Mycobacterium pyrenivorans sp. nov., a novel polycyclic-aromatic-hydrocarbon-degrading species

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The taxonomic position of a polycyclic-aromatic-hydrocarbon-degrading bacterium, strain 17A3^T, isolated from contaminated soil was determined using a combination of phenotypic and genotypic properties. The isolate showed phenotypic properties that were diagnostic for species of the genus *Mycobacterium*. Comparative 16S rRNA gene sequence analysis assigned 17A3^T to the 16S rRNA gene subgroup that contains *Mycobacterium aurum*, *Mycobacterium austroafricanum*, *Mycobacterium vaccae* and *Mycobacterium vanbaalenii*, but it could clearly be distinguished from these species using a combination of physiological, chemotaxonomic markers and internal rRNA gene spacer analyses. The data showed that strain 17A3^T (= DSM 44605^T = NRRL B-24244^T) merits recognition as the type strain of a novel species of the genus *Mycobacterium*. The name *Mycobacterium pyrenivorans* sp. nov. is proposed for the species because of its ability to use pyrene as a sole source of carbon and energy.

The *Corynebacterineae*, a suborder of the order *Actinomycetales* (Stackebrandt *et al.*, 1997), is the taxon that harbours most xenobiotic degraders among Gram-positive bacteria. Namely, species of the genera *Gordonia*, *Rhodococcus* and *Mycobacterium* are well known for their capacity to degrade environmentally hazardous chemicals (Bell *et al.*, 1998; Williamsen & Karlson, 1997). In this paper, we describe a novel pyrene-degrading, rapidly growing isolate that represents a novel species of the genus *Mycobacterium*.

Strain 17A3^T was isolated from an enrichment culture obtained from soil that was highly contaminated with polycyclic aromatic hydrocarbons (PAHs). The soil sample was collected on the site of a former coking plant at Übach-Palenberg, Germany. An enrichment culture of indigenous bacteria was extracted from soil with 0.2% tetrasodium pyrophosphate and was cultivated in mineral medium amended with phenanthrene, anthracene, pyrene, fluoranthene and benzo[a]pyrene (100 mg l⁻¹ each) as sole sources of carbon and energy (Schwiening, 1999). 17A3^T

was purified by alternately streaking dilutions of the sample on R2A agar plates and cultivating isolated colonies in mineral medium with PAHs. Purity was confirmed by plating on R2A agar and the recovered single isolate was maintained by repeated transfer to mineral medium with PAH or pyrene (50 mg l⁻¹) and R2A agar plates.

Gram staining, acid–alcohol-fastness, colony morphology, the ability to grow at various temperatures, pigment production and photoreactivity were determined after 2 weeks of growth on R2A agar plates at 28–30 °C using the methods described by Vincent Lévy-Frébault & Portaels (1992) and Wayne *et al.* (1974). The catalase test was performed as described by Kubica & Pool (1960). Nitrate reductase and Tween 80 hydrolysis were detected as described by Wayne *et al.* (1974) and by BBL Taxo nitrite test strips. The protocols used to determine growth on xylose, trehalose, mannitol and sorbitol as sole sources of carbon were those of Silcox *et al.* (1981). The type strains of the closely related species *Mycobacterium austroafricanum* (DSM 44191^T) (Tsukamura *et al.*, 1983), *Mycobacterium aurum* (DSM 43999^T) (Tsukamura, 1966) and *Mycobacterium vaccae* (DSM 43292^T) (Bönike & Juhasz, 1964) were tested under identical conditions. *Mycobacterium vanbaalenii* DSM 7251^T (Khan *et al.*, 2002) could not be included in the tests because it was described after performing the experiments.

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Abbreviations: PAH, polycyclic aromatic hydrocarbon; RISA, rRNA gene internal spacer analysis.

The GenBank/EMBL/DDBJ accession number of the 16S rRNA gene sequence of *Mycobacterium pyrenivorans* 17A3^T is AJ431371.

The capacity of strain 17A3^T to degrade different PAHs in liquid batch experiments was tested over a period of 8 weeks by using mineral medium amended with phenanthrene, anthracene, fluoranthene, pyrene and benzo[a]pyrene (20 mg l⁻¹ each). Remaining PAHs were extracted four times with ethyl acetate by vigorous mixing on a stirring plate. Extracts were analysed by GC, which was performed on a Hewlett Packard 5890 Series II gas chromatograph equipped with a flame-ionization detector. A fused silica column (HP-5, cross-linked 5% PhMe-Silicone, 30 × 0.25 mm, 0.25 µm film thickness) with an uncoated deactivated precolumn (Hewlett Packard; Retention Gap, 5 m × 0.32 mm) was used. GC conditions were: injector temperature 270 °C; detector temperature 285 °C; the column temperature program was initial temperature 40 °C for 4 min followed by a linear gradient of 10 °C min⁻¹ to a final temperature of 280 °C, which was maintained for 15 min.

The strain was grown for fatty acid and mycolic acid analysis (HPLC) for 7 days at 35 °C in Petri dishes on Middlebrook 7H10 medium supplemented with glycerol and OADC (DSMZ medium 645; DSMZ, 2001). Three to four inoculation loops of cell material were scraped from the plates and used directly for the analyses. TLC analyses of mycolic acids were performed with whole-cell methanolysates from freeze-dried cells as described previously (Springer *et al.*, 1995).

Fatty acids were analysed as methyl esters obtained from cells after saponification, methylation and extraction by GC as described previously (Sasser, 1990; Schröder *et al.*, 1997). For mycolic acid analyses by HPLC, the cells were saponified by KOH and transferred to their bromophenacyl esters as described previously by Butler *et al.* (1996) and Miller (1997). Mycolic acid bromophenacyl esters were analysed by HPLC operated by the Sherlock System software (MIDI Inc.).

rRNA gene internal spacer analyses (RISA) were carried out with strain 17A3^T and *M. vanbaalenii* DSM 7251^T, *M. aurum* DSM 43999^T and *M. austroafricanum* DSM 44191^T as references using methods described previously (Garcia-Martinez *et al.*, 1999; Gürtler & Stanisich, 1996; Jensen *et al.*, 1993; Ranjard *et al.*, 2000).

Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA gene and purification of PCR products were carried out as described previously (Rainey *et al.*, 1996). The ae2 editor (Maidak *et al.*, 1999) was used to align the almost complete 16S rRNA gene sequence of strain 17A3^T (1451 nt) against the 16S rRNA gene sequences of type strains of the genus *Mycobacterium*. Phylogenetic analyses (De Soete, 1983; Felsenstein, 1993) followed described methods.

Cells of strain 17A3^T were Gram-positive and acid-fast. The scotochromogenic, rough colonies were yellow, which intensified after exposure to light. In liquid media, the cells

clumped together or showed biofilm formation on glass or PAH crystal surfaces. This is probably due to their hydrophobic cell-wall surface. On TSB (trypticase soy broth) agar plates, growth appeared within 7 days at 35 °C. In the semi-quantitative catalase test, the foam height was >45 mm. The nitrate reduction test showed a weak positive reaction. Strain 17A3^T was not able to hydrolyse Tween 80 within 10 days. Mannitol could be used for growth. Xylose, trehalose and sorbitol could not be assimilated. The results of physiological tests are listed in Table 1.

In mineral salt medium, strain 17A3^T was able to mineralize phenanthrene, fluoranthene and pyrene aerobically. Decreases in anthracene and benzo[a]pyrene concentrations in the PAH-amended culture medium were not detected. In addition, pyrene was used as a sole source of carbon and energy. No loss of degradability was observed after subcultivating the strain on composed media like R2A agar for an extended period.

Separation of whole-organism acid methanolysates by TLC developed in two dimensions produced a multispot pattern composed of alpha-, epoxy and omega-dicarboxy mycolates plus a spot of alcohols (wax-ester mycolates). This pattern was shared by *M. aurum*, *M. austroafricanum*, *M. vaccae* and a few other mycobacteria (Vincent Lévy-Frébault & Portaels, 1992; Häggblom *et al.*, 1994; Hinrikson & Pfyffer, 1994) (data not shown).

Analyses of mycolic acids by HPLC revealed a characteristic UV-HPLC chromatogram of *Mycobacterium* species that have separated, triple-peak clusters with prominent peaks in the early cluster that emerge prior to 5.0 min. Strain 17A3^T shares this pattern with its relatives *M. aurum*, *M. austroafricanum*, *M. vaccae* (Butler & Guthertz, 2001) and *M. vanbaalenii* (Khan *et al.*, 2002). Strain 17A3^T can easily

Table 1. Physiological properties of strain 17A3^T and closely related species

Reference strains: 1, *M. austroafricanum* DSM 44191^T; 2, *M. aurum* DSM 43999^T; 3, *M. vaccae* DSM 43292^T. +, Positive; -, negative; ±, weakly positive; S, scotochromogenic; P, photochromogenic. All strains were positive for growth on mannitol and negative for growth on sorbitol.

Characteristic	17A3 ^T	1	2	3
Formation of pigment	S	S	S	P
Growth at 42 °C	-	-	-	+
Tween 80 hydrolysis	-	+	+	+
Nitrate reduction	±	+	-	-
Catalase activity (semi-quantitative)	+†	-	+	+
Growth on:				
Xylose	-	+	+	-
Trehalose	-	-	+	-

*Hydrolysis within 10 days.

†Foam height >45 mm.

be separated from these species by quantitative differences (Table 2).

Analyses of fatty acids by GLC revealed the expected diagnostic pattern for members of the genus *Mycobacterium*, i.e. straight-chain saturated and unsaturated fatty acids together with a diagnostic amount of tuberculostearic acid (10-methyl-branched octadecanoic acid). The alcohols that were already detected by TLC eluted together with the fatty acids and could be identified as octadecanol (C18:0 alcohol) and eicosanol (C20:0 alcohol) (Table 3). Taking the qualitative and quantitative composition of the

Table 2. Comparison of the mycolic acid pattern (HPLC) of *Mycobacterium* sp. 17A3^T with related species

Reference strains: 1, *M. aurum* DSM 43999^T; 2, *M. austroafricanum* DSM 44191^T; 3, *M. vaccae* DSM 43292^T; 4, *M. vanbaalenii* DSM 7251^T. ECL is equivalent chain-length of mycolic acid in comparison to low (ECL 34·000) and high (ECL 110·000) RIBI molecular internal standards. tr, Traces.

ECL	17A3 ^T	1	2	3	4
37·497	—	—	—	—	1·48
40·092	0·49	—	—	—	0·12
41·831	—	—	0·98	—	0·41
44·984	3·42	—	10·58	1·19	8·14
47·035	1·65	—	—	—	—
48·165	16·14	23·49	27·50	4·85	16·64
49·515	—	6·82	—	—	18·24
50·278	8·78	—	—	—	—
51·145	—	23·35	—	—	—
51·874	13·00	—	24·52	12·18	10·35
52·929	19·44	—	—	—	—
54·957	3·41	7·03	3·90	11·73	2·44
55·778	3·12	—	—	—	—
58·528	—	—	0·39	4·47	0·69
60·703	—	—	1·44	2·22	0·92
62·063	1·25	—	—	—	—
64·459	4·34	4·13	9·65	5·72	8·32
67·378	12·49	26·01	15·14	7·47	16·54
69·996	—	2·28	2·75	2·87	3·91
72·841	9·30	—	—	0·68	2·78
75·080	3·18	0·78	1·09	0·80	1·39
77·271	—	0·72	0·45	0·71	0·97
79·157	—	0·29	—	—	—
81·801	—	0·43	0·99	3·58	3·41
82·352	tr	—	—	—	1·93
83·963	—	1·44	0·61	9·14	—
84·617	tr	—	—	—	—
86·369	—	2·51	—	17·17	0·84
86·810	tr	—	—	—	—
88·477	—	0·75	—	11·75	0·47
90·346	—	—	—	3·20	—
92·956	—	—	—	0·35	—

Table 3. Comparison of the fatty acid pattern of *Mycobacterium* sp. 17A3^T with those of related species

Reference strains: 1, *M. aurum* DSM 43999^T; 2, *M. austroafricanum* DSM 44191^T; 3, *M. vaccae* DSM 43292^T; 4, *M. vanbaalenii* DSM 7251^T. Abbreviations are exemplified by: *cis*-7-16:1, *cis*-7 hexadecenoic acid (palmitoleic acid); 10-methyl-18:0, 10-methyl-octadecanoic acid (tuberculostearic acid). Values are percentages of total fatty acids. Major fatty acids (>15%) are in bold.

Fatty acid	17A3 ^T	1	2	3	4
10:0	—	—	—	—	0·05
12:0	—	—	—	0·40	0·09
14:0	2·93	3·43	2·55	1·67	2·71
15:0	0·23	0·56	0·33	0·78	0·80
<i>cis</i> -7-16:1	2·41	0·79	0·89	—	0·95
<i>cis</i> -9-16:1	0·66	0·94	10·65	5·29	—
<i>cis</i> -10-16:1	7·93	6·85	—	6·35	10·34
16:0	25·20	23·35	27·59	23·49	29·11
8-methyl-16:0	0·15	—	—	1·15	0·79
10-methyl-16:0	—	—	0·42	—	—
17:0	0·22	0·35	0·25	0·76	0·57
18:2	0·15	—	0·18	1·37	0·23
<i>cis</i> -9-18:1	30·43	19·74	8·85	28·31	10·52
<i>cis</i> -11-18:1	0·22	—	0·37	1·48	0·48
18:0	3·20	1·59	2·75	1·74	1·81
10-methyl-18:0	7·47	12·62	14·24	10·35	12·30
20:0	0·62	—	0·29	—	0·19
18:0 alcohol	12·54	12·59	15·66	12·74	16·31
20:0 alcohol	5·64	15·19	15·01	4·12	12·78

fatty acids into consideration, this pattern resembles those of *M. aurum*/*M. austroafricanum*. The fatty acid pattern of strain 17A3^T was identified by the Microbial Identification System (MIDI Inc.) as *M. aurum* but with only a low correlation.

In order to characterize strain 17A3^T further and to compare it with closely related strains on the DNA level, we conducted RISA analysis. Spacer regions between the 16S and 23S rRNA genes show a certain degree of length variation (Gürtler & Stanisich, 1996; Garcia-Martinez *et al.*, 1999). These spacers can be easily amplified by means of universal PCR primers with similarity to the conserved flanking sequences. RISA can be used for both molecular characterization of a bacterial species and ecological studies of the diversity of prokaryotic communities (e.g. Ranjard *et al.*, 2000; Sigler *et al.*, 2002). Length polymorphisms occur to a minor degree within a species. The diversity of spacer length is greater if different species or genera are compared, although distantly related species may produce the same RISA pattern (Sonderkamp *et al.*, 2001). Fig. 1 shows the patterns of the intergenic rRNA gene spacer amplicates from strain 17A3^T, *M. vanbaalenii* DSM 7251^T, *M. aurum* DSM 43999^T and *M. austroafricanum* DSM 44191^T.

With intragenic 16S rRNA gene sequence similarities

Fig. 1. RISA patterns of *Mycobacterium* species. Lanes: 1, *Mycobacterium* sp. 17A3^T; 2, *M. vanbaalenii* DSM 7251^T; 3, *M. austroafricanum* DSM 44191^T; 4, *M. aurum* DSM 43999^T; M, *Pvu*II-restricted λ DNA.

ranging between 94.6 and 98.6%, strain 17A3^T is a member of the genus *Mycobacterium*, closely related to *M. austroafricanum* DSM 44191^T (98.6%), *M. vanbaalenii* DSM 7251^T (98.5%), *M. aurum* DSM 43999^T (98.5%) and *M. vaccae* DSM 43292^T (97.8%). Both distance-matrix and maximum-likelihood analysis gave consistent results in that strain 17A3^T clustered adjacent to type strains of *M. vanbaalenii*, *M. austroafricanum*, *M. vaccae* and *M. aurum*, though the bootstrap value for the branching point of this lineage was only 58% (Fig. 2; distance-matrix tree). The presence of clear differences in genomic (RISA, 16S rRNA gene sequence), chemotaxonomic (mycolic acids, fatty acids) and physiological properties in the five members of the *M. aurum* sublineage of the genus *Mycobacterium* and the finding that 16S rRNA gene similarity values determined for these five type strains are similar or even lower than those found for several other pairs of type strains of *Mycobacterium* species (e.g. *M. aurum* DSM 43999^T and *M. vanbaalenii* DSM 7251^T, 98.7%; *M. vaccae* DSM 43292^T and *M. vanbaalenii* DSM 7251^T, 99.3%; or *Mycobacterium fortuitum* DSM 46621^T and *Mycobacterium senegalense* DSM 43656^T, 99.4%) justifies the description of a novel species for strain 17A3^T, for which the name *Mycobacterium pyrenivorans* sp. nov. is proposed.

Fig. 2. Phylogenetic relatedness of the pyrene-metabolizing strain 17A3^T among closely related type strains of *Mycobacterium* species, based upon 16S rRNA gene sequence comparison. More distantly related type strains of the genus served as the root. The dendrogram was generated by distance matrix analysis (De Soete, 1983). Numbers within the dendrogram indicate the percentages of occurrence of the branching order in 100 bootstrapped trees (only values of 50% and above are shown). The scale bar represents 1 nucleotide substitution per 100 nucleotides.

Description of *Mycobacterium pyrenivorans* sp. nov.

Mycobacterium pyrenivorans (py.re.ni.vo'rans. N.L. n. *pyrenum* pyrene; L. v. *vorare* to devour; L. part. adj. *vorans* devouring, digesting; N.L. part. adj. *pyrenivorans* digesting pyrene).

Cells are strictly aerobic, Gram-positive, acid-fast rods. The rough colonies show a yellow colour, which intensifies after exposure to light. In liquid media, the cells clump together or show biofilm formation on glass. On TSA, growth appears within 7 days at 35 °C. Cells grow well between 24 and 37 °C but not at 42 °C. Classified as a scotochromogenic, rapidly growing mycobacterium. Catalase-positive. Nitrate reduction test shows a weak reaction. Does not hydrolyse Tween 80 within 10 days. Mineralizes phenanthrene, fluoranthene and pyrene but not anthracene or benzo[a]pyrene. Can use mannitol as a sole source of carbon but can not grow on xylose, trehalose or sorbitol. The fatty acid pattern from whole-cell methanolysates is composed of tetradeanoic acid (3%), *cis*-7-hexadecenoic acid (3%), palmitic acid (25%), oleic acid (31%), stearic acid (3%), tuberculostearic acid (8%) and eicosanoic acid (1%). Significant amounts of alcohols 2-octadecanol (13%) and eicosanol (6%) are also present. TLC of mycolic acid methanolysates reveals alpha-mycolates, epoxy-mycolates and omega-carboxymycolates plus 2-eicosanol (wax-ester mycolates). The mycolic acid HPLC elution profile is unique and can be used for differentiation from the closely related species *M. aurum*,

M. austroafricanum, *M. vaccae* and *M. vanbaalenii* and all other mycobacteria.

The type strain, 17A3^T (= DSM 44605^T = NRRL B-24244^T), was isolated from soil of a former coking plant at Übach-Palenberg, Germany.

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