrofulaceum*, *M. gastri*, *M. szulgai*, *M. malmoense*, *M. intracellulare*, *M. paratuberculosis*, and *M. avium* (15, 21, 23). The bar indicates 10-nucleotide differences.

formally described yet (21). *M. phlei*, *M. flavescens*, *M. smegmatis*, and *M. thermoresistibile* have often been grouped together in numerical classification schemes, showing 75 to 84% similarity (17, 24, 25). These organisms are represent thermotolerant rapid growers which grow at 45°C. Although strain 1389/90^T does not grow at 45°C, its ability to grow at 41°C places it with the thermotolerant species. Strain 1389/90^T and the closely related thermotolerant fast-growing species share a common molecular signature within the 16S rRNA sequence. These species have a guanine nucleotide inserted in helix 10, resulting in a helix containing 11 base

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      10      20      30      40      50      60
1  CCCTGCACTT TGGGATAAGC CTGGGAAACT GGGTCTAATA CCGAATACAC CCTTCTGATC
61 GCATGGTCTG GGAGGGGAAA GCTTTTTCGG TGTGGGATGG GCGCGCGGCC TATCAGCTTG
121 TTGGTGGGGT GATGGCTAC CAAGGCGACG ACGGGTAGCC GGCCTGAGAG GGTGTCCGGC
181 CACACTGGGA CTGAGATACG GCCCAGACTC CTACGGGAGG CAGCAGTGGG GAATATTGCA
241 CAATGGGCGC AAGCCTGATG CAGCGACGCC GCGTGGGGGA TGACGGCCTT CGGGTTGTAA
301 ACCTCTTTCA GCATCGACGA AGCGCAAGTG ACGGTAGATG CAGAAGAAGC ACCGGCCAAC
361 TACGTGCCAG CAGCCGCGGT AATACGTAGG GTGCGAGCGT TGTCCGGAAT TACTGGGCGT
421 AAAGAGCTCG TAGGTGGTTT GTCACGTTGT TCGTGAAAAC TCACAGCTTA ACTGTGGGCG
481 TGCGGGCGAT ACGGGCAGAC TAGAGTACTG CAGGGGAGAC TGGAATTCCT GGTGTAGCGG
541 TGGAATGCGC AGATATCAGG AGGAACACCG GTGGCGAAGG CGGGTCTCTG GGCAGTAACT
601 GACGCTGAGG AGCGAAAGCG TGGGGAGCGA ACAGGATTAG ATACCTGGT AGTCCACGCC
661 GTAAACGGTG GGTACTAGGT GTGGGTTTCC TTCCTTGGGA TCCGTGCCGT AGCTAACGCA
721 TTAAGTACCC CGCCTGGGGA GTACGGCCGC AAGGCTAAAA CTCAAAGGAA TTGACGGGGG
781 CCCGCACAAG CGGCGGAGCA TGTGGATTAA TTCGATGCAA CGCGAAGAAC CTTACCTGGG
841 TTTGACATGC ACAGGACGCT GGTAGAGATA TCAGTTCCCT TGTGGCCTGT GTGCAGGTGG
901 TGCAATGGCTG TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA
961 CCCTTGCTCTC ATGTTGCCAG CACGTAATGG TGGGGACTCG TGAGAGACTG CCGGGGTCAA
1021 CTCGGAGGAA GGTGGGGATG ACGTCAAGTC ATCATGCCCC TTATGTCCAG GGCTTCACAC
1081 ATGCTACAAT GGCCGGTACA AAGGGCTGCG ATGCCGTGAG GTGGAGCGAA TCCTTTCAAA
1141 GCCGGTCTCA GTTCGGATCG GGTCTGCAA CTCGACCCCG TGAAGTCGGA GTCGCTAGTA
1201 ATCGCAGATC AGCAACGCTG CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA
1261 CGTCATGAAA GTCGGTAACA CCCGAAGCCA GTGGCCTAAC CCTTGTGGGA GGGAGCTGTC
1321 GAAGGTGGGA TCGGC

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FIG. 3. 16S rRNA sequence of strain 1389/90^T (total length, 1,335 nucleotides). The first and last nucleotide correspond to *E. coli* 16S rRNA positions 135 and 1481, respectively. The positions of alignment uncertainty that were omitted for the phylogenetic analysis are underlined.

TABLE 2. Hamming distances derived from 16S rRNA sequences^a

Organism	Hamming distances										
	<i>M. senegalense</i>	<i>M. neoaurum</i>	<i>M. chelonae</i> subsp. <i>abscessus</i>	<i>M. fallax</i>	<i>M. aurum</i>	Strain 1389/90 ^T	<i>M. smegmatis</i>	<i>M. flavescens</i>	" <i>M. chromogen</i> "	<i>M. thermoresistibile</i>	<i>M. phlei</i>
<i>M. fortuitum</i>	12	34	46	24	36	41	30	26	41	45	36
<i>M. senegalense</i>		34	44	26	37	41	35	31	43	46	40
<i>M. neoaurum</i>			45	34	48	41	34	41	44	44	54
<i>M. chelonae</i> subsp. <i>abscessus</i>				52	48	66	64	63	67	62	66
<i>M. fallax</i>					52	37	34	33	32	40	39
<i>M. aurum</i>						53	48	44	55	59	58
Strain 1389/90 ^T							18	25	26	34	29
<i>M. smegmatis</i>								12	21	23	27
<i>M. flavescens</i>									20	27	26
" <i>M. chromogen</i> "										22	30
<i>M. thermoresistibile</i>											40

^a Sequences were obtained from references 15 and 21.

pairs; all other mycobacteria have a helix that contains 10 base pairs (21).

A recent phylogenetic analysis placed *M. fallax* within the branch containing the thermotolerant rapid growers (21), although the authors noted that no overt phenotypic characteristics suggest such a relationship (11). However, phylogenetic trees are dynamic, and their topology changes with the organisms selected. The selection of the organisms used in our analysis allowed us to redefine the phylogenetic position of *M. fallax* and to place this organism outside the branch containing the thermotolerant rapid growers, a position that reflects the inability of *M. fallax* to grow at temperatures of more than 37°C. Given the short branch length that separates *M. fallax*, *M. aurum*, and the thermotolerant group of mycobacteria, any attempt to describe the order of the divergence of these organisms will require additional supportive comparative data.

Taxonomic description of *Mycobacterium confluentis* sp. nov. *Mycobacterium confluentis* (con.flu.en'tis. L. gen.n. *confluentis*, referring to Koblenz, the source of the strain on which the description of the species is based). Cells are acid-alcohol-fast coccobacilli (0.5 to 0.8 by 0.7 to 1.7 µm). Does not form spores, capsules, or aerial hyphae. Visible growth from dilute inocula occurs within 2 to 4 days; colonies on Löwenstein-Jensen agar are eugonic, colorless, smooth, and 3 to 5 mm in diameter; after prolonged culture (8 to 12 weeks) a dark pigmentation that is not dependent on light (a variable trait) may be observed. Growth occurs at 22, 31, 37, and 41°C; optimum growth occurs at temperatures between 31 and 37°C. Susceptible in vitro to antituberculous drugs. Biochemical characteristics are shown in Table 1. *M. confluentis* produces a simple, unique mycolate pattern. The phylogenetic position, based on an evaluation of 16S rRNA sequences, is within the genus *Mycobacterium*, most closely related to the thermotolerant rapid growers (Fig. 2). Type strain 1389/90 was isolated from human sputum; a culture of this strain has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 44017.

Characteristics which differentiate *M. confluentis* from other related mycobacteria. *M. confluentis* can be differentiated from other thermotolerant mycobacteria by its inability to grow at temperatures of more than 41°C. A striking property which separates it from other rapidly growing mycobacteria is its susceptibility to antituberculous drugs. A

unique 16S rRNA sequence characterizes *M. confluentis*. Direct sequence determination of amplified 16S rDNA sequences has been introduced recently for differentiation of *Mycobacterium* species (14).

Phenotypically, a certain similarity between *M. confluentis* and *M. thermoresistibile* is apparent. However, *M. confluentis* can easily be differentiated from *M. thermoresistibile* by its inability to grow at 52°C and its lack of Tween hydrolysis and β-esterase activity, as well as its lack of tolerance to 5% NaCl. Furthermore, *M. confluentis* has acetamidase and acid phosphatase activities.

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