Isoptericola hypogeus sp. nov., isolated from the Roman catacomb of Domitilla

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In order to clarify the taxonomic position of an actinobacterium from the Roman catacomb of Domitilla, a combination of phenotypic characterization, phylogenetic analysis based on the 16S rRNA gene sequence and DNA–DNA relatedness studies was used. The results from the polyphasic taxonomic study of this organism showed that strain HKI 0342^T (=DSM 16849^T=NCIMB 14033^T) should be considered as the type strain of a novel species of the genus *Isoptericola*, for which the name *Isoptericola hypogeus* sp. nov. is proposed.

The genus *Isoptericola* has been proposed by Stackebrandt *et al.* (2004) for the misclassified species *Cellulosimicrobium variabile* Bakalidou *et al.* 2002 and is currently based on this single species. The type strain, which is the only representative of *Isoptericola variabilis* at the time of writing, was isolated from the hindgut of the Australian termite *Mastotermes darwiniensis* (Frogatt). *I. variabilis* is phylogenetically closely related to members of the genera *Xylanimonas* (Rivas *et al.*, 2003), *Xylanibacterium* (Rivas *et al.*, 2004) and *Xylanimicrobium* (Stackebrandt & Schumann, 2004). The latter genus has been recently established to harbour the misclassified species *Promicromonospora pachnodae* (Cazemier *et al.*, 2003, 2004), the type strain of which was isolated from the hindgut of larvae of the scarab beetle *Pachnoda marginata*. Together, these four genera constitute a phylogenetically distinct cluster within the suborder *Micrococcineae*, and have cellulolytic and xylanolytic activities in common.

Strain HKI 0342^T was isolated from a sample of tufa collected in the burial chamber of the first arcosolium behind the entrance to the Roman catacomb of Domitilla (Rome, Italy); PY-BHI agar (Yokota *et al.*, 1993) and a standard dilution-plate procedure were used. General laboratory cultivation, morphological studies, determination of optimal growth parameters (temperature, pH, oxygen requirements) and antibiotic-susceptibility tests were performed, using solid or liquid organic medium 79 (Prauser & Falta, 1968) and an incubation temperature of 28 °C. Cell morphology and cell dimensions were examined by using phase-contrast microscopy with a Zeiss Axioscope 2 microscope equipped with image-analysing software (Axio Vision 2.05). The colony morphology of 2–10-day-old cultures was studied using a stereo microscope (Olympus). Standard physiological tests were carried out according to the methods described by Cowan & Steel (1965), Gordon *et al.* (1974), Lanyi (1987) and Smibert & Krieg (1994). Acid production from carbon sources and enzyme activities were studied using the API 50 CHB/E kit (incubation times of up to 7 days) and API ZYM galleries (bioMérieux). Additionally, the utilization of carbon sources was tested using Biolog GP2 MicroPlates and MicroLog computer software (Biolog Identification System). Xylanolytic activity was determined on medium II, described by Cazemier *et al.* (2003), and incubation times of up to 28 days. Susceptibility to antibiotics was examined by placing antibiotic discs (Difco) on agar plates that were seeded with suspensions of the test strains grown in a soft agar layer for 24 h at 28 °C. Oxygen requirements were studied with the GENbag microaer and GENbag anaer incubation systems (bioMérieux). The pH range for growth was established by using liquid medium adjusted to pH values between 4 and 11 with either 1 M HCl or 20 % (w/v) Na_2CO_3 solution and incubated at 28 °C for up to 10 days. The reference strains used for comparisons in physiological tests and DNA–DNA pairing studies were *I. variabilis* DSM 10177^T, *Xylanimicrobium pachnodae* DSM 12657^T, *Xylanimonas cellulosilytica* DSM 15894^T and *Myceligenerans xiligouense* DSM 15700^T. Biomass for chemotaxonomic

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain HKI 0342^T is AJ854061.

Detailed physiological characteristics of strain HKI 0342^T are available as supplementary material in IJSEM Online.

Table 1. Differential physiological characteristics of strain HKI 0342^T and *I. variabilis* DSM 10177^T

Characteristic	HKI 0342^T	DSM 10177^T
Decomposition of:		
Urea	−	+
Utilization of:		
Aconitate	−	+
Citrate	−	+
Malate	+*	−
Growth characteristics		
GENbag anaer	−	+†
Growth at 10 °C	+	−
Growth at 42 °C	−	+
Growth in the presence of 10·0 % NaCl	−	+
Susceptibility to streptomycin (10 μg)	+	−
Enzyme assay (API ZYM)		
α-Galactosidase	−	+
API 50 CH/BE		
Methyl β-xyloside	−	+
Methyl α-D-glucoside	−	+
Melibiose	−	+
Melezitose	−	+
D-Raffinose	−	+
Biolog GP2 MicroPlate (24 h incubation)		
Glycogen	−	+
N-Acetyl-D-mannosamine	−	+
Amygdalin	−	+
L-Fucose	−	+
Gentiobiose	−	+
D-Gluconic acid	−	+
α-D-Lactose	−	+
Lactulose	−	+
D-Mannitol	−	+
D-Melezitose	−	+
D-Melibiose	−	+
Methyl α-D-galactoside	−	+
Methyl β-D-galactoside	−	+
Methyl α-D-glucoside	−	+
Methyl β-D-glucoside	−	+
Methyl α-D-mannoside	−	+
Palatinose	−	+
D-Raffinose	−	+
L-Rhamnose	−	+
D-Sorbitol	−	+
Stachyose	−	+
Sucrose	−	+
D-Trehalose	−	+
Turanose	−	+
Acetic acid	−	+
γ-Hydroxybutyric acid	+	−
L-Lactic acid	−	+
Monomethyl succinate	−	+
Propionic acid	−	+
L-Glutamic acid	−	+
L-Serine	−	+
2′-Deoxyadenosine	−	+
Inosine	−	+
Thymidine	−	+
Adenosine 5′-monophosphate	−	+

*Delayed reaction.

†Data from Cui *et al.* (2004).

and molecular systematic studies was prepared by growing the strain in shake flasks containing liquid organic medium 79 or Bacto tryptic soy broth (Sigma–Aldrich) for 24–48 h. Stock cultures of strain HKI 0342^T in liquid organic medium 79 supplemented with 5 % DMSO were maintained in either the vapour phase of liquid nitrogen or at −80 °C by adding a 1 : 1 mixture of glycerol and a medium that consisted of K_2HPO_4 (1·26 %), KH_2PO_4 (0·36 %), $MgSO_4$ (0·01 %), sodium citrate (0·09 %), $(NH_4)_2SO_4$ (0·18 %) and glycerol (8·8 %).

On medium 79, strain HKI 0342^T formed wrinkled, circular, smooth, pale-yellow colonies about 1–4 mm in diameter. In young cultures (8–24 h), a well-developed primary mycelium (width 0·6–0·7 μm) was observed which fragmented, in the stationary growth phase (48–72 h), into irregular, non-motile, short rods and cocci (diameter 0·8–1·0 μm). In contrast to stationary-phase cultures of *I. variabilis*, both cell types were arranged mainly as short flexible chains or as clusters. Furthermore, strain HKI 0342^T and *I. variabilis* DSM 10177^T exhibited different growth characteristics (Table 1). The catacomb isolate was an aerobic to microaerophilic organism and did not grow in an anaerobic atmosphere, unlike *I. variabilis* DSM 10177^T, which is a facultatively anaerobic organism (Bakalidou *et al.*, 2002). *I. variabilis* grew at temperatures up to 45 °C, but not at 10 °C, while strain HKI 0342^T did not grow above 40 °C, but grew well at 10 °C. Both strains had in common the ability to hydrolyse xylan. However, they could be readily distinguished from one another by means of a broad spectrum of physiological properties. Clearly differential characteristics of the two strains under study are listed in Table 1. Full detailed results (classical physiological tests, API kits and Biolog test) are provided as supplementary tables in IJSEM Online.

For sequence analysis of the 16S rRNA gene, bacterial DNA was extracted according to the method described by Marmur (1961). The 16S rRNA gene was amplified by a PCR using the conserved primers 27F (5′-AGAGTTTGATCC-TGGCTCAG-3′) and 1522R (5′-AAGGAGGTGATCCAG-CCGCA-3′). PCR thermal conditions were as follows: 95 °C for 1 min; 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 2 min; and a final extension cycle at 72 °C for 10 min. Forward and reverse strands of the amplified DNA fragment

were sequenced in an ABI 3700 sequencer (Applied Biosystems). The 16S rRNA gene sequences of the strains studied were aligned manually with nucleotide sequences obtained from the GenBank and EMBL databases. The algorithm of Jukes & Cantor (1969) was applied in order to transform sequence dissimilarities into evolutionary distances. Phylogenetic dendrograms were reconstructed by using the method of De Soete (1983) and the neighbour-joining method (Felsenstein, 1993).

The binary 16S rRNA gene sequence similarity values between strain HKI 0342^T and its nearest phylogenetic neighbours range between 95·0 and 98·8 %. A dendrogram of relationships based on the Jukes & Cantor (1969) corrections of similarity values (Fig. 1) shows strain HKI 0342^T as branching next to *I. variabilis* DSM 10177^T (98·8 %), whereas members of *Xylanimonas* and relatives, *Cellulosimicrobium* and *Promicromonospora* are less closely related. To determine the genomic relatedness between strains DSM 10177^T and HKI 0342^T, DNA–DNA similarity studies were performed.

For DNA–DNA relatedness studies, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970) under consideration of the modifications described by Huß *et al.* (1983), using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an *in-situ* temperature probe (Varian). The DNA G + C content was determined as recommended by Mesbah *et al.* (1989).

Strains HKI 0342^T and DSM 10177^T shared a mean DNA–DNA relatedness value of 31·7 % (individual values of 33·4 and 30·0 %), a value which clearly indicates that the two strains belong to distinct genomic species.

The following chemotaxonomic characteristics were determined: the structure of the peptidoglycan, as described by Schleifer & Kandler (1972), Schleifer (1985), MacKenzie (1987), Groth *et al.* (1996) and Frank *et al.* (1980); the acyl type of the muramic acid, as described by Uchida *et al.* (1999); the whole-cell sugars present, as described by Becker *et al.* (1965) and Saddler *et al.* (1991); the menaquinones present, as described by Groth *et al.* (1996); the polar lipids present, as described by Minnikin *et al.* (1979) and Collins & Jones (1980); the mycolic acids present, as described by Minnikin *et al.* (1975); and the fatty acid profile (MIDI system; Agilent).

The chemotaxonomic characteristics of strain HKI 0342^T were most similar to those of *I. variabilis* and supported the affiliation of this organism to the genus *Isoptericola*. The peptidoglycan contained *N*-acetylated muramic acid and corresponded to type A4α, L-Lys–D-Glu (A11.33 according to http://www.dsmz.de/species/murein.htm). Strain HKI 0342^T differs from *I. variabilis* in having D-Glu instead of D-Asp as the interpeptide bridge. Glucose, rhamnose, galactose and a minor amount of mannose were found in whole-cell hydrolysates. Strain HKI 0342^T was characterized by the presence of menaquinones with nine isoprene units, which differs from *I. variabilis* in that the major components were MK-9(H_2) and MK-9 (peak areas 42 : 29, respectively) instead of MK-9(H_4) (peak area 10). The phospholipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol mannoside, phosphatidylinositol, two unknown phospholipids and an unknown glycolipid. In addition to the polar lipid components reported by Stackebrandt *et al.* (2004) for *I. variabilis*, phosphatidylinositol mannoside was also detected in strain DSM 10177^T in this study. The fatty acid profile consisted of anteiso-$C_{15:0}$ (44·0 mol%), iso-$C_{16:0}$ (22·6 mol%), iso-$C_{15:0}$ (18·1 mol%), iso-$C_{14:0}$ (8·4 mol%) and anteiso-$C_{17:0}$ (3·1 mol%). Mycolic acids were not detected.

It is evident from Fig. 1 that strain HKI 0342^T is most closely related to the type strain of *I. variabilis*. The low level of DNA–DNA relatedness (about 30 %) between the two strains, together with the numerous differences in chemotaxonomic, morphological and a broad range of physiological characteristics (Table 1), clearly indicates that strain HKI 0342^T represents a novel species of the genus

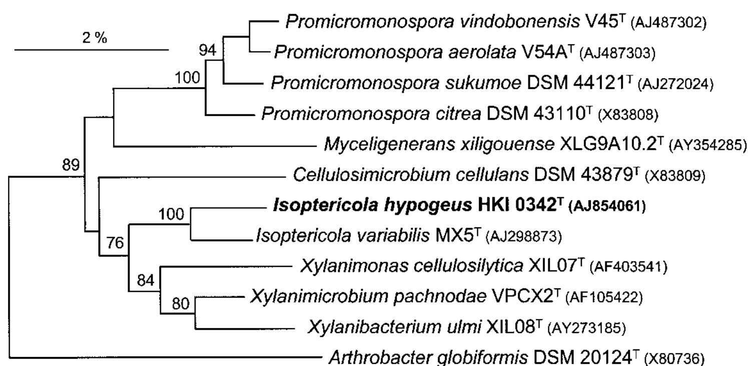

Fig. 1. Phylogenetic relatedness among members of the genera *Isoptericola*, *Xylanimonas*, *Xylanimicrobium*, *Xylanibacterium*, *Cellulosimicrobium*, *Myceligenerans* and *Promicromonospora*, based on 16S rRNA gene sequence comparison. *Arthrobacter globiformis* DSM 20124^T (X80736) was used as the outgroup. The dendrogram was generated by neighbour-joining analysis (Felsenstein, 1993). 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). Bar, 2 substitutions per 100 nt.

Isoptericola, for which the name *Isoptericola hypogeus* is proposed.

Description of *Isoptericola hypogeus* sp. nov.

Isoptericola hypogeus (hy.po.ge'us. L. masc. adj. *hypogeus* underground, referring to the site of isolation).

Gram-positive, aerobic to microaerophilic actinomycete with a well-developed primary mycelium (diameter of hyphae 0·6–0·7 μm) that undergoes fragmentation into short, irregular, non-motile rods and cocci (diameter 0·8–1·0 μm) in the stationary growth phase. Aerial mycelium is absent. Colonies on organic medium 79 are wrinkled, circular, smooth and pale yellow (diameter about 1–4 mm). Growth occurs between 10 and 40 °C (optimal growth at 28 °C) and at pH values in the range of 6–9. NaCl in the culture medium is tolerated at concentrations up to 10 %. Physiological characteristics relating to the utilization of carbohydrates, enzyme activities and susceptibility to antibiotics are listed in Table 1 and in the supplementary data available in IJSEM Online. The peptidoglycan type is A4α, L-Lys–D-Glu. The whole-cell sugars comprise glucose, rhamnose, galactose and a minor amount of mannose. The acyl type is acetyl. The menaquinones comprise MK-9(H_2), MK-9 and MK-9(H_4) (peak areas 42 : 29 : 10) and the major fatty acids are anteiso-$C_{15:0}$ (44·0 mol%), iso-$C_{16:0}$ (22·6 mol%), iso-$C_{15:0}$ (18·1 mol%), iso-$C_{14:0}$ (8·4 mol%) and anteiso-$C_{17:0}$ (3·1 mol%). The phospholipids comprise diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol mannoside, phosphatidylinositol, two unknown phospholipids and an unknown glycolipid. Mycolic acids are absent. The G + C content of the DNA of the type strain is 73·8 mol%.

The type strain, HKI 0342^T (= DSM 16849^T = NCIMB 14033^T), was isolated from a tufa sample from a burial chamber in the catacomb of Domitilla, Rome, Italy.

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