Agromyces aureus sp. nov., isolated from the rhizosphere of *Salix caprea* L. grown in a heavy-metal-contaminated soil

Erika Corretto, Livio Antonielli, Angela Sessitsch, Stéphane Compant, Markus Gorfer, Melanie Kuffner† and Günter Brader

Correspondence
Günter Brader
guenter.brader@ait.ac.at

Health and Environment Department, Bioresources Unit, AIT Austrian Institute of Technology, Konrad-Lorenz-Straße 24, A-3430 Tulln, Austria

A Gram-reaction-positive, motile, yellow-pigmented and rod-shaped bacterial strain, designated AR33^T, was isolated from the rhizosphere of *Salix caprea* L. growing in a former zinc/lead mining and processing site in Austria. A polyphasic approach was applied to determine its taxonomic position. 16S rRNA gene sequence analysis, and morphological and chemotaxonomic properties showed that strain AR33^T belongs to the genus *Agromyces*. Strain AR33^T had peptidoglycan type B2γ and the major menaquinones were MK-11, MK-10 and MK-12. The main branched-chain fatty acids were anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. Strain AR33^T showed catalase and oxidase activity and multiple heavy metal resistances to zinc, lead and cadmium. The DNA G+C content was 70.1 mol%. Levels of 16S rRNA gene sequence similarity with closely related recognized species of the genus *Agromyces* ranged between 98 and 99 %. However, DNA–DNA hybridization between strain AR33^T and the type strains of three *Agromyces* species showed values lower than 42 % relatedness. Therefore, differential phenotypic characteristics together with DNA–DNA relatedness suggested that strain AR33^T can be recognized as representing a distinct *Agromyces* species, for which the name *Agromyces aureus* sp. nov. is proposed. The type strain is AR33^T (=DSM 101731^T=LMG 29235^T).

The genus *Agromyces* was proposed by Gledhill & Casida (1969) and the description was later amended by Zgurskaya *et al.* (1992). The genus includes non-spore-forming, microaerophilic to aerobic, filamentous and non-filamentous Gram-positive bacteria affiliated to the phylum *Actinobacteria*. Members of the genus have variable catalase and oxidase activity and B2γ-type peptidoglycan. Major fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$, and MK-11 and MK-12 are the predominant menaquinones (Akimov & Evtushenko, 2012). *Agromyces* species have been isolated from various environments such as soil (Yoon *et al.*, 2008; Lee *et al.*, 2011), rhizosphere (Takeuchi & Hatano, 2001; Jung *et al.*, 2007), caves and rocks (Jurado *et al.*, 2005a, b, c), plant tissues (Dorofeeva *et al.*, 2003; Rivas *et al.*, 2004) and sea sediments (Hamada *et al.*, 2014).

Strain AR33^T was isolated from *Salix caprea* L. rhizosphere samples collected in a former zinc/lead mining and processing site (Arnoldstein, Austria) (Wenzel & Jockwer, 1999). The rhizosphere soil was shaken with 0.8 % (w/v) NaCl solution for 30 min. Dilutions of the suspension were plated on R2A (Difco) containing 1 mM Cd $(NO_3)_2$, 1 mM Pb $(NO_3)_2$ and 1 mM $Zn(NO_3)_2$ and incubated at room temperature (Kuffner *et al.*, 2008). For the description of morphological, physiological and biochemical characteristics, the strain was routinely cultivated at 27 °C in Landy medium (per litre: 20 g glucose, 5 g glutamate, 0.25 g $MgSO_4$, 0.25 g KCl, 0.5 g KH_2PO_4, 150 µg $FeSO_4$, 5 mg $MnSO_4$, 160 µg $CuSO_4$, 1 g yeast extract, pH 7.2). The following type strains were included for comparative studies: *Agromyces lapidis* DSM 1639^T, *Agromyces terreus* DSM 22016^T, *Agromyces allii* DSM 21551^T, *Agromyces italicus* DSM 16388^T and *Agromyces salentinus* DSM 16198^T.

Cell morphology, dimensions, pigmentation and motility were examined using a confocal laser scanning microscope [Olympus Fluoview FV1000 with multi-line laser FV5-LAMAR-2 and HeNe(G) laser FV10-LAHEG230-2]. Cells were grown overnight at 28 °C in Landy medium and subsequently stained with the green fluorescent nucleic acid stain SYTO9 at 3 µM (ThermoFisher) for 15 min. Images were

†Present address: Austrian Agency for Health and Food Safety, Spargelfeldstraße 191, 1220, Vienna, Austria.

The GenBank/EMBL/DDJB accession numbers for the two variants (v1, v2) of the complete 16S rRNA gene sequence of strain AR33T are KU141338 and KU141339, respectively.

Three supplementary figures and one supplementary table are available with the online Supplemenatry Material.

taken at 405, 488 and 633 nm with a 40× objective, then merged (RGB) using Image J software (Schneider *et al.*, 2012). Z Project Stacks were then used to create the pictures (as described by Campisano *et al.*, 2014). Flagella were stained using the 'Ryu staining' method as described by Ferooz & Letesson (2010). The Gram reaction was determined by using the Gram-colour set (Merck) according to the manufacturer's instructions. Growth at 4, 10, 15, 25, 30 and 37 °C was assessed on solid Landy medium after 7 days. The pH range for growth was determined using liquid Landy medium adjusted to values between pH 4 and 11 with 1 M HCl and 20 % (w/v) Na_2CO_3 and incubated at 27 °C for 10 days. Solid Landy medium amended with 2, 3, 4 and 5 % (w/v) NaCl was used to determine halotolerance. Methyl red and Voges–Proskauer tests were performed as described by Lanyi (1987). Microaerophilic growth was assessed using anaerobic jars containing a pack of Microbiology Aerocult A mini (Merck).

The API 50CH (API 50CHB medium, 48 h, 28 °C) and API 20E systems (bioMérieux) were used to test the ability to assimilate single carbon sources, produce acid from certain substrates and other physiological characteristics; enzyme activities were assessed using the API ZYM system (bioMérieux). Catalase activity was demonstrated by using 3 % (v/v) hydrogen peroxide (Cappuccino & Sherman, 2002). Oxidase activity was tested on filter paper moistened with oxidase reagent (bioMérieux). Hydrolysis of hippurate was determined using hippurate discs (Sigma) and ninhydrin reagent (Lanyi, 1987). Chitinase activity was tested on Landy medium plates supplemented with chitin azure (Sigma) and incubated at 28 °C for 7 days. Hydrolysis of adenine, L-tyrosine, xanthine and hypoxanthine was determined on nutrient agar (Fluka) as described by Gordon *et al.* (1974). Hydrolysis of casein, Tween 20 and Tween 80 was assessed as described by Cowan & Steel (1965). Resistance to heavy metals was tested on solid Landy medium supplemented with different concentrations of zinc sulfate (1–10 mM), lead nitrate (1–10 mM) and cadmium nitrate (0.5–2 mM). Susceptibility to antibiotics was tested on Landy medium plates using antibiotic discs containing the following antibiotics: ampicillin (10 μg), carbenicillin (100 μg), chloramphenicol (30 μg), erythromycin (10 μg), gentamicin (30 μg), kanamycin (30 μg), nalidixic acid (30 μg), neomycin (30 μg), rifampicin (5 μg), streptomycin (10 μg), tetracycline (30 μg) and vancomycin (30 μg).

Genomic DNA was extracted using a phenol–chloroform-based protocol and the whole genome was sequenced using an Illumina MiSeq platform (300 bp, paired-end reads). The reads were assembled with SPAdes (Bankevich *et al.*, 2012) and the gaps within the contigs were closed via PCR. The assembly of the complete genome resulted in 4.37 Mb with 259.63±45.98× coverage (CP013979). The G+C content was calculated using Qualimap (García-Alcalde *et al.*, 2012). Recently, Kim *et al.* (2015) demonstrated that determination of the G+C content using genomic information seems to be more accurate than the classic experimental methods. This is especially true for genome sequences having a high-quality assembly and a coverage value of at least 16×. Using RNAmmer (Lagesen *et al.*, 2007), we were able to identify three copies of the 16S rRNA gene with two different sequences (accession nos. KU141338, KU141339). To identify the closest described relatives, the 16S rRNA gene sequences were subjected to BLAST analysis using the GenBank database. 16S rRNA gene sequences were aligned with MUSCLE (Edgar, 2004), poorly aligned positions were removed using Gblocks (Castresana, 2000) and phylogenetic reconstruction was inferred in MEGA6 (Tamura *et al.*, 2013) using the neighbour-joining method (Saitou & Nei, 1987) (Fig. 1) and the maximum-likelihood method (Fig. S1, available in the online Supplementary Material) (Tamura *et al.*, 2004). Tree topology robustness was verified by a bootstrap test with 1000 reiterations. DNA–DNA relatedness analysis was performed with the closely related strains (*A. lapidis* DSM 1639^T, *A. terreus* DSM 22016^T, *A. allii* DSM 21551^T, *A. italicus* DSM 16388^T, *A. salentinus* DSM 16198^T). DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977) and the 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).

The peptidoglycan structure was determined as follows: cells were disrupted by shaking with glass beads in a Vibrogen cell mill (Johanna Otto) and processed according to the method of Schleifer (1985). The whole-cell sugars were analysed by TLC on cellulose plates according to Staneck & Roberts (1974). Cellular fatty acids were extracted using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988) and subsequently separated by GC. Polar lipids were extracted from freeze dried cell material (modified after Bligh & Dyer, 1959), then separated by two-dimensional silica gel TLC and detected with molybdatophosphoric acid stain as described by Tindall *et al.* (2007). Respiratory menaquinones were extracted using the two-stage method described by Tindall (1990a, b), separated into their different classes by TLC on silica gel and further analysed by HPLC.

Cells of strain AR33^T were Gram-reaction-positive, motile (monotrichous) and had a rod shape: 0.3–0.5 μm wide, 1.0–2.0 μm long and up to 6 μm when forming curved branching hyphae (Fig. S2). A slight natural auto-fluorescence was registered when the cells were exited with blue light (350 nm). Growth occurred between 10 and 30 °C with the optimum being 28 °C. Colonies were characterized by a bright yellow pigmentation. Growth was observed under aerobic as well as under microaerophilic conditions. Strain AR33^T showed tolerance to up to 6 mM zinc sulfate and lead nitrate and up to 1 mM cadmium nitrate. Cells tolerated the presence of NaCl in the growth medium up to a concentration of 3 % (w/v). Strain AR33^T exhibited catalase and oxidase activity, but no chitinase activity. The methyl red test showed a positive result only after 4 days of growth. No acetoin production was detected with the Voges–Proskauer test.

Phylogenetic analysis was carried out using the two variants of the complete 16S rRNA gene sequences obtained from

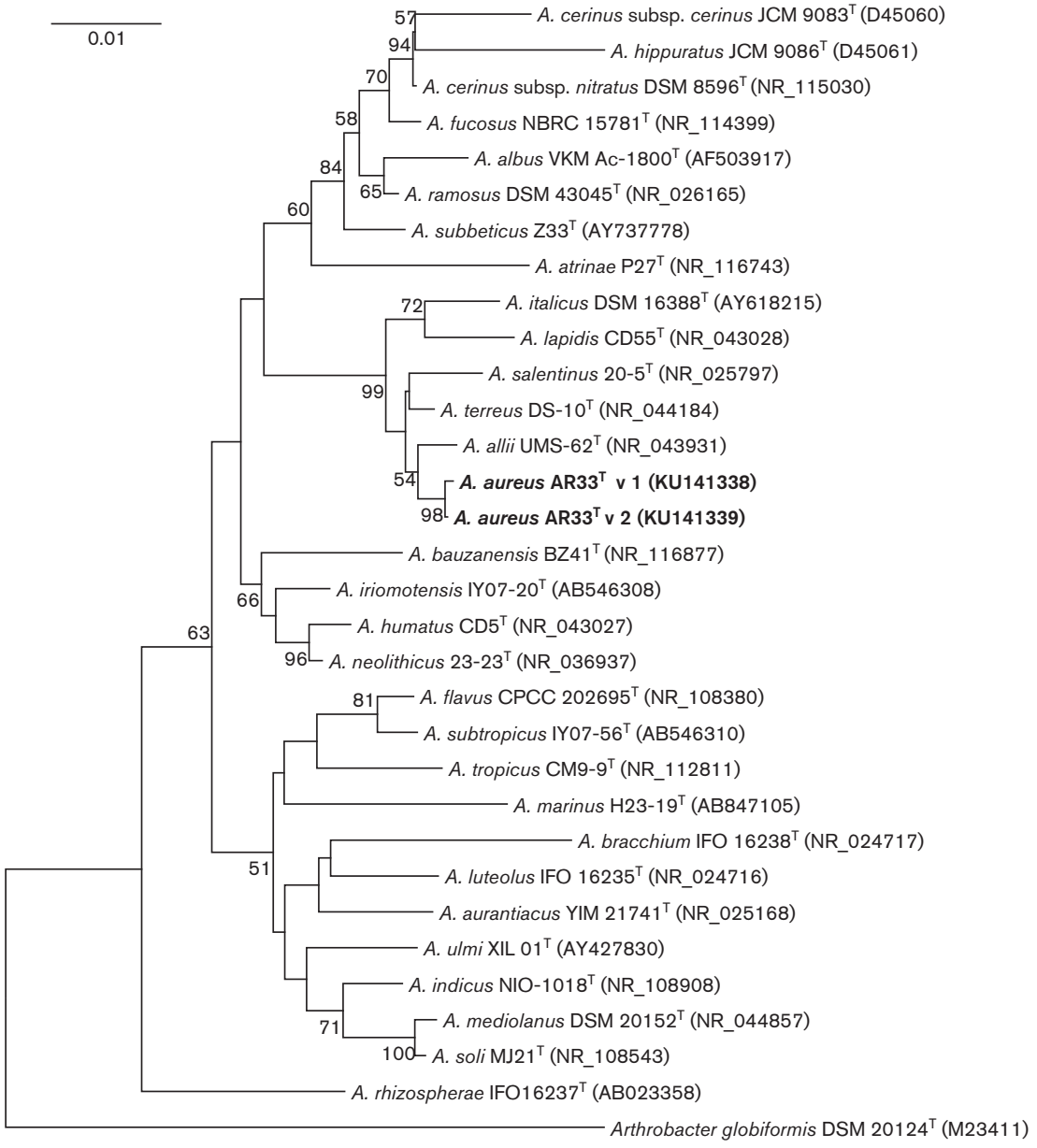

Fig. 1. Neighbour-joining tree showing the phylogenetic position of strain AR33^T and previously described *Agromyces* species based on 16S rRNA gene sequences. The two variants of the complete 16S rRNA gene sequence of strain AR33^T (v1, v2) were used. *Arthrobacter globiformis* DSM 20124^T (M23411) was used as an outgroup. Bootstrap values (expressed as percentages of 1000 replications) are given at branch points; only values >50% are shown. Bar, 0.01 changes per nucleotide position.

the whole genome analysis (Fig. 1). Comparison with the most closely related described strains showed high levels of similarity to *A. lapidis* DSM 1639^T (98 %), *A. terreus* DSM 22016^T (99 %), *A. allii* DSM 21551^T (99 %), *A. italicus* DSM 16388^T (98 %), *A. salentinus* DSM 16198^T (98 %). Since these values are above the generally accepted threshold of 97 % similarity (Tindall *et al.*, 2010), DNA–DNA hybridization was performed as described above. Results showed values lower than the accepted cut-off value of 70 % relatedness (32.8 % with *A. lapidis* DSM 16390^T, 41.9 % *A. terreus* DSM 22016^T, 35.7 % *A. allii* DSM 21551^T, 17.2 % *A. italicus* DSM 16388^T, 11 % *A. salentinus* DSM 16198^T), suggesting that strain AR33^T represents a distinct species. The DNA G+C content of strain AR33^T was 70.1 mol%, which is consistent with values calculated with classic experimental methods used previously to describe other *Agromyces* species (Table 1).

As for other members of the genus *Agromyces*, the peptidoglycan structure of strain AR33^T belonged to the B2γ type (Schleifer & Kandler, 1972), containing the following amino

Table 1. Differential phenotypic characteristics between strain AR33^T and phylogenetically related *Agromyces* species

Strains: 1, AR33^T; 2, *A. lapidis* DSM 1639^T; 3, *A. terreus* DSM 22016^T; 4, *A. allii* DSM 21551^T; 5, *A. italicus* DSM 16388^T; 6, *A. salentinus* DSM 16198^T. +, Positive; −, negative; ±, weakly positive; V, variable. All strains were grown in Landy medium. All are positive in catalase and oxidase tests and are susceptible to ampicillin (10 μg), carbenicillin (100 μg), chloramphenicol (30 μg), tetracycline (30 μg) and vancomycin (30 μg). All are able to produce acid from cellobiose, D-fructose, D-galactose and D-glucose.

Characteristic	1	2	3	4	5	6
DNA G+C content (mol%)	70.1*	70.4	71.1	71.1	70.8	72.3
Temperature range (°C):	10–30	10–37	10–35	4–33	10–37	10–37
Temperature optimum (°C)	28	28	25	30	28	20–28
Halotolerace (NaCl; %, w/v)	3	4	6	3	4	4
Resistance to heavy metals (mM):						
Zinc	6	2	2	6	1	2
Lead	6	1	2	2	1	2
Cadmium	1	−	−	−	−	−
Production of H_2S	−	+	−	−	+	+
Utilization of citrate	±	−	−	−	−	−
Reduction of nitrate	−	+	+	+	+	−
Hydrolysis of:						
Casein	−	+	−	+	+	+
Gelatine	−	V	+	+	+	−
Hippurate	+	+	−	+	+	+
Hypoxanthine	+	−	+	−	+	−
Tween 20	+	+	+	−	+	+
Tween 80	+	−	+	−	−	−
Tyrosine	±	+	+	−	+	+
Urea	−	−	−	−	−	+
Xanthine	+	−	−	−	+	−
Acid production from (API 50 CH B/E):						
Amygdalin	+	+	−	+	±	+
D-Lactose	−	−	−	+	±	−
D-Mannitol	−	−	+	−	−	−
D-Mannose	±	+	+	+	+	+
Melibiose	−	−	+	+	−	−
Raffinose	+	±	±	−	−	±
D-Ribose	−	−	−	+	−	+
Sucrose	+	+	+	+	−	+
Trehalose	+	−	−	+	−	+
Turanose	±	−	−	±	−	+
D-Xylose	+	−	+	+	+	+
Gentibiose	±	−	−	±	−	±
Inulin	+	+	+	−	−	+
L-Arabinose	+	−	+	+	+	+
L-Rhamnose	+	−	+	+	−	+
Methyl α-D-mannopyranoside	−	+	−	−	−	−
N-Acetylglucosamine	+	+	−	−	−	−
Potassium 5-ketogluconate	−	−	+	−	−	−
Salicin	+	+	±	+	+	+
Enzyme activity (API ZYM):						
Alkaline phosphatase	−	+	−	−	+	+
Lipase (C14)	−	−	+	−	−	−
Valine arylamidase	±	+	−	+	+	+
Cystine arylamidase	±	+	−	+	+	+
α-Chymotrypsin	−	+	−	V	−	−
α-Mannosidase	−	−	+	−	−	−

Table 1. cont.

Characteristic	1	2	3	4	5	6
N-Acetyl-β-glucosaminidase	+	−	±	+	−	+
Antibiotic suceptibility:						
Erythromycin (10 μg)	+	+	+	+	+	−
Gentamicin (30 μg)	+	±	+	+	+	−
Kanamycin (30 μg)	±	−	±	+	−	+
Nalidixic acid (30 μg)	−	+	−	−	−	+
Neomycin (30 μg)	±	±	+	+	+	−
Rifampicin (5 μg)	±	+	−	−	+	−
Streptomycin sulfate (10 μg)	±	−	+	±	−	+

*The DNA G+C content of strain 1 was determined based on the genomic content.

acids: alanine/glycine/glutamic acid/2,4-diaminobutyric acid (0.5 : 0.6 : 1.0 : 1.2). Cell-wall sugar analysis revealed the presence of galactose, rhamnose, ribose and fucose. The main branched-chain fatty acids were anteiso-$C_{15:0}$ (43.9 % of the total), anteiso-$C_{17:0}$ (39.6 %) and iso-$C_{16:0}$ (11 %); the most abundant saturated fatty acid was $C_{16:0}$ (3.4 %). Interestingly, the cell-wall sugar composition and the high abundance of branched-chain fatty acid anteiso-$C_{17:0}$ seems to be a unique characteristic of isolate AR33^T compared with the other type strains considered in this study (Table S1). The most abundant saturated fatty acid was $C_{16:0}$ (3.4 %). MK-11, MK-10 and MK-12 at peak area ratios of approximately 53, 24 and 11 %, respectively, were the main menaquinones. The major polar lipids were diphosphatidylglycerol, glycolipid and phosphatidylglycerol (Fig. S3, Table S1).

Based on the data presented, strain AR33^T is considered to represent a novel species of the genus *Agromyces*, for which the name *Agromyces aureus* sp. nov. is proposed.

Description of *Agromyces aureus* sp. nov.

Agromyces aureus (au′re.us. L. masc. adj. *aureus* referring to the bright yellow colony colour).

Cells are Gram-reaction-positive, microaerophilic to aerobic, motile and rod shaped, 0.3–0.5×1.0–6.0 μm. Growth occurs between 10 and 30 °C with the optimum being 28 °C. Optimal pH for growth is between 6.5 and 7.5. Adenine is not hydrolysed. Cells are resistant up to 6 mM zinc and lead, and up to 1 mM cadmium. API ZYM assays show acid phosphatase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, α-glucosidase and β-glucosidase activity, whereas esterase (C4), esterase lipase (C8) and β-galactosidase are weakly active. Trypsin, α-galactosidase and α-fucosidase activity is not found. Acid is produced from starch, arbutine, D-arabinose, maltose, aesculin, glycerol, glycogen and L-fucose, but not from D-adonitol, D-arabitol, D-fucose, D-lyxose, melezitose, D-sorbitol, D-tagatose, dulcitol, erythritol, inositol, L-arabitol, L-sorbose, L-xylose, methyl α-D-glucopyranoside, methyl β-D-xylopyranoside, potassium 2-ketogluconate, potassium gluconate or xylitol. Tryptophan deaminase activity is found, but arginine decarboxylase, lysine decarboxylase and ornithine decarboxylase are not detected. Indole is not produced. The following substrates are assimilated: amygdalin, D-glucose, sucrose, L-arabinose and L-rhamnose; D-mannitol, melibiose, D-sorbitol and inositol are not assimilated. The cell-wall peptidoglycan is type B2γ (D-Glu–L-DAB) and the cell-wall sugars are galactose, rhamnose, ribose and fucose. MK-11, MK-10 and MK-12 are the main menaquinones. Major cellular fatty acids are anteiso-$C_{15:0}$, anteiso-$C_{17:0}$ and iso-$C_{16:0}$. Diphosphatidylglycerol, glycolipid and phosphatidylglycerol are the predominant polar lipids.

The type strain, AR33^T (=DSM 101731^T=LMG 29235^T), was isolated from the rhizosphere of willow trees (*Salix caprea* L.) grown in a former mining site in Arnoldstein, Austria. The G+C content of the type strain is 70.1 mol%.

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