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Mycobacterium interjectum, a New Species Isolated from a Patient with Chronic Lymphadenitis

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***Mycobacterium*-like organisms, isolates 2081/92 and 4185/92, were recovered from a lymph node of a child with chronic lymphadenitis. The growth characteristics, acid-fastness, and mycolic acids of the isolate were consistent with those for *Mycobacterium* species. The isolates were biochemically distinct from described *Mycobacterium* species, although they most closely resembled *M. scrofulaceum*. Comparative 16S rDNA sequencing showed that these isolates represent a new slow-growing *Mycobacterium* species which is named *M. interjectum*. Our results demonstrate the importance of 16S rDNA sequencing for recognizing the diversity of species within the genus *Mycobacterium*.**

Mycobacteria are aerobic, nonmobile bacteria that are widespread in nature and that range from soil-dwelling saprophytes to pathogens of humans and animals (23, 32). Cervical lymphadenitis is among the most common manifestation of infection with nontuberculous mycobacteria in children. The mycobacteria isolated represent mainly *M. avium*, *M. scrofulaceum*, and *M. malmoense* (8, 33). In this report, we describe a new *Mycobacterium* species, *M. interjectum*, that was isolated from a child with chronic lymphadenitis.

CASE REPORT

The patient was an 18-month-old boy who presented with local swelling at the right submandibular region which had been increasing in size for 1 week. Physical examination revealed a 1-by-1-cm firm, indolent lymph node under the right mandible with lividly colored overlying skin. Further examination disclosed no abnormalities. The child had not received *M. bovis* BCG vaccination. The medical and family history were unremarkable.

Ultrasonic inspection of the swelling showed several enlarged, slightly confluent, sonographically hypodense lymph nodes. Laboratory data including sedimentation rate and blood cell counts were normal. Chest roentgenogram and computed tomography scan as well as abdominal ultrasonic examination revealed no abnormalities. Within 2 weeks the swelling had almost completely disappeared spontaneously.

Eight months later the boy was admitted because of a relapse at the same location. According to the mother, the boy looked pale, was tired, and had no appetite. Physical examination showed a 3-by-3.5-cm firm, movable, indolent submandibular lymph node. Further physical examination was unremarkable.

Ultrasonic examination revealed an echoreduced, solid structure that was sharply separated from the surroundings at a size of 2 by 2.4 by 2 cm with no evidence of central colliquation. Chest X rays were repeatedly normal. The sedimentation rate was 30/71, and the leukocyte count was slightly elevated with deviation to the left. Under standard antibiotic therapy, the swelling increased within the next 10 days. Tuberculin testing (Tubergen 10 units; Behring, Marburg, Germany) was positive (three indurations of 3 to 4 mm in diameter). Surgical exploration showed a massive inflammation of the lymph nodes. Only a partial resection was possible. Histopathology showed granulomatous inflammation, caseating necrosis, and scattered acid-fast bacilli. The excised tissue was cultured for mycobacteria (isolate 2081/92), and therapy with a triple regimen that included isoniazid, rifampin, and pyrazinamide was started under the impression that the child had a tuberculous lymphadenitis. Three months later, the involved lymph nodes, including a fistula that developed in the meantime, could be totally removed by surgery, isolate 4185/92 was recovered, and therapy was changed to clarithromycin, isoniazid, and protionamide. No relapses occurred thereafter.

MATERIALS AND METHODS

Growth and biochemical tests. Surgical specimens were processed by standard procedures (23) and were inoculated onto Löwenstein-Jensen (LJ) slants. Two isolates, strains 2081/92 and 4185/92, were recovered.

Colony morphology and the ability to grow at various temperatures (22, 31, 37, 41 and 45°C) were determined after 4 weeks of incubation on LJ slants. The following properties were determined, as described previously (16): production of nicotinic acid (26); acetamidase, benzamidase, urease, isonicotinamidase, nicotinamidase, pyrazinamidase, and succinidamidase activities (2); nitrate reduction (3); Tween hydrolysis (31); acid phosphatase activity (10); production of esterases (11) and arylsulfatases (19); catalase activity (17); β-galactosidase (13); and resistance to isoniazid (0.25 μg/ml), streptomycin (4.0 μg/ml), ethambutol (5.0 μg/ml), and rifampin (2.0 μg/ml) (27). The other strains used in the present study were obtained from the strain collection of the National Reference Laboratory for Mycobacteria, Forschungsinstitut Borstel.

Lipid analyses. Mycolic acids were analyzed by thin-layer chromatography of whole organism methanolysates (20, 22). Fatty acids were analyzed by using fatty acid methyl esters obtained from wet biomass (approximately 40 mg) by sapon-

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TABLE 1. Distinguishing characteristics of selected *Mycobacterium* species[a]

Characteristic	Strain 4185/92	Strain 2081/92	*M. kansasii*[b]	*M. simiae*[b]	*M. malmoense*[b]	*M. scrofulaceum*[b]
Enzymatic activity as production of:						
Nicotinic acid	0	0	0	±	0	0
Acetamidase	0	0	0	0	0	0
Benzamidase	0	0	0	0	0	0
Urease	+	+	+	+	±	+
Isonicotinamidase	0	0	0	0	0	0
Nicotinamidase	+	+	+	±	+	+
Pyrazinamidase	+	+	0	±	+	+
Succinidamidase	0	0	0	0	0	0
Tween hydrolysis (10 days)	0	0	+	0	+	0
Nitrate reductase	0	0	+	0	0	0
Arylsulfatase (10 days)	(+)	0	+	0	0	0
Acid Phosphatase	0	0	+	0	0	0
α-Esterase	+	+	0	+	(+)	+
β-Esterase	0	0	0	+	(+)	+
Catalase at 22°C	+	+	+	+	+	+
Catalase at 68°C	+	+	+	+	0	+
β-Galactosidase	±	±	0	0	0	0
Photochromogenic	0	0	+	+	0	0
Growth at						
22°C	0	0	+	+	±	+
31°C	+	+	+	+	+	+
37°C	+	+	+	+	±	+
41°C	0	0	+	+	0	+
45°C	0	0	0	0	0	0
Susceptibility to						
Isoniazid	R	R	R	R	R	R
Streptomycin	R	S	R	R	R	R
Ethambutol	R	R	S	R	S	S
Rifampin	S	S	S	S/R	S/R	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.

ification, methylation, and extraction as described previously (18, 20, 21). The fatty acid methyl ester mixtures were separated and analyzed by using a model 5898A microbial identification system (Microbial ID, Newark, Del.) which consisted of a Hewlett-Packard model 5980 gas chromatograph fitted with a 5% phenylmethyl silicone capillary column (0.2 mm by 25 m), a flame ionization detector, a Hewlett-Packard model 3392 integrator, a Hewlett-Packard model 7673A automatic sampler, and a Hewlett-Packard model 900/300 computer (Hewlett-Packard Co., Palo Alto, Calif.). The gas chromatographic parameters were as follows: carrier gas, ultra-high-purity hydrogen; column head pressure, 60 kPa; injection volume, 2 μl; column split ratio, 100:1; septum purge, 5 ml/min; column temperature, 170 to 270°C at 5°C/min; injection port temperature, 250°C; and detector temperature, 300°C. Peaks were automatically integrated, and fatty acid names were determined and percentages were calculated. The results of the fatty acid analyses of the isolates were compared by principal component analysis with the data of the mycobacteria species stored in the MIS fatty acid data base. The entry of each *Mycobacterium* species into the MIDI fatty acid library is based on about 20 strains of this species. The results of the comparison of the unknown isolate are given as similarity indices. Similarity indices of 0.5 to 0.9 can be taken for reliable species identification.

rRNA sequencing and phylogenetic analysis. A colony from an LJ slant was dissolved in 500 μl of 10 mM Tris–1 mM EDTA (TE). The bacterial pellet was harvested by centrifugation in a microcentrifuge and was redispersed in 100 μl of TE containing acid-washed glass beads with a diameter of 100 μm (Sigma, Deisenhofen, Germany). A tissue disintegrator (H. Mickle, Gomshall, Surrey, United Kingdom) was used to disrupt the cells (2 min at maximum speed). After centrifugation for 5 min, 5 μl of the supernatant was used for amplification of the 16S rRNA gene.

Amplification by the polymerase chain reaction was performed as described previously (6, 15, 24) by using two pairs of primers that yielded two overlapping DNA fragments containing the almost complete 16S rRNA gene. The sequences of the oligonucleotide primer pairs were AGAGTT TGATCCTGGCTCAG (positions 8 to 28) in combination with TGCACACAGGCCACAAGGGA (positions 1046 to 1026) and GTGTGGGTTTCCTTCCTTGG (positions 830 to 847) in combination with AAGGAGGTGATCCAGCCGCA (positions 1542 to 1522). The nucleotide positions correspond to the numbering of the prokaryotic 16S rRNA sequence in *Escherichia coli.*

Typically, one of the primers used in the amplification reaction was biotinylated to allow for single-stranded DNA sequencing (9). The single-stranded DNA template was prepared with Dynabeads (Dynal, Hamburg, Germany) essentially as described by the manufacturer. For both isolates, the nucleotide sequence of the signature region (24) was determined. For isolate 4185/92 we determined the nucleotides of the 16S rRNA gene at 1,439 contiguous

positions. The sequence that was obtained was aligned with selected 16S rRNA sequences (25, 29, 30) as described previously (25). For the phylogenetic analysis, regions of alignment uncertainty were omitted (positions 45 to 56, 178 to 181, and 784 to 796 of the sequence, corresponding to *E. coli* positions 83 to 95, 212 to 217, and 841 to 851; additionally, positions 1 to 7 and 1427 to 1439 of the sequence were omitted, because most of the published sequences [25] lack this part of the molecule). Pairwise distances were calculated by weighing nucleotide differences and insertions-deletions equally (Hamming distances). The phylogenetic tree was constructed by using the neighborliness method (28) as described previously (25).

Nucleotide sequence accession number. The EMBL nucleotide sequence accession number for strain 4185/92^T is X70961.

RESULTS

Growth characteristics and biochemical features. Cells of isolates 4185/92 and 2081/92 grown on LJ slants presented as acid-alcoholic-fast, rod-shaped (0.6 to 1.0 μm by 0.7 to 2.0 μm) coccobacilli. They were often polymorphic with few filaments (up to 6.0 μm in length), but no spores, capsules, or aerial hyphae were observed. On LJ slants the isolates grew as dysgonic, smooth, and scotochromogenic yellowish colonies of 1 to 2 mm in diameter within 21 to 28 days when incubated at temperatures ranging from 31 to 37°C. A comparison of the properties of isolates 4185/92 and 2081/92 with the properties of other slow-growing species of mycobacteria is shown in Table 1. The pattern of enzymatic activities and metabolic properties demonstrates that the isolated strains differ from described species, although they resemble *M. scrofulaceum* (32). In particular, the isolates possess urease, nicotinamidase, α-esterase, and pyrazinamidase activities and a heat-stable catalase. Results for arylsulfatase and β-galactosidase tests were variable. The tests for niacin production; nitrate reduction; Tween hydrolysis; and β-esterase, acetamidase, benzamidase, isonicotinamidase, and succinidamidase activities were negative. In contrast to *M. scrofulaceum*, the isolates did not grow at 22 and 41°C and showed a negative β-esterase test. The isolates were susceptible to rifampin and were resistant to isoniazid and ethambutol.

Lipid analyses. Two-dimensional thin-layer chromatography revealed that isolates 4185/92 and 2081/92 contain α-, keto-, ω-carboxy- and epoxy-mycolates, a pattern which is also found in *M. avium* and *M. scrofulaceum* (22).

Fatty acid analysis by gas-liquid chromatography of isolates 2081/92 and 4185/92 revealed a pattern similar to that for *M. scrofulaceum* (Table 2). In contrast to *M. scrofulaceum*, isolates 2081/92 and 4185/92 contained low amounts of dodecanoic acid and lacked *cis*-9-hexadecenoic acid. The isolates differed from *M. simiae* by the presence of *cis*-10-hexadecenoic acid and heptadecenoic acid and the absence of *cis*-11-hexadecenoic acid.

16S rRNA sequencing. Partial 16S rRNA sequence determination of a region with a species-specific character for mycobacteria (24) revealed a sequence that differed from those of all described species of slow-growing mycobacteria (1, 7, 12, 14–16, 20, 24, 25, 29, 30; unpublished sequences; see Fig. 1). Isolate 4185/92 was chosen to determine the nearly complete 16S rRNA sequence (1439 positions covering 96% of the molecule). Sequence analysis disclosed a sequence that is unique among the more than 30 published mycobacterial sequences, including *M. kansasii*, *M. scrofu-*

TABLE 2. MIS whole-cell fatty acid analysis of strains 4185/92 and 2081/92

Strain	Peak area (%)														10 ME 18:0 TBSA[a]	MIS Identif. (similarity index)
	12:0	2-Me-12:0	14:0	2-Me-14:0	2,4-DiMe-14:0	15:0	16:1 B *cis* 6	16:1 *cis* 9	16:1 *cis* 10	16:1 *cis* 11	16:0	17:1	17:0	18:2 *cis* 9 12	18:1 *cis* 9	18:1 *cis* 11	18:0		
Strain 2081/92	0.47		6.27			1.57	1.61		6.14		23.31	13.44	1.40	0.34	22.41		6.78	11.09	Not in data base[b]
Strain 4185/92	0.36		6.37			2.13	2.50		6.27		25.35	8.13	1.02	0.23	22.99		7.04	14.20	Not in data base[b]
M. malmoense[c]			4.49			2.34	1.98	1.01	7.67		34.64		0.93	1.81	22.03		5.76	15.41	Not in data base[b,d]
M. kansasii[c]	0.29	0.7	3.40	0.36	4.12	0.29	0.90	2.13		5.25	38.88	0.43	0.60	0.55	30.22	0.85	4.32	5.63	*M. kansasii* (0.730)
M. simiae[c]	0.34		7.50			0.36	1.28	1.68		4.55	39.68		0.57	0.34	28.96		6.63	7.21	M. simiae (0.863)
M. scrofulaceum[c]			4.71			0.39	2.26	1.91	8.38		28.72	9.97	0.26	0.46	21.85		2.74	5.20	M. scrofulaceum (0.558)

[a] TBSA, tuberculostearic acid (10-*methyl*-octadecenoic acid).
[b] Similarity index too low for species identification.
[c] Strains were obtained from the strain collection of the Forschungsinstitut Borstel.
[d] *M. malmoense* is not included in the MIS data base.

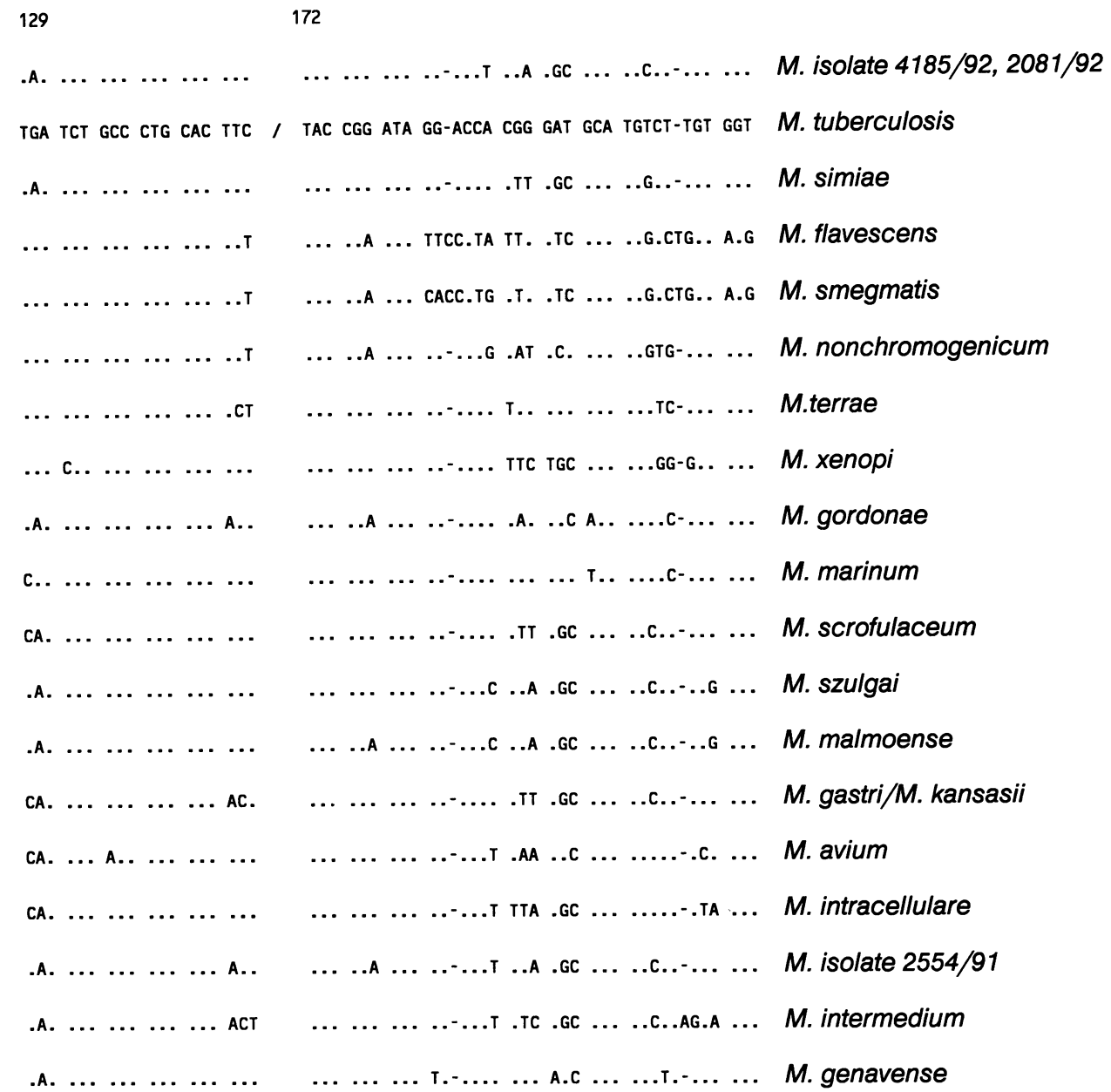

FIG. 1. Alignment of selected mycobacterial 16S rRNA sequences. *M. tuberculosis* was used as the reference sequence. Only nucleotides different from those in the *M. tuberculosis* sequence are shown; dashes indicate deletions. The respective *E. coli* 16S rRNA positions are indicated.

laceum, *M. simiae*, and *M. malmoense*. A phylogenetic tree based on 16S rRNA sequences positions the isolated mycobacterium within the slow-growing mycobacteria next to *M. simiae* (Fig. 2) with which our isolate shares a 16S rRNA sequence homology of 98.9% (Table 3).

DISCUSSION

Strains 2081/92 and 4185/92 were presumptively identified as *Mycobacterium* species by morphologic, i.e., acid-fastness, and growth characteristics. This identification was confirmed by lipid analysis and comparative 16S rRNA sequencing. Biochemical test results and whole-cell fatty acid methyl esters resembled those of *M. scrofulaceum*. The inability to grow at 22 and 41°C, a negative β-esterase test, the presence of low amounts of dodecanoic acid, and the lack of *cis*-9-hexadecenoic acid distinguished our isolates from *M. scrofulaceum*. Mycolic acid analysis by two-dimensional thin-layer chromatography showed the presence of α-, keto-, ω-carboxy-, and epoxy-mycolates, a pattern which is found in *M. avium* and *M. scrofulaceum* but differs from the patterns for most other mycobacteria which possess α-, keto-, and methoxy-mycolates. A phylogenetic tree obtained by comparative 16S rRNA sequencing revealed that strain 4185/92^T is phylogenetically related to *M. simiae* (Fig. 2). Together with *M. simiae*, strain 4185/92^T occupies an intermediate position between slow- and fast-growing mycobac-

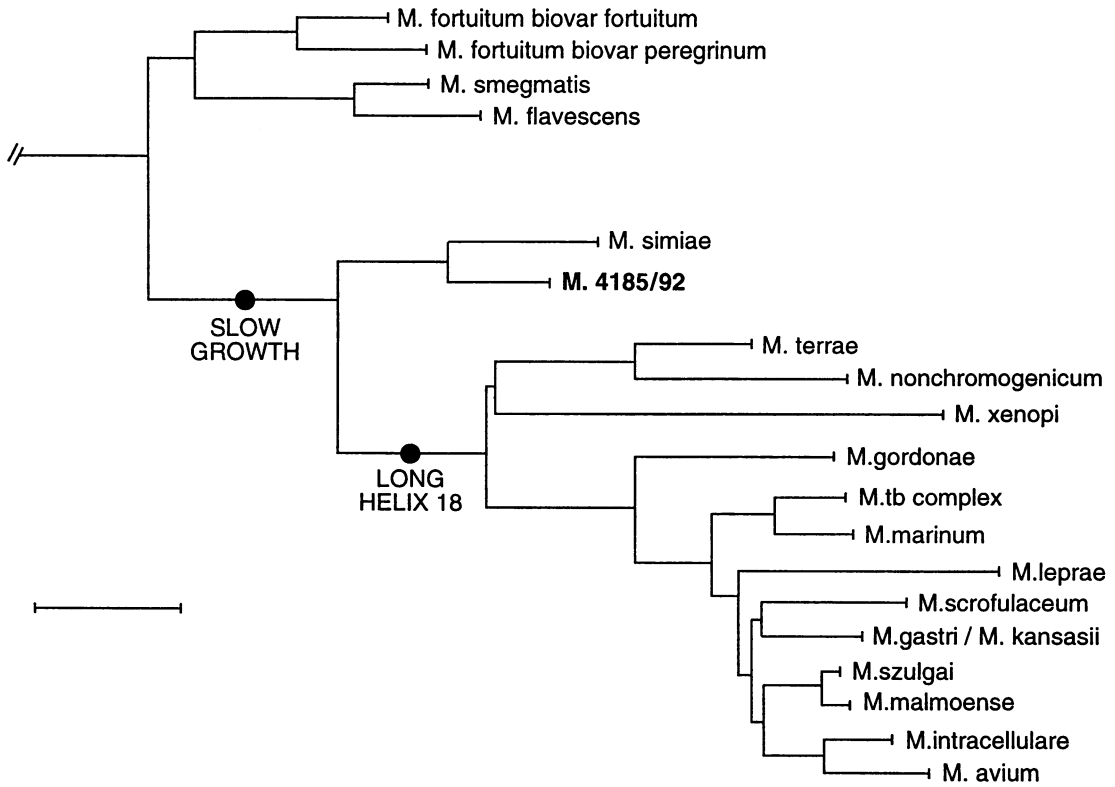

FIG. 2. Phylogenetic tree based on 16S rRNA sequences illustrating the position of strain 4185/92^T. The tree was rooted by using *Nocardia asteroides* as the outgroup. The bar indicates 10 nucleotide differences.

teria. The structural division between the slow-growing and fast-growing mycobacteria is marked by length and sequence variation within helix 18 of the molecule. An extended helix is found among members of slow-growing species, while rapidly growing mycobacteria are characterized by a short helix 18 (25, 29). Yet, a recently described and steadily expanding group of mycobacteria including *M. simiae*, *M. intermedium* (20), "*M. genavense*" (5), strain 2554/91 (7), and *M. interjectum* is characterized by slow growth at the phenotypic level and the simultaneous presence of the molecular signature of fast-growing mycobacteria, i.e., a short helix 18.

TABLE 3. Sequence homology of selected mycobacterial 16S rRNA sequences with *Mycobacterium* sp. strain 4185/92

Strain	% Homology[a]
M. tuberculosis	96.3
M. simiae	98.9
M. flavescens	96.6
M. smegmatis	96.7
M. nonchromogenicum	95.3
M. terrae	96.0
M. xenopi	94.2
M. gordonae	97.0
M. marinum	96.3
M. scrofulaceum	97.2
M. szulgai	97.2
M. malmoense	97.2
M. gastri/*M. kansasii*	96.7
M. avium	96.6
M. intracellulare	96.7
2554/91	99.0
M. intermedium	98.4
"*M. genavense*"	98.7

[a] Percent homology to strain 4185/92 is based on 1391 of the 1456 determined 16S rRNA sequence positions, after omission of the regions of alignment uncertainty.

Strains 2081/92 and 4185/92 were isolated from a child with chronic lymphadenitis, the most common manifestation of infection with nontuberculous mycobacteria in childhood. The finding of a mycobacterium in surgically obtained, primarily sterile tissue in connection with a particular clinical entity and the pathognomonic histopathologic changes strongly argues for a pathogenic role for our isolates.

With the recognition of more species of mycobacteria, it is possible that correct identification by current biochemical and phenotypic tests may become more difficult, because the biochemical reactions used for the identification of mycobacteria (23) may result in erroneous identifications (7). As demonstrated for *M. interjectum*, which phenotypically and biochemically most closely resembled *M. scrofulaceum* but was shown by 16S rDNA sequencing to represent a previously undescribed species, the standard biochemical reactions used for identification of mycobacteria may underestimate the complexity of the genus *Mycobacterium*, because genetically distinct species may exhibit similar or identical biochemical reaction patterns. Our data point to the importance of performing additional studies of a *Mycobacterium* organism that is isolated from a patient and does not precisely fit any one identification. The phenotypic homogeneity and concomitant lack of differential characteristics among members of the genus *Mycobacterium* stand in contrast to the phylogenetic diversity now recognized. Partial

16S rRNA sequence determination is an alternative rapid and convenient method for the identification of mycobacteria (15, 24) that can be implemented into routine laboratories. Indeed, automated systems for amplification, sequencing, and data analysis are now available and may form the next generation of automated microbial identification systems. In contrast to standard phenotypic and biochemical methods for identification of mycobacteria, which are laborious and time-consuming and which often give ambiguous and misleading results, direct 16S rDNA sequence determination is rapid (completion within 2 days) and results in definitive and unambiguous identification. It is particularly suited for prompt recognition and characterization of previously unrecognized species (4, 15, 16).

Description of *Mycobacterium interjectum* sp. nov. *M. interjectum* (in.ter.jec'tum, L. neut. adj. of *interjectus*, corresponding to the phylogenetic position between rapid and slow-growing mycobacteria) is an acid-alcohol-fast, rod-shaped coccobacillus (0.6 to 1.0 μm by 0.7 to 2.0 μm) which may exhibit few filaments (up to 6.0 μm). It does not form spores, a capsule, or aerial hyphae. Visible growth from a dilute inoculum requires 3 to 4 weeks; colonies on LJ agar are dysgonic, smooth, scotochromogenic, and 1 to 2 mm in diameter. Growth occurs at temperatures of between 31 and 37°C. The organism is susceptible in vitro to rifampin; it is resistant in vitro to isoniazid and ethambutol. It is positive in reactions for urease, nicotinamidase, α-esterase, pyrazinamidase, and heat-stable catalase. It is negative in reactions for niacin production, nitrate reduction, Tween hydrolysis, β-esterase, acetamidase, benzamidase, isonicotinamidase, and succinidamidase. *M. interjectum* synthesizes α-, keto-, ω-carboxy-, and epoxy-mycolates. Phylogenetic analysis, based on an evaluation of 16S rRNA sequences, places *M. interjectum* in an intermediate position between rapid and slow-growing mycobacteria. The type strain of *M. interjectum* is strain 4185/92. A culture of this strain has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 44064. It was isolated from a child with cervical lymphadenitis.

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