

Agromyces italicus sp. nov., *Agromyces humatus* sp. nov. and *Agromyces lapidis* sp. nov., isolated from Roman catacombs

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A polyphasic study was carried out to clarify the taxonomic positions of three Gram-positive isolates from the Catacombs of Domitilla, Rome (Italy). 16S rRNA gene sequence comparisons placed these strains within the genus *Agromyces*. The morphological and chemotaxonomic characteristics of these isolates were consistent with the description of the genus *Agromyces*. The three isolates could be readily distinguished from one another and from representatives of all *Agromyces* species with validly published names by a broad range of phenotypic characteristics and DNA–DNA relatedness studies. Therefore, these isolates are proposed to represent three novel species of the genus *Agromyces*, *Agromyces italicus* sp. nov. (type strain CD1^T = HKI 0325^T = DSM 16388^T = NCIMB 14011^T), *Agromyces humatus* sp. nov. (type strain CD5^T = HKI 0327^T = DSM 16389^T = NCIMB 14012^T) and *Agromyces lapidis* sp. nov. (type strain CD55^T = HKI 0324^T = DSM 16390^T = NCIMB 14013^T).

Strains of the genus *Agromyces* are common inhabitants of different soils ranging from fertile meadows to deserts [*Agromyces ramosus* Gledhill and Casida 1969, *Agromyces cerinus* subsp. *cerinus* Zgurskaya *et al.* 1992, *Agromyces cerinus* subsp. *nitratus* Zgurskaya *et al.* 1992, *Agromyces fucosus* Zgurskaya *et al.* 1992 emend. Ortiz-Martinez *et al.* 2004, *Agromyces hippuratus* (Zgurskaya *et al.* 1992) Ortiz-Martinez *et al.*, 2004, *Agromyces mediolanus* Suzuki *et al.* 1996 and *Agromyces aurantiacus* Li *et al.* 2003]. The rhizosphere of plants harbours a wide diversity of these organisms, as has been shown by Takeuchi & Hatano (2001) with the description of three species, *Agromyces brachium*, *Agromyces luteolus* and *Agromyces rhizosphaerae*, from mangrove rhizosphere soil. Dorofeeva *et al.* (2003) isolated *Agromyces albus* from the leaves and inflorescences of members of the Primulaceae, while *Agromyces ulmi* was isolated from the decayed stump of an elm tree, *Ulmus nigra* (Rivas *et al.*, 2004). Recently, two novel species of this genus, *Agromyces salentinus* and *Agromyces neolithicus*, have been isolated from the Grotta dei Cervi, a cave in southern Italy with singular neolithic rock art paintings (Jurado *et al.*, 2005).

Strains CD1^T and CD5^T were obtained from the wall of a tomb located in the Little Apostle cubicle, Domitilla Catacombs, Rome, Italy (Sanchez-Moral *et al.*, 2004). Strain CD55^T was isolated by touching the stone wall of a tomb (first arcosolium on the left side behind the entrance of Domitilla Catacombs) with a sterile cotton swab and suspending the attached bacteria in 1:10 diluted organic medium 79 (OM79) (Prauser & Falta, 1968; Jurado *et al.*, 2005). The strains were isolated on nutrient agar (Difco) or, in the case of strain CD55^T, on peptone/yeast extract/brain heart infusion agar (Yokota *et al.*, 1993), using a standard dilution plate procedure.

All the methods used in this study have been recently described by Jurado *et al.* (2005). Range of pH for growth was established using liquid OM79 medium adjusted to initial pH values between 4 and 11 with either 1 M HCl or 20% (w/v) Na₂CO₃ solution and incubated at 28 °C for up to 10 days. The following type strains were used as references for comparative studies: *A. albus* VKM Ac-1800^T, *A. cerinus* subsp. *cerinus* IMET 11525^T, *A. fucosus* IMET 11529^T, *A. mediolanus* VKM Ac-1388^T, *A. neolithicus* DSM 16197^T, *A. ramosus* IMET 11027^T and *A. salentinus* DSM 16198^T. Morphological and physiological traits are summarized in the species descriptions and Table 1.

Differences referring to the compositions of whole-cell sugars, menaquinones and polar lipids are shown in Table 2. Cell-wall amino acids in strains CD1^T, CD5^T and CD55^T

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains CD1^T, CD5^T and CD55^T are AY618215, AY618216 and AY618217, respectively.

Table 1. Characteristics that differentiate strains CD1^T, CD5^T and CD55^T and their closest relatives within the genus *Agromyces*

Strains: 1, *A. italicus* sp. nov. CD1^T; 2, *A. humatus* sp. nov. CD5^T; 3, *A. lapidis* sp. nov. CD55^T; 4, *A. albus* VKM Ac-1800^T; 5, *A. cerinus* subsp. *cerinus* IMET 11525^T; 6, *A. fucosus* IMET 11529^T; 7, *A. mediolanus* VKM Ac-1388^T; 8, *A. neolithicus* DSM 16197^T; 9, *A. ramosus* IMET 11027^T; 10, *A. salentinus* DSM 16198^T. Data for *A. cerinus* subsp. *cerinus* and *A. ramosus* were taken from Groth *et al.* (1996). –, Negative; +, positive; (+) weakly positive; v, variable; ND, not determined. Strains CD1^T, CD5^T and CD55^T share the following properties. They produce acid from starch, arbutin, D-fructose, glycogen, maltose, D-mannose, but not from adonitol, D-arabitol, L-arabitol, dulcitol, erythritol, D-fucose, gluconate, 2-ketogluconate, 5-ketogluconate, β -gentiobiose, inositol, D-lyxose, melibiose, melezitose, sorbitol, L-sorbose; D-tagatose, L-xylose, methyl β -xyloside or xylitol. They produce alkaline phosphatase, esterase (C1), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -glucosidase and β -glucosidase, but not α -galactosidase, *N*-acetyl- β -glucosaminidase, lipase (C14), trypsin or α -fucosidase. They are sensitive to chloramphenicol (30 μ g), imipenem (10 μ g), ofloxacin (10 μ g), oxytetracycline hydrochloride (30 μ g), rifampicin (5 μ g) and vancomycin hydrochloride (30 μ g), but not to kanamycin (30 μ g), lincomycin (2 μ g), norfloxacin (10 μ g) or sulfonamide (200 μ g). They hydrolyse aesculin and starch, but not adenine or Tween 80. Voges-Proskauer, methyl red and indole tests give negative results. They are positive for H₂S production.

Characteristic	1	2	3	4	5	6	7	8	9	10
DNA G+C content (mol%)	70.8	70.6	70.4	69.0	70.5	70.6	72.3	65.3	68.9	72.3
Growth at 10 °C	(+)	–	(+)	(+)	(+)	+	(+)	–	+	+
Growth in 4.0% NaCl (w/v)	+	–	+	(+)	–	+	+	–	(+)	(+)
Microaerophilic growth	+	–	+	–	–	+	+	+	+	(+)
Decomposition or hydrolysis of:										
Casein	+	+	+	+	–	+	+	+	–	+
Gelatin	+	+	v	+	–	+	–	+	–	–
Hippurate	+	+	+	+	–	+	+	+	+	+
Hypoxanthine	+	(+)	–	–	(+)	+	+	–	–	–
Tyrosine	+	+	+	–	+	+	+	+	–	+
Urea	–	–	–	+	–	–	–	–	–	+
Xanthine	+	–	–	–	–	+	+	–	–	–
Biochemical tests										
Nitrate reduction	+	+	+	–	–	–	–	+	v	–
Catalase reaction	+	+	+	+	+	+	+	+	–	+
Oxidase test	+	–	v	+	+	+	v	v	–	v
Acid production from (API 50 CH B/E):										
Amygdalin	(+)	–	+	–	+	+	+	–	+	(+)
D-Arabinose	+	–	+	+*	+	+	(+)*	+	+	+
L-Arabinose	+	+	–	+	–	+	–	+	+	+
Cellobiose	+	v	+	–	+	+	+	+	–	+
L-Fucose	+	–	+	–	(+)	+	–	+	+	+
Galactose	+	+	+	(+)	+	+	+	+	–	+
D-Glucose	+	+	+	(+)	+	+	+	+	–	+
Glycerol	+	+	+	–	+	+	+	+	+	+
Inulin	–	+	+	–	–	(+)	–	–	+	+
Lactose	(+)	–	–	–	+	–	+	–	–	–
Methyl α -D-mannoside	–	–	+	–	–	+	–	–	–	–
Methyl α -D-glucoside	–	–	–	–	–	+	–	–	–	–
Mannitol	–	+	–	+	–	–	–	+	(+)	–
N-Acetylglucosamine	–	v	+	–	–	+	–	(+)*	+	–
D-Raffinose	–	+	(+)	+*	–	+	–	+	+	(+)
Rhamnose	–	+	–	+	+	+*	+	–	+	+
Ribose	–	–	–	+*	–	–	+	–	–	+
Salicin	+	–	+	–	+	+	(+)	–	–	+
Sucrose	–	+	+	(+)	–	+	+	(v)	+	+
Trehalose	–	–	–	–	–	(+)*	+	–	–	+
D-Turanose	–	–	–	–	–	–	(+)	–	–	+
D-Xylose	+	–	–	+*	–	–	–	–	–	+
Utilization of:										
Aconitate	–	–	–	–	+	–	–	–	–	–

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9	10
Citrate	–	–	–	–	+	–	–	–	–	–
Malate	–	+	–	+	+	–	–	–	+	–
Succinate	–	+	–	+	–	–	–	–	+	–
Enzyme assays (API ZYM):										
α -Chymotrypsin	–	–	+	–	–	–	–	+	–	–
α -Galactosidase	–	–	–	–	+	–	–	+	–	–
β -Galactosidase	+	+	+	+	+	+	+	–	–	+
β -Glucuronidase	–	+	–	–	–	–	–	+	–	–
<i>N</i> -Acetyl- β -glucosaminidase	–	–	–	+	–	+	+	+	–	+
α -Mannosidase	–	+	–	–	–	–	–	+	–	–
Antibiotic susceptibility:										
Ampicillin (10 μ g)	+	+	+	+	+	–	+	+	+	+
Ciprofloxacin (5 μ g)	+	+	+	+	V	+	–	+	+	+
Kanamycin (30 μ g)	–	–	–	+	+	+	–	–	+	+
Methicillin (5 μ g)	+	+	+	–	ND	–	–	+	ND	+
Nalidixic acid (30 μ g)	–	–	+	+	–	–	–	–	–	+
Norfloxacin (10 μ g)	–	–	–	–	–	–	–	+	–	+
Novobiocin (5 μ g)	+	+	+	+	+	+	–	+	–	+
Penicillin G (10 IU)	+	–	+	–	–	–	–	+	+	+
Polymyxin B (300 IU)	–	+	–	+	V	–	–	+	+	+
Streptomycin sulfate (10 μ g)	–	+	–	+	+	+	–	–	+	+
Sulfonamide (200 μ g)	–	–	–	–	–	–	–	–	–	+

*Delayed response.

were diamino butyric acid, glycine, glutamic acid and alanine. Acyl type for these three strains was acetyl. The predominant fatty acids of strains CD1^T, CD5^T and CD55^T were anteiso-C_{15:0} (50.9, 41.5 and 48.1%, respectively), anteiso-C_{17:0} (15.3, 34.0 and 13.0%), iso-C_{16:0} (14.7, 15.5 and 16.5%), iso-C_{15:0} (14.2, 5.4 and 10.5%) and C_{16:0} (1.7, 1.3 and 5.9%).

Sequence comparisons of 16S rRNA genes from strains CD1^T, CD5^T and CD55^T showed a close phylogenetic relationship to *A. ramosus*, *A. cerinus* subsp. *cerinus*, *A. salentinus*, *A. neolithicus*, *A. albus*, *A. mediolanus* and *A. fucus* with percentages of similarity ranging from 94 to 98%. Strains CD1^T and CD55^T showed high similarity

(97%). The recently described species *A. salentinus* (Jurado *et al.*, 2005) was highly related to strains CD1^T and CD55^T with 97% similarity. *A. neolithicus* was the closest relative to strain CD5^T (98%). A phylogenetic tree generated by the neighbour-joining method showing the relationships between members of the genus *Agromyces* and the three novel isolates, CD1^T, CD5^T and CD55^T, is shown in Fig. 1.

The DNA G + C contents of strains CD1^T, CD5^T and CD55^T were 70.8, 70.6 and 70.4 mol%, respectively. DNA–DNA relatedness studies showed significant differences between the three isolates as well as with their closest phylogenetic neighbours. In all cases, differences in melting temperatures were >7.7 °C (roughly <51% DNA–DNA relatedness;

Table 2. Chemotaxonomic characteristics of strains CD1^T, CD5^T and CD55^T

Components are listed in decreasing order of abundance.

Characteristic	Strain CD1 ^T	Strain CD5 ^T	Strain CD55 ^T
Whole-cell sugars*	Gal, Rib, Glc, Man	Glc, Gal, Rha, Man	Glc, Gal, Man, Rib
Major menaquinones	12, 13	13, 12	12, 13
Polar lipids†	DPG, PG, 2 PL, GL	DPG, PG, PL, 2 GL	DPG, PG, PL, 2 GL

*Gal, Galactose; Glc, glucose; Man, mannose; Rha, rhamnose; Rib, ribose.

†DPG, Diphosphatidylglycerol; GL, unknown glycolipid; PG, phosphatidylglycerol; PL, unknown phospholipid.

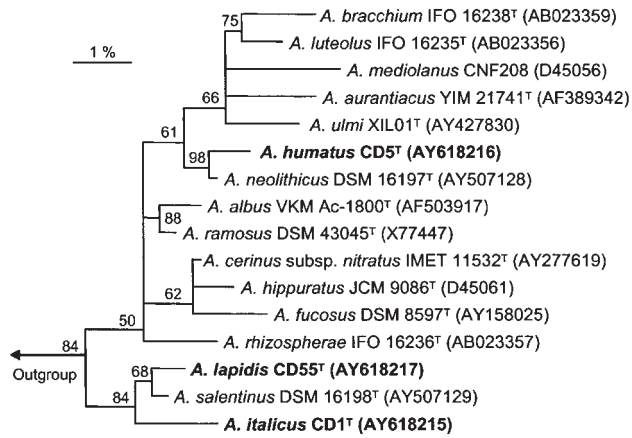


Fig. 1. Neighbour-joining phylogenetic tree showing the relationships between species of the genus *Agromyces* and isolates CD1^T, CD5^T and CD55^T based on 16S rRNA gene sequences. Bar, 1% sequence divergence. The outgroup (not shown) was *Corynebacterium* sp. QSSC3-5 (AF170740).

Rosselló-Mora & Amann, 2001), which is well above the 5 °C cut-off point recommended for the delineation of species (Stackebrandt & Goebel, 1994).

The genotypic and phenotypic characteristics of strains CD1^T, CD5^T and CD55^T are consistent with their classification in the genus *Agromyces* (Gledhill & Casida, 1969; Zgurskaya *et al.*, 1992). The isolates can be readily distinguished from representatives of *Agromyces* species with validly published names, and from one another, by a broad range of phenotypic properties (Table 1) and composition of whole-cell sugars (Table 2). DNA–DNA relatedness data support the above distinctions between strains CD1^T, CD5^T and CD55^T and their closest relatives within the genus *Agromyces*. Based on the results of this polyphasic approach, we propose that the three studied isolates are classified within novel species of the genus *Agromyces*, *Agromyces italicus* sp. nov. (strain CD1^T), *Agromyces humatus* sp. nov. (strain CD5^T) and *Agromyces lapidis* sp. nov. (strain CD55^T).

Description of *Agromyces italicus* sp. nov.

Agromyces italicus (i.ta'li.cus. L. masc. adj. *italicus* from Italy, the origin of the type strain).

Gram-positive, aerobic to microaerophilic actinomycete that forms branching hyphae (width 0.4–0.6 µm) which break up into irregular diphtheroid and rod-like, non-motile fragments. Colonies are circular, convex, smooth and cream. Colony diameter is about 1 mm. Growth occurs between 10 and 37 °C (optimal growth at 28 °C) and at pH 5–9.5. NaCl is tolerated up to 4% (w/v), but not at 6% (w/v). Phenotypic characteristics including antibiotic susceptibility and enzymic activities are reported in Table 1. Chemotaxonomic characteristics are reported in Table 2. Cell-wall amino acids are diaminobutyric acid, glycine,

glutamic acid and alanine. Acyl type is acetyl. Predominant fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}; mycolic acids are absent. G + C content is 70.8 mol%.

The type strain is CD1^T (= HKI 0325^T = DSM 16388^T = NCIMB 14011^T), isolated from the wall of a tomb located in the Little Apostle cubicle, Domitilla Catacombs, Rome, Italy.

Description of *Agromyces humatus* sp. nov.

Agromyces humatus (hu.ma'tus. L. masc. part. adj. *humatus* buried).

Gram-positive, aerobic actinomycete that forms branching hyphae (width 0.3–0.5 µm) which break up into irregular diphtheroid and rod-like, non-motile fragments. Colonies are circular, convex, smooth and yellow. Colony diameter is about 1 mm. Growth occurs between 15 and 37 °C (optimal growth at 28 °C) and at pH 5–9.5. NaCl is tolerated up to 2% (w/v), but not at 4% (w/v). Phenotypic characteristics including antibiotic susceptibility and enzymic activities are reported in Table 1. Chemotaxonomic characteristics are reported in Table 2. Cell-wall amino acids are diamino-butyric acid, glycine, glutamic acid and alanine. Acyl type is acetyl. Predominant fatty acids are anteiso-C_{15:0} and anteiso-C_{17:0}; mycolic acids are absent. G + C content is 70.6 mol%.

The type strain is CD5^T (= HKI 0327^T = DSM 16389^T = NCIMB 14012^T), isolated from the wall of a tomb located in the Little Apostle cubicle, Domitilla Catacombs, Rome, Italy.

Description of *Agromyces lapidis* sp. nov.

Agromyces lapidis (la.pi'dis. L. gen. n. *lapidis* of a stone).

Gram-positive, aerobic to microaerophilic actinomycete that forms branching hyphae (width 0.4–0.6 µm) which break up into irregular diphtheroid and rod-like, non-motile fragments. Colonies are circular, convex, smooth and yellow. Colony diameter is about 1 mm. Growth occurs between 10 and 37 °C (optimal growth at 28 °C) and at pH 5–9.5. NaCl is tolerated up to 4% (w/v), but not at 6% (w/v). Phenotypic characteristics including antibiotic susceptibility and enzymic activities are reported in Table 1. Chemotaxonomic characteristics are reported in Table 2. Cell-wall amino acids are diaminobutyric acid, glycine, glutamic acid and alanine. Acyl type is acetyl. Predominant fatty acids are anteiso-C_{15:0} and iso-C_{16:0}; mycolic acids are absent. G + C content is 70.4 mol%.

The type strain is CD55^T (= HKI 0324^T = DSM 16390^T = NCIMB 14013^T), isolated from a carved stone wall of the Domitilla Catacombs, Rome, Italy.

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