

Mycobacterium holsaticum sp. nov.

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Strains of a novel species of the rapidly growing mycobacteria, *Mycobacterium holsaticum* sp. nov., were isolated from various clinical specimens. The isolates grew at 22–40 °C, were positive for nitrate and tellurite reduction, had phosphatase, urease, nicotinamidase and pyrazinamidase activities, were resistant to isoniazid and rifampin and were susceptible to streptomycin and ethambutol. Analyses of the 16S rRNA gene and a fragment of the heat-shock protein gene *hsp65* revealed unique nucleotide sequences. A phylogenetic analysis based on the comparison of the 16S rDNA sequence with that of other mycobacterial species allocated the strain to the rapidly growing mycobacteria. A conspicuous characteristic of the novel species is the similarity of the species-specific sequence of the 16S rRNA gene to the sequence of the *Mycobacterium tuberculosis* complex, resulting in a cross-reaction with the AccuProbe for the *M. tuberculosis* complex when performed with a 5 min selection step. The type strain of the novel species is strain 1406^T (= DSM 44478^T = CCUG 46266^T). Another strain of *M. holsaticum* sp. nov., strain 5050, which differed in the internal transcribed spacer sequence, was deposited as DSM 44479 (= CCUG 46267).

Keywords: *Mycobacterium holsaticum* sp. nov., 16S rRNA gene, internal transcribed spacer (ITS), heat-shock protein (*hsp65*), AccuProbe

INTRODUCTION

In recent years, the number of non-tuberculous mycobacteria (NTM) isolated from clinical specimens has increased greatly, partly due to opportunistic infections accompanying immunosuppression, but also as a result of improved culture and identification techniques (Hanna *et al.*, 1999). On the other hand, an increasing number of mycobacterial species that exert very similar if not identical biochemical characteristics renders identification by classical methods more and more ineffective. The use of molecular methods such as DNA sequencing of the 16S rRNA gene overcomes these deficiencies, since most mycobacterial species are characterized by a unique 16S rRNA gene sequence (Harmsen *et al.*, 1999; Kirschner *et al.*, 1993; Springer

et al., 1996). However, one consequence of 16S rDNA sequence determination in a reference laboratory is the finding of 16S rDNA sequences not allocated to validly described species (Tortoli *et al.*, 2001). Based on these 'new' sequences, the assumption can be drawn of a novel, so far undescribed mycobacterial species, which can be confirmed by additional biochemical characteristics and molecular features that differentiate these isolates from established species.

During recent years, several rapidly growing mycobacterial strains were collected at the German National Reference Centre for Mycobacteria. A group of isolates was characterized by an identical 16S rDNA sequence, which was so far not released to nucleotide databases. In this study, we describe the phenotypic and genetic characteristics of these strains and propose a novel species, *Mycobacterium holsaticum* sp. nov.

METHODS

Strains. A total of nine mycobacterial strains characterized by an identical signature sequence of the 16S rRNA gene was included in this study. They were isolated from clinical specimens [sputum ($n = 6$), urine (1), gastric fluid (1),

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Abbreviations: ITS, internal transcribed spacer; LJ, Löwenstein–Jensen; TCH, thiophene-2-carboxylic acid hydrazide; TSC, thiosemicarbazone.

The EMBL accession numbers for the 16S rRNA, ITS1 and part of the *hsp65* gene sequence of strain DSM 44478^T are AJ310467–AJ310469. The EMBL accession number for the ITS1 sequence of strain DSM 44479 is AJ310470.

unknown (1)]. All strains were isolated from different patients and originated from different regions in Germany. In the scope of this study, multiple isolates from one patient were not recorded. Strain 1406^T (= DSM 44478^T = CCUG 46266^T) was isolated from sputum and strain 5050 (= DSM 44479 = CCUG 46267) was isolated from urine.

Colony morphology, growth requirements and biochemical characterization. Colony morphology, pigmentation and the ability to grow at various temperatures (22, 31, 37, 40 and 45 °C) on Löwenstein–Jensen (LJ) slants were determined and the following biochemical and physiological tests were performed on all nine strains: nitrate and tellurite reduction, hydrolysis of Tween 80, iron uptake, degradation of *p*-aminosalicylic acid, growth on MacConkey agar (without crystal violet) and on LJ slants in the presence of sodium chloride (5%), thiophene-2-carboxylic acid hydrazide (TCH, 2 µg ml⁻¹) and thiosemicarbazone (TSC, 0.5, 1 and 8 µg ml⁻¹) (Deutsches Institut für Normung, 1996; Kent & Kubica, 1985; Szabo & Vandra, 1963; Wayne *et al.*, 1974). In addition, activities of arylsulfatase, acetamidase, benzamidase, urease, nicotinamidase, phosphatase, pyrazinamidase, allantoinamidase, succinamidase and both heat-stable and semi-quantitative catalase were recorded (Bönicke, 1961; Kent & Kubica, 1985; Kubica & Pool, 1960; Marks & Trollope, 1960). Growth on LJ slants in the presence of isoniazid (0.25 and 1.0 µg ml⁻¹), streptomycin (4.0 and 8.0 µg ml⁻¹), ethambutol (1.0 and 2.0 µg ml⁻¹), rifampin (16 and 32 µg ml⁻¹), protionamide (16 and 32 µg ml⁻¹) and ofloxacin (0.5, 1, 2 and 4 µg ml⁻¹) was determined (Deutsches Institut für Normung, 1996).

Lipid analyses. Mycolic acids were analysed by TLC (Minnikin, 1993; Minnikin *et al.*, 1984) and cellular fatty acids by GLC (Luquin *et al.*, 1991) from two strains. Samples were processed as described elsewhere (Brown *et al.*, 1999).

AccuProbe assays. AccuProbe assays (Gen-Probe) for the *Mycobacterium tuberculosis* complex were performed according to the manufacturer's instructions, except that the selection step was 5 min instead of 10 min. In addition, AccuProbe assays for the *M. tuberculosis* complex were performed with a 10 min selection step, as recommended, from five strains.

Measurement of G + C content of DNA. Mycobacteria were cultured on LJ slants. DNA extraction, purification and degradation and G + C content determination by HPLC were performed as described by Mesbah *et al.* (1989), except that a Waters 486 tuneable absorbance detector plus a Waters 746 data module (Millipore/Waters) were used.

Preparation of DNA. Strains were grown on LJ slants or in liquid medium (BACTEC 460TB 12B medium). From solid media, one loopful of bacteria was suspended in distilled water, sonicated for 15 min and boiled in a waterbath for 15 min. The suspension was used directly for PCR. If cells grew in liquid medium, 2 ml culture medium were centrifuged (12000 g, 10 min, room temperature) and the pellet was resuspended in distilled water, centrifuged again and finally resuspended in 100 µl distilled water prior to sonication and heating.

Nucleic acid analyses. For identification of mycobacteria, amplification of the 5' part of the 16S rRNA gene using primers A (Edwards *et al.*, 1989) and 264 (Bödinghaus *et al.*, 1990) and additional sequencing of the hypervariable regions A and B (Kirschner *et al.*, 1993) was performed from all nine isolates. Based on identical sequences resulting from this determination, four strains were chosen for complete

16S rRNA analysis. For this, amplification of an approximately 2100 bp DNA fragment comprising the entire 16S rRNA gene and the internal transcribed spacer (ITS) was performed using primers targeting the promoter sequence of the rRNA operon (primer P2; 5'-GTGTTGTTGAG-AACTCAATAGT-3') and the 5'-region of the 23S rRNA gene (primer ITS2; Richter *et al.*, 1999). The complete PCR product was sequenced on an automated DNA sequencer (ABI 377; Applied Biosystems) by cycle sequencing using the Big Dye RR Terminator cycle sequencing kit (Applied Biosystems) according to the manufacturer's instructions, using a set of internal primers [forward primers 14 (5'-CGAGTGGCGAACGGGTGAGTAACA-3'), 10 (5'-GAGCTCGTAGGTGGTTTGTGTC-3') and 19 (5'-TTACCTGGGTTTGACATG-3'); reverse primers 4 (5'-GCCGTATCTCAGTCCCAGTG-3'), 247 and 264 (Bödinghaus *et al.*, 1990)] and the PCR primers. The resulting sequences were aligned and compared to the sequences of the International Nucleotide Sequence Database Collaboration (Altschul *et al.*, 1997).

The ITS sequence was analysed from the remaining five isolates for which the 16S rDNA sequence was not completely determined. For amplification, primers were used that targeted the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene, as described previously (Richter *et al.*, 1999). Both PCR primers were also used as sequencing primers. The resulting sequences were aligned and compared to the sequences of the International Nucleotide Sequence Database Collaboration.

Amplification of part of the heat-shock protein gene *hsp65* of all nine strains was performed according to Telenti *et al.* (1993). The resulting PCR product was sequenced using the PCR primers and analysed for restriction sites as well as used for RFLP analysis following digestion with the restriction enzymes *Bst*EII and *Hae*III (Telenti *et al.*, 1993).

Sequence and phylogenetic analyses. The resulting 16S rDNA sequence was added to the rDNA sequence database of the Technical University Munich (release December 1998) using the program package ARB (Strunk *et al.*, 2001). The tool ARB_ALIGN was used for automatic sequence alignment. The resulting alignments were checked and corrected manually, considering the secondary structure of the rRNA molecule. The ARB database was supplemented by importing several 16S rDNA sequences from mycobacteria. Tree topologies were evaluated by performing maximum-parsimony, neighbour-joining and maximum-likelihood analysis. Sequences were used for treeing only if they were at least 90% complete. Alignment positions at which fewer than 50% of the sequences of the entire dataset shared the same residue were excluded from the calculations.

RESULTS AND DISCUSSION

Microscopy and phenotypic characteristics

Cells of the novel isolates were acid–alcohol-fast and appeared as short or coccoid rods. Colony morphology was dependent on the incubation temperature: at 22 °C, the colonies were very dysgonic and transparent, whereas at higher temperatures, the strains grew in smooth, moist, shiny, off-white to yellow-pigmented colonies within 7 days at temperatures up to 40 °C. All of the novel strains were homogeneous in their biochemical properties: they were positive for nitrate and tellurite reduction, tolerated 5% NaCl, 2 µg TCH ml⁻¹ and 8 µg TSC ml⁻¹ and exhibited

Table 1. Differential characteristics of *M. holsaticum* sp. nov. and selected rapidly growing mycobacteria

Common characters for all taxa: resistance to isoniazid and rifampin, positive 8-day tellurite reduction, growth at 31 and 37 °C, presence of urease, nicotinamidase and pyrazinamidase and presence of α -mycolic acids. Data for reference taxa were taken from Wayne & Kubica (1986). +, Positive; +/-, variable; -, negative; ND, not determined; PAS, *p*-aminosalicylic acid.

Characteristic	<i>M. holsaticum</i>	<i>M. fortuitum</i>	<i>M. chelonae</i>	<i>M. smegmatis</i>
Pigmentation	+/-	-	-	+
Growth at:				
22 °C	+	+	+	ND
40 °C	+	-*	-	+
45 °C	-	-	-	+
Growth on MacConkey	-	+	+	-
Nitrate reduction	+	+	-	+
Tellurite reduction 3 days	-	+	+	+
Tween hydrolysis	+/-	-	-	-
Iron uptake	-	+	-	+
PAS degradation	-	-	+	-
NaCl tolerance	+	+	-	+
TSC tolerance	+	ND	ND	ND
Arylsulfatase	-	+	+	-
Phosphatase	+	+	+	-
Amidases:				
Acetamidase	-	+	+	+
Benzamidase	-	-	-	+
Allantoinamidase	-	+	-	+
Succinamidase	-	-	-	+
Catalase (68 °C)	-	+	-	-
Mycolic acids	methoxy, keto	α' , epoxy, methoxy	α'	α' , epoxy
Susceptibility to:†				
Streptomycin	S	R	R	S
Ethambutol	S	R	R	S
Protionamide	S	R	R	R
Ofloxacin	S	S	R	S

* Poor growth is sometimes observed.

† R, Resistant; S, susceptible.

phosphatase, urease, nicotinamidase and pyrazinamidase activities. Some isolates were weakly positive for Tween 80 hydrolysis. The strains did not take up iron or degrade *p*-aminosalicylic acid. Arylsulfatase, acetamidase, benzamidase, allantoinamidase, succinamidase and stable catalase activities were leaking. They did not grow on MacConkey agar. The strains were resistant to isoniazid and rifampin, but susceptible to streptomycin, ethambutol, protionamide and ofloxacin. Characteristics that differentiate the novel isolates from other rapidly growing mycobacteria often isolated are summarized in Table 1.

Lipid analysis

Major mycolic acids were α -, methoxy- and keto-mycolic acids. Cellular fatty acids ranged from C10:0 to C20:0, the major components being C16:0 (24.7%) and C18:1 ω 9c (26.4%). Minor components were C10:0 (< 1%), C11:0 (< 1%), C12:0 (1.8%), C14:0 (4.1%), C15:0 (< 1%), C16:1 ω 9c (4.2%), C16:1 ω 7c

(2.2%), C16:1 ω 6c (5.1%), C17:1 ω 7c (7.4%), C17:0 (< 1%), C18:2 ω 6,9c (< 1%), C18:1 ω 7c (< 1%), C18:0 (3.3%), tuberculostearic acid (10Me-18:0; 5.8%) C20:0 ALC (12.7%) and C20:0 (< 1%).

G + C content of DNA

The G + C content of the DNA was 68.4 mol %.

16S rRNA sequence analysis

Analysis of the complete 16S rRNA gene of the novel isolates, representing *M. holsaticum* sp. nov., revealed a unique sequence. In the International Nucleotide Sequence database, there was no entry with an identical sequence. The 16S rRNA gene of *M. holsaticum* sp. nov. is characterized by a short helix in the hypervariable region 18, a characteristic of rapidly growing mycobacteria. With regard to the mycobacterial species-specific region V2 of helix 10, the sequence of *M. holsaticum* sp. nov. is remarkably similar

	176
<i>M. tuberculosis</i>	CCGGATAGGA CCACGGGATG CATGTCTTGT GGTGGAAAGC GCTTTAGCGG
<i>M. holsaticum</i>	...A..... .T..... -T.....
<i>M. celatum</i>T..... -T...T....

Fig. 1. Species-specific region V2 of helix 10 of the 16S rRNA genes of *M. tuberculosis*, *M. holsaticum* sp. nov. and *M. celatum*. Numbering corresponds to the *Escherichia coli* 16S rRNA gene. For *M. holsaticum* and *M. celatum*, only bases that differ from those of *M. tuberculosis* are given; dots indicate identity with *M. tuberculosis*, deletions are marked by dashes.

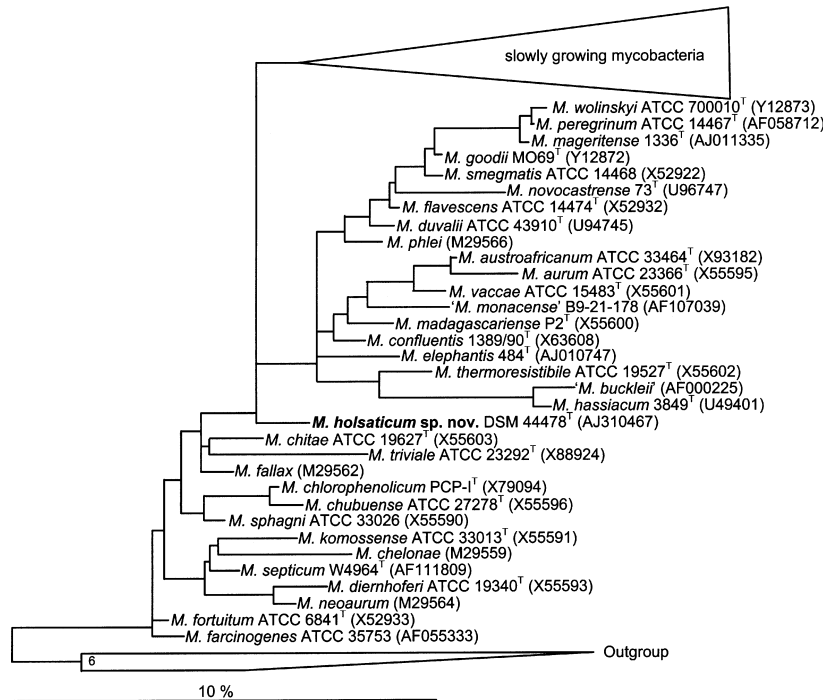


Fig. 2. Maximum-likelihood tree based on 1427 positions of nearly full-length 16S rRNA sequences derived from 200 *Mycobacterium* species. The dendrogram of slowly growing mycobacteria was condensed. Selected sequences from Gram-positive organisms with high G+C content were taken to root the tree. Trees constructed with other tree reconstruction algorithms (neighbour-joining and parsimony) generally resulted in the same overall tree topology. Bar, 10% estimated sequence divergence.

to the sequences of *M. tuberculosis* and *Mycobacterium celatum*, differing in only a few bases (Fig. 1).

Phylogenetic analysis

Phylogenetic sequence analysis allocated the species into the group of rapidly growing mycobacteria. Trees were reconstructed only on nearly full-length sequences (> 1300 bases) using maximum-parsimony, neighbour-joining and maximum-likelihood analyses in combination with different filters. The tree shown in Fig. 2 is based on maximum-likelihood analysis and was corrected by taking into consideration the different results of various tree-reconstruction algorithms. Bifurcations indicate branching that appeared stable by all three methods and were well separated from neighbouring branches in all cases. Multifurcations indicate tree topologies that could not be resolved significantly on the basis of the dataset available.

Sequence analysis of the ITS

Interspecific polymorphism of the 16S–23S rRNA ITS can be used for differentiation of mycobacteria (Kraus *et al.*, 2001; Park *et al.*, 2000; Roth *et al.*, 1998, 2000). Analysis of the ITS of *M. holsaticum* sp. nov. revealed

two sequences that differed in an insertion of 10 bp, in spite of identical 16S rDNA sequences. Strain-to-strain variation in this region is known from several mycobacteria (Roth *et al.*, 1998, 2000; Richter *et al.*, 1999), particularly from environmental species (Frothingham, 1999). Database analyses using both sequences revealed no identical entries.

hsp65 sequence and RFLP analysis

Analysis of part of the mycobacterial *hsp65* gene is a widely applied method for identification of mycobacterial species (Devallois *et al.*, 1997; Ringuet *et al.*, 1999; Telenti *et al.*, 1993; Steingrube *et al.*, 1995). Sequencing of the *hsp65* PCR product from *M. holsaticum* sp. nov. resulted in a unique nucleotide sequence compared with the sequences stored in the International Nucleotide Sequence Database (Altschul *et al.*, 1997). The *M. holsaticum* *hsp65* fragment contains no restriction site for *Bst*EII, but six restriction sites for *Hae*III, resulting in seven restriction fragments with the following fragment sizes: 139, 96, 58, 51, 36, 36 and 23 bp. By gel electrophoresis of the RFLP assay using *Hae*III, a pattern of four fragments of approximately 140, 100, 60 and 30 bp can be visualized. Absence of a *Bst*EII restriction site discri-

minates the strains clearly from most rapidly growing mycobacteria, particularly from the *Mycobacterium fortuitum* complex, the *hsp65* PCR fragment of which contains at least one restriction site for *Bst*EII (Devallois *et al.*, 1997).

AccuProbe results

In clinical mycobacteriology laboratories, commercially available RNA probes (AccuProbes), targeting rRNA and known to be highly sensitive and specific (Lebrun *et al.*, 1992), are widely used to identify the *M. tuberculosis* complex. However, *M. celatum*, an NTM species, has been reported to cross-react with the *M. tuberculosis* complex probe, due to similarities in the very part of the 16S rRNA gene (Fig. 1) that is targeted by the RNA probe, thus leading to a false-positive result (Bux-Gwehr *et al.*, 1998; Somoskövi *et al.*, 2000). As a consequence, an improved protocol with a prolonged selection step is recommended to circumvent this cross-reactivity. However, false-positive results are still reported, probably because of incorrect performance of the test.

Due to the high similarity of the species-specific 16S rDNA sequences of *M. holsaticum* sp. nov. and the *M. tuberculosis* complex (Fig. 1), cross-reactivity can also be observed when performing an AccuProbe assay with the *M. tuberculosis* complex probe on *M. holsaticum* strains with a 5 min selection step (RLU values ranging from 40090 to 168662). In contrast, with a 10 min selection step, the results for all strains investigated were negative (1377 to 1890 RLU). Thus, in the case of a positive *M. tuberculosis* complex AccuProbe result, combined with rapid growth in culture or atypical morphology, *M. holsaticum* has to be taken into account.

Combined molecular and phenotypic features

Analysis of the entire 16S rRNA gene, revealing a unique sequence, and determination of unique sequences of the ITS and the *hsp65* PCR fragment provide genetic evidence for a novel species belonging to the rapidly growing mycobacteria, as demonstrated by phylogenetic analyses. Phenotypic features, like susceptibility to streptomycin and ethambutol, the inability to take up iron and the mycolic acid pattern, confirm this assessment of a novel species.

Characteristics that differentiate *M. holsaticum* sp. nov. from other rapidly growing mycobacteria

M. holsaticum sp. nov. can be differentiated easily from the *M. fortuitum* complex by its morphology, absence of arylsulfatase and allantoinase, absence of growth on MacConkey agar and susceptibility to streptomycin and ethambutol. It differs from many rapidly growing mycobacteria in the inability to take up iron and the absence of acetamidase. Furthermore, *M. holsaticum* is characterized by a unique 16S rDNA

sequence, placing the species in the rapidly growing mycobacteria, and a unique *hsp65* sequence with a unique RFLP pattern.

Description of *Mycobacterium holsaticum* sp. nov.

Mycobacterium holsaticum (hol.sa'ti.cum. L. adj. *holsaticum* referring to the German region of Holstein, the location of the institute in which the strains were first analysed).

Cells are acid-alcohol-fast, short or coccoid rods. Colonies are dysgonic and transparent at 22 °C, but smooth, moist, shiny and off-white- to yellow-pigmented at higher temperatures. Isolates grow within 7 days at temperatures up to 40 °C. Strains are positive for nitrate and tellurite reduction, tolerate 5% NaCl, 2 µg TCH ml⁻¹ and 8 µg TSC ml⁻¹, have phosphatase, urease, nicotinamidase and pyrazinamidase activities and are weakly positive for Tween 80 hydrolysis. They do not take up iron and can not degrade *p*-aminosalicylic acid. There is no arylsulfatase, acetamidase, benzamidase, allantoinamidase, succinamidase or heat-stable catalase activity. The strains are resistant to isoniazid and rifampin, but susceptible to streptomycin, ethambutol, protionamide and ofloxacin. Cell walls contain predominantly α-, methoxy- and ketomycolates. Major cellular fatty acids are C16:0, C18:1ω9c and tuberculostearic acid. The G+C content of the DNA is 68.4 mol%. Analysis of the entire 16S rRNA gene provides genetic evidence for a novel species. Phylogenetic analysis based on the 16S rDNA sequence allocates this species to the rapidly growing mycobacteria. Sequences of ITS and the *hsp65* PCR fragment are also unique.

The type strain, 1406^T (= DSM 44478^T = CCUG 46266^T), has a long ITS sequence. Strain 5050 (= DSM 44479 = CCUG 46267) has a short ITS sequence.

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