***Mycobacterium bohemicum* sp. nov., a new slow-growing scotochromogenic mycobacterium**

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A new, slow-growing, scotochromogenic mycobacterium was isolated from sputum of a 53-year-old patient with Down's syndrome suffering from tuberculosis. Growth occurred at temperatures between 25 and 40 °C with an optimum at 37 °C. This strain had surprisingly few enzymic activities (only positive for 68 °C heat-stable catalase and weakly positive for urease) and was sensitive to prothionamide, cycloserine, clarithromycin, gentamicin and amikacin but showed resistance to isoniazid, streptomycin, ethambutol, rifampin and ciprofloxacin. These characteristics assign this organism to a novel mycobacterial species characterized by a unique 16S rDNA nucleotide sequence. The name *Mycobacterium bohemicum* sp. nov. is proposed for this new, slow-growing, scotochromogenic mycobacterium. The type strain is DSM 44277^T.

Keywords: *Mycobacterium bohemicum* sp. nov., 16S rDNA sequence, phylogenetic tree

INTRODUCTION

For decades the term mycobacteria was used as a synonym of *Mycobacterium tuberculosis*; this was true until other mycobacteria (atypical mycobacteria or mycobacteria other than *Mycobacterium tuberculosis*) were detected. The clinical significance of the latter is difficult to estimate because some are able to infect humans, but others have never been related to disease. Atypical mycobacteria are present in the human environment (Wolinsky, 1979) and may therefore be isolated from clinical specimens. For this reason, the isolation of atypical mycobacteria represents a clinical and taxonomic problem. The clinical significance has to be assessed (Corpe *et al.*, 1963) and unambiguous microbial identification is needed. This, however, becomes more and more difficult with the increasing number of new mycobacterial species as some of these organisms may behave more or less alike when

conventional methods are used. In such cases 16S rDNA analysis can provide definite identification (Springer *et al.*, 1993). In strain DSM 44277^T presented here, the lack of most of the enzymic activities characteristic of slow-growing mycobacteria rendered it easily identifiable by standard methods and analysis of the 16S rRNA gene yielded a characteristic unique sequence.

METHODS

Mycobacterial strain, cellular features, growth temperatures and biochemical tests. Strain DSM 44277^T was isolated (Deutsches Institut für Normung, 1991; Deutsches Zentralkomitee zur Bekämpfung der Tuberkulose, 1991) from three consecutive sputum specimens. Growth was observed on Löwenstein-Jensen (LJ) medium and on 12 B medium (Kubica & Wayne, 1984).

Besides microscopic and morphological investigations, the following features were studied: pigmentation, growth at 25, 31, 37, 40 and 45 °C, growth in the presence of 5% NaCl, niacin accumulation and nitrate reduction (Goodfellow &

The GenBank accession number for the sequence of the 16S rDNA of DSM 44277^T is U84502.

Table 1. Selected criteria for the differentiation of slow-growing, pigmented mycobacteria

Data are from Goodfellow & Wayne (1982), Kent & Kubica (1985), Kubica & Wayne (1984), Runyon *et al.* (1980) and Shinnick & Good (1994). Symbols: –, less than 15 % of the isolates were positive; +, more than 85 % of the isolates were positive; v, 16–85 % of the isolates were positive; ±, usually absent or a weak reaction was possible; S, scotochromogenic; P, photochromogenic. Blank spaces indicate that the information is not currently available or that the property is unimportant.

Species	Pigmentation	Growth at (°C)					Niacin	Nitrate reduction	Semi-quantitative catalase activity	Catalase activity (68 °C)	Tolerance to 5 % NaCl	Tween hydrolysis	Aryl sulfatase (10 d)	Acid phosphatase	Amides									
		25	31	37	40	45									Acetamide	Benzamide	Urea	Nicotinamide	Pyrazinamide	Succinamide	Allantoin	β-Galactosidase activity	α-Esterase activity	β-Esterase activity
<i>Mycobacterium bohemicum</i>	S	+	+	+	+	–	–	–	–	+	–	–	–	–	–	–	±	–	–	–	–	–	–	–
<i>Mycobacterium cookii</i>	S	+	–	–	–	–	–	–	+	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
<i>Mycobacterium farcinogenes</i>	S	–	–	–	–	–	–	+	+	–	–	–	–	–	–	–	v	–	–	–	–	–	–	–
<i>Mycobacterium gordonae</i>	S	+	+	+	–	–	–	–	+	+	–	+	v	v	–	–	±	–	–	–	–	–	+	+
<i>Mycobacterium hiberniae</i>	S	+	–	–	–	–	–	+	–	–	–	v	+	–	–	–	–	–	–	–	–	–	–	–
<i>Mycobacterium interjectum</i>	S	–	–	–	–	–	–	–	+	–	–	–	v	–	–	–	+	–	–	–	–	–	–	–
<i>Mycobacterium scrofulaceum</i>	S	+	+	+	±	–	–	–	+	+	–	–	v	–	–	–	+	+	±	–	–	–	v	v
<i>Mycobacterium szulgai</i>	S	+	+	+	–	–	–	+	+	+	–	±	v	–	–	–	+	–	–	–	–	–	+	–
<i>Mycobacterium xenopi</i>	S	–	–	+	+	+	–	–	–	+	–	–	+	+	–	–	–	+	+	–	–	–	+	+
<i>Mycobacterium asiaticum</i>	P	–	+	+	–	–	–	–	+	+	–	+	+	–	–	–	–	–	v	–	–	–	+	+
<i>Mycobacterium intermedium</i>	P	–	–	–	–	–	–	–	+	–	–	+	+	+	–	–	+	–	–	–	–	+	–	v
<i>Mycobacterium kansasii</i>	P	+	+	+	+	–	–	+	+	+	–	+	±	–	–	–	+	+	±	–	–	–	+	–
<i>Mycobacterium simiae</i>	P	+	+	+	±	–	+	–	+	+	–	–	–	–	–	–	+	–	±	–	–	–	–	+

*Visible growth was noted on LJ medium after 6 weeks incubation

†Visible growth was noted on LJ medium after 4 weeks incubation.

Wayne, 1982; Kent & Kubica, 1985; Kubica & Wayne, 1984; Runyon *et al.*, 1980; Shinnick & Good, 1994). The biochemical properties examined are shown in Table 1. Tests for drug susceptibility were performed on LJ medium and were interpreted by the modified proportion method (Deutsches Institut für Normung, 1991; Deutsches Zentralkomitee zur Bekämpfung der Tuberkulose, 1991). The properties of strain DSM 44277^T and other slow-growing, pigmented mycobacteria are compared in Table 1.

Culture conditions for fatty acid analysis. Strain DSM 44277^T was cultivated on Middlebrook 7H10 agar (DSM medium No. 645; Deutsche Sammlung von Mikroorganismen und Zellkulturen, 1993) at 37 °C for 1 week. To obtain sufficient cell material for analysis of chemical markers, the strain was grown in GPHF liquid medium (DSM medium 553; Deutsche Sammlung von Mikroorganismen und Zellkulturen, 1993).

Analyses of cell wall amino acids and sugars. Amino acids and sugars of whole-cell hydrolysates were analysed by TLC as described by Stanek & Roberts (1974).

Extraction and analysis of isoprenoid quinones. Isoprenoid quinones were extracted and purified by the small-scale integrated procedure described by Minnikin *et al.* (1984). Once menaquinone was isolated and lyophilized, the preparation was dissolved in 200 µl 2-propanol. Portions (1–10 µl) were separated by HPLC without further purification using an RP-18 column (250 × 4 mm) filled with Lichrospher 100 (particle size, 5 mm). The column temperature was kept at 40 °C. Acetonitrile/2-propanol (65:35, v/v) was used as mobile phase. The flow rate was 1 ml min⁻¹ (Kroppenstedt, 1985; Kroppenstedt *et al.*, 1981).

Extraction and analysis of fatty acids. Fatty acid methyl esters were obtained from 40 mg (wet wt) of cells by saponification, methylation and extraction using minor modifications (Kuykendall *et al.*, 1988) of the method of Miller (1982). The fatty acid methyl ester mixtures were analysed by using a model 5898A Microbial Identification System (Microbial ID). Peaks were automatically integrated and fatty acid names and percentages were determined by the Microbial Identification System standard software (Microbial ID). GC parameters were as follows: the carrier gas was ultrahigh purity hydrogen; column head pressure, 60 kPa; injection volume, 2 µl; column split ratio, 100:1; septum purge, 5 ml min⁻¹; column temperature increased from 170 to 270 °C at a rate of 5 °C min⁻¹; injection port temperature, 250 °C; detector temperature, 300 °C.

Extraction and analysis of mycolic acids. Freeze-dried bacteria (50 mg) were degraded by treatment at 75 °C with 3 ml of a methanol/toluene/sulphuric acid mixture (30:15:1, by vol.) for 16 h (Minnikin *et al.*, 1980). Long-chain components were extracted with hexane and traces of acid were removed by passage through a short column of ammonium hydrogen carbonate. The hexane extracts were examined by TLC and two-dimensional chromatography as described previously (Schröder *et al.*, 1997).

Determination of the DNA G + C content. DNA was isolated by using procedures described by Visuvanathan *et al.* (1989). Approximately 20 µg DNA was hydrolysed and dephosphorylated as described by Mesbah *et al.* (1989). Nucleosides were separated by reversed-phase HPLC as described previously (Schröder *et al.*, 1997) using the solvent system of Tamaoka & Komagata (1984). The retention times of the nucleosides were determined with synthetic compounds.

Non-methylated λ phage DNA with a G + C content of 49.858 mol% (Sanger *et al.*, 1982) was used as a calibration reference. The G + C contents were calculated automatically with a Shimadzu integrator.

PCR-based amplification of 16S rDNA. A single colony of the mycobacterial strain was collected from LJ medium and chromosomal DNA was extracted as previously described (Reischl *et al.*, 1994). A 1 µg portion of bacterial DNA was subjected to PCR with each of the following primer pairs: M285 and M264 (Kirschner *et al.*, 1993) to obtain a 1030 bp amplification product of the 16S rDNA 5' end; R247 (5' GTAGTCCACGCCGTAAACGG 3') and M261 (Kirschner *et al.*, 1993) to obtain an approximately 760 bp amplification product of the 16S rDNA 3' end. Broad-range eubacterial primers M285 and M261 were combined with genus-specific primers M264 and R247 to produce a 246 bp overlap between the two amplicons. Standard amplification mixtures were incubated at 95 °C for 2 min, 68 °C for 1 min, 95 °C for 1 min, 68 °C for 1 min and 95 °C for 1 min, followed by 35 cycles of the following temperature profile: 95 °C for 40 s, 55 °C for 40 s and 72 °C for 1.5 min. The PCR mixtures were extracted with phenol and subjected to agarose gel electrophoresis. Extraction and purification of the amplicons were performed with a QiaEx-II purification kit (Qiagen).

DNA sequencing. Cycle sequencing reactions of the 16S rDNA amplicons were carried out as described in the PRISM Ready Reaction Dye Deoxy Terminator Cycle Sequencing Kit protocol (Applied Biosystems). Optimal results were obtained with the following forward and reverse sequencing primers: M285 (Kirschner *et al.*, 1993) and new primer R928 (5' CCTTTGAGTTTTAGCCTTGC 3') for the 1030 bp amplicon; new primer R249 (5' GGATCCGTGCCGTAGCTAACGC 3') and M261 (Kirschner *et al.*, 1993) for the 760 bp amplicon. Both strands of the specific amplicons originating from different amplification reactions were sequenced in duplicate to rule out the possibility of *Taq* DNA polymerase-induced errors. The fluorescence-labelled reaction products were analysed with a model 373A automated DNA sequencer (Applied Biosystems). The interpretation of the results was accomplished with the SeqEd 1.03 software package (Applied Biosystems).

Sequence and phylogenetic analysis. The sequence of the new strain was aligned with 30 16S rDNA reference sequences from closely related mycobacterial species by using Genetic Data Environment software version 2.2 (Larsen *et al.*, 1993). No gaps were removed, while probable sequencing errors within the reference sequences were not weighted. A phylogenetic tree was constructed by using the neighbour-joining method (Saitou & Nei, 1987), applied to distances corrected for multiple hits and for unequal transition and transversion rates according to Kimura's two-parameter model (Kimura, 1980), thus omitting parts of uncertain alignment at both ends of the gene. Tree positions were confirmed by parsimony analysis; bootstrapping was not performed due to the high degree of similarity of mycobacterial 16S rDNA.

RESULTS

Microscopy and culture

Strain DSM 44277^T showed acid-fastness like other slow-growing mycobacteria (Goodfellow & Wayne, 1982; Kubica & Wayne, 1984; Runyon *et al.*, 1980).

Table 2. Fatty acids of *Mycobacterium bohemicum*

Fatty acid	Area (%)
14:0	2.55
15:0	0.53
16:1 <i>cis</i> 9	1.60
16:1 <i>cis</i> 10	6.13
16:0	26.93
16:1 <i>cis</i> 7	2.15
17:0	0.52
18:1 <i>cis</i> 9	24.42
18:0 10-methyl	7.48
18:2	0.39
20:0	0.67
18:0 alcohol	3.46
20:0 alcohol	20.34

The strain was scotochromogenic and grew at 25–40 °C within 4–6 weeks after inoculation. At 37 °C, the colonies were about 1–2 mm in diameter. Temperatures between 37 and 40 °C seemed to favour growth.

The strain did not grow on NaCl. In general, enzymic

activity was remarkably low; no accumulation of niacin, no reduction of nitrate, no hydrolysis of Tween 80 and no arylsulfatase and acid phosphatase activities were observed. In contrast, catalase activity at 37 °C (43 mm) was detected and was not inactivated by heating to 68 °C. No benzamidase, acetamidase, nicotinamidase, pyrazinamidase, succinamidase and allantoinamidase activities were observed. The activities of β -esterase, α -esterase and β -galactosidase were negative while urease activity was weak.

The strain was susceptible to cycloserine (16 mg l⁻¹), prothionamide (32 mg l⁻¹), clarithromycin (2 mg l⁻¹), gentamicin (2 mg l⁻¹) and amikacin (2 mg l⁻¹), but resistant to isoniazid (1 mg l⁻¹), rifampin (32 mg l⁻¹), streptomycin (8 mg l⁻¹), ethambutol (2 mg l⁻¹) and ciprofloxacin (4 mg l⁻¹).

Chemotaxonomic analyses

The cell walls of strain DSM 44277^T contained arabinose and galactose as major cell wall sugars; *meso*-diaminopimelic acid was the only diamino acid. MK-9(H₂) was the predominant menaquinone and small amounts of MK-8(H₂) and MK-10(H₂) were also detected. Analysis of the fatty acids from whole-cell

Region A

(129)	(177)	
5' TGA TCT GCC CTG CAC TTC	CGG ATA GG-ACCA CGG GAT GCA TGT CT- TGT GGT GGA	<i>M. tuberculosis</i>
5' CA. A.	 T. AA . C C.	<i>M. avium</i>
5' .A.	 T TTA .GC TA	<i>M. intracellulare</i>
5' CA. AC.	 TT .GC C	<i>M. kansasii</i>
5' .A.	.A C .A .GC G	<i>M. malmoense</i>
5' CA.	 TT .GC C GG- G.	<i>M. scrofulaceum</i>
5' . . . C.	 TTC TGC GG- G.	<i>M. xenopi</i>
5' .A.	 TT .GC C	<i>M. simiae</i>
5' .A.	 T .A .GC C	<i>M. interjectum</i>
5' T	 T. .C .C G TG- . . . A.	<i>M. farcinogenes</i>
5' CA.	 T. . . . T .A .GC C	<i>M. bohemicum</i>

Region B

(533)	
5' CCT CTT TCA CCA TCG ACG AAG G-- TC CGG GTT CTC TCG GAT TGA CGG TAG GTG GAG AAG AAG CAC CGG	<i>M. tuberculosis</i>
5' T.	<i>M. avium</i>
5' T.	<i>M. intracellulare</i>
5' T.	<i>M. kansasii</i>
5' T.	<i>M. malmoense</i>
5' CT -- -AC T.T GT. .G.	<i>M. scrofulaceum</i>
5' G.C C-- .G T. TGG G. C.	<i>M. xenopi</i>
5' G. GG. C-- G. A-- G C C. C.	<i>M. simiae</i>
5' G. GG. C-- G. A-- G C C. C.	<i>M. interjectum</i>
5' AT. GG. C-- G. A-- G C C.A T.	<i>M. farcinogenes</i>
5' G. T.	<i>M. bohemicum</i>

Fig. 1. Comparison of 16S rDNA signature sequences (hypervariable regions A and B; Kirschner *et al.*, 1993) of selected species of the genus *Mycobacterium* including *Mycobacterium bohemicum*. Only nucleotides different from *Mycobacterium tuberculosis* are shown; dashes indicate deletions. The corresponding positions of the *E. coli* 16S rRNA gene are shown in parentheses for reference. The GenBank accession numbers for sequences are as follows: *Mycobacterium tuberculosis*, X52917; *Mycobacterium avium*, X52918; *Mycobacterium intracellulare*, X52927; *Mycobacterium kansasii*, X15916; *Mycobacterium malmoense*, X52930; *Mycobacterium scrofulaceum*, X52924; *Mycobacterium xenopi*, X52929; *Mycobacterium simiae*, X52931; *Mycobacterium interjectum*, X70961; *Mycobacterium farcinogenes*, X55592; *Mycobacterium bohemicum*, U84502.

extracts revealed a pattern of straight-chain, saturated and unsaturated fatty acids along with significant amounts of tuberculostearic acid (10-methyloctadecanoic acid). Two alcohols, octadecanol and eicosanol were also present (Table 2). Strain DSM 44277^T could not be precisely identified on the basis of its fatty acid pattern by using the MIDI mycobacterial database. However, the fatty acid pattern resembled a species of the *Mycobacterium avium*–*intracellulare*–*scrofulaceum* (MAIS) complex.

One-dimensional TLC of whole-organism acid methanolsates of strain DSM 44277^T indicated that this organism synthesizes mycolic acids that include α -mycolates and wax esters. Two-dimensional TLC resulted in a more precise separation of the components, especially of the α -mycolmethoxyketo-wax esters and the ketomycolates, but only α -mycolates and alcohols could be detected.

G + C content

The G + C content of strain DSM 44277^T was 63.5 mol %.

16S rRNA sequence analysis

An almost complete nucleotide sequence of the gene encoding the small 16S rDNA subunit was determined. When compared to all publicly available mycobacterial 16S rDNA sequences in the GenBank sequence database, the sequence of strain DSM 44277^T was found to be unique. Detailed examination of the 16S rDNA sequence demonstrated complete homology in genus-specific regions, but revealed a number of differences in species-specific regions (Fig. 1). Thus, the sequence showed the characteristics of a slow-growing mycobacterium with a long stretch in helix 18 (Springer *et al.*, 1996). With regard to the variable region in helix 10, the sequence clustered with the *Mycobacterium avium*–*intracellulare* complex and other slow-growers such as *Mycobacterium szulgai* and *Mycobacterium kansasii*. However, significant differences within this region allow easy discrimination of the novel species.

Phylogenetic analysis

Comparing the novel 16S rDNA sequence to related 16S rDNA sequences from well established mycobacterial taxa, the phylogenetic position of the novel species could be determined (Kirschner *et al.*, 1993; Rogall *et al.*, 1990). A phylogenetic tree inferred from the results of the sequence comparisons is shown in Fig. 2. Some landmarks on this tree have been shown in a previously published study on mycobacterial phylogeny (Rogall *et al.*, 1990). When calculating the homology score between all similar mycobacterial 16S rDNA sequences, *Mycobacterium kansasii* ranked at the top (Fig. 2).

Fig. 2. Phylogenetic tree indicating the relationship of *Mycobacterium bohemicum* to selected mycobacterial species. The tree was inferred using the neighbour-joining method applied to distances corrected for multiple hits and for unequal transition and transversion rates by the Kimura's two parameter model. The tree was rooted by using *Nocardia asteroides* as an outgroup.

DISCUSSION

When detecting acid-fast bacilli in human specimens, it is of clinical and epidemiological importance to determine whether they belong to the highly pathogenic *Mycobacterium tuberculosis* complex. In the case of atypical mycobacteria, this finding often raises the question of real infection or colonization which often depends on the host's immune status; an answer to this question is needed since some atypical mycobacteria may be of clinical importance. In that situation, an unambiguous identification of the isolate may be necessary. Strain DSM 44277^T was isolated from consecutive sputum specimens from a 53-year-old patient with Down's syndrome who suffered from tuberculosis. The identification of an atypical mycobacterium isolated from a patient with known infection with *Mycobacterium tuberculosis* is crucial as it may have impact on treatment considerations.

Mycobacterium bohemicum is not readily distinguishable from other mycobacteria by conventional biochemical tests, as shown in Table 1, and is characterized by remarkably low enzymic activity. Within the extended panel of enzyme reactions tested, only heat-stable catalase and weak urease activities were recorded, while tests for thermolabile catalase were negative, thus allowing the discrimination of *Myco*-

bacterium bohemicum from other slow-growing scotochromogenic or photochromogenic mycobacteria.

Within the group of slow-growing mycobacteria, only *Mycobacterium haemophilum* has, as yet, a similar 'poor' spectrum of biochemical activities; since it is non-photochromogenic, it can be easily distinguished from *Mycobacterium bohemicum* visually. Among the rapid growers, the recently reported *Mycobacterium hassiacum* (Schröder *et al.*, 1997) also shows a similar spectrum of biochemical activities; however, its thermophilic properties (rapid growth even at 65 °C) render it clearly different from the strain presented here.

With regard to HPLC analysis, the strain shows typical features which are characteristic for all species of the genus *Mycobacterium*. These include *meso*-diaminopimelic acid and arabinose plus galactose in whole-cell hydrolysates, quinone MK-9(H₂), a fatty acid pattern composed of unbranched saturated and unsaturated fatty acids plus tuberculostearic acid and mycolic acids having a chain length of about 90 carbon atoms. The occurrence of two alcohols, 18:0 and 20:0, in the fatty acid pattern (free of wax esters) places the strain into a cluster of species which also includes *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum*, *Mycobacterium terrae* and *Mycobacterium phlei*.

Sequence analysis of the 16S rRNA gene is a reliable method for mycobacterial identification and has proven its potential in the detection of new species (Böddinghaus *et al.*, 1990; Kirschner *et al.*, 1993; Schröder *et al.*, 1997; Springer *et al.*, 1993, 1996). Furthermore, dysgonic species such as *Mycobacterium genavense* could be misidentified without sequence analysis (Haas *et al.*, 1993). However, some other species are easily distinguished by conventional procedures but cannot be differentiated at the 16S rDNA level: *Mycobacterium tuberculosis* and *Mycobacterium bovis*, and *Mycobacterium kansasii* and *Mycobacterium gastri* share the same 16S rDNA sequence while species such as *Mycobacterium malmoense* and *Mycobacterium szulgai* show a very high 16S rDNA sequence similarity of 99.9% (Rogall *et al.*, 1990).

Primers targeting the genus-specific regions of the 16S rDNA can be used to amplify the entire 16S rRNA gene of about 1500 bp of any mycobacterium (Böddinghaus *et al.*, 1990; Springer *et al.*, 1996). Overall 16S rDNA homology in mycobacteria exceeds 90%. Therefore, sequencing errors might easily lead to the description of 'novel species'. However, the finding of a novel signature sequence in the so-called variable regions for mycobacteria (position 129–266, *Escherichia coli* numbering; Kirschner *et al.*, 1993) together with distinct phenotypical properties leads to the assumption that a new species has been detected. Characteristic mismatches of the *Mycobacterium bohemicum* 16S rDNA signature sequence to the sequences of similar species, such as *Mycobacterium*

kansasii, *Mycobacterium avium*, *Mycobacterium intracellulare* or *Mycobacterium szulgai*, may therefore provide a target for specific DNA probes or other nucleic-acid-based diagnostic procedures for its specific recognition (Fig. 1).

Description of *Mycobacterium bohemicum* sp. nov.

Mycobacterium bohemicum (bo.he'mi.cum. M.L. adj. *bohemicum* referring to the Czech Republic where the organism was first isolated).

Rod-shaped cells show acid-alcohol fastness. Colonies grown on LJ medium are scotochromogenic and 1–2 mm in diameter. Grows at temperatures between 25 and 40 °C and does not grow on NaCl. Susceptible to cycloserine, gentamicin, amikacin, prothionamide and clarithromycin, but resistant to isoniazid (1 mg l⁻¹), rifampin (32 mg l⁻¹), streptomycin (8 mg l⁻¹), ethambutol (2 mg l⁻¹) and ciprofloxacin (4 mg l⁻¹). Positive reactions for heat-stable catalase (68 °C) and urease. Urease activity is weak. Negative reactions for niacin, nitrate reductase, arylsulfatase (10 d), acid phosphatase, hydrolysis of Tween 80, acetamidase, benzamidase, nicotinamidase, pyrazinamidase, succinamidase and allantoinamidase. Strain does not show α -esterase, β -esterase or β -galactosidase activities. Cell wall contains arabinose and galactose as major sugars and *meso*-diaminopimelic acid is the only diamino acid. MK-9(H₂) is the predominant menaquinone and small amounts of MK-8(H₂) and MK-10(H₂) are also found. Analysis of the fatty acids from the whole cells reveals a pattern of straight-chain, saturated and unsaturated fatty acids along with significant amounts of tuberculostearic acid. The strain cannot be precisely identified on the basis of its fatty acid pattern by using the MIDI mycobacterial database, but resembles species of the MAIS complex. TLC of mycolic acid methanolysates indicates the presence of α -mycolates and wax esters. Only α -mycolates and alcohols are detected by two-dimensional TLC. Combined results from HPLC and conventional biochemical testing are supportive of a novel species. Conclusive genetic evidence of a novel species has been demonstrated with 16S rDNA analysis. The unique sequence has molecular signatures of slow-growing mycobacteria and *Mycobacterium bohemicum* seems to be phylogenetically near to the slow-growing *Mycobacterium kansasii*, *Mycobacterium gastri*, *Mycobacterium szulgai* and *Mycobacterium avium*-*intracellulare* complex. Type strain is DSM 44277^T (deposited in the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

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