Microbacterium yannicii sp. nov., isolated from *Arabidopsis thaliana* roots

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Bacterial strain G72^T was isolated from surface-sterilized roots of *Arabidopsis thaliana* growing in its natural habitat. This Gram-positive, rod-shaped, non-motile, microaerophilic and aerobically growing isolate was characterized by using a polyphasic approach. On the basis of 16S rRNA gene sequence similarity, strain G72^T belongs to the genus *Microbacterium*. However, reassociation values in a DNA–DNA hybridization analysis with closely related strains were between 45.1 and 15.9 %. The DNA G+C content was 70.1 mol%. Strain G72^T possessed a peptidoglycan type based on B2 β with partial substitution of glutamic acid by 3-hydroxy glutamic acid (Glu/Hyg–Gly–D–Orn). The isolate contained MK-13, MK-12 and MK-11 as major respiratory quinones and anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} as predominant fatty acids. The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and three unidentified glycolipids. Based on its physiological and biochemical traits, as well as genotypic results, strain G72^T is considered to represent a novel species, for which the name *Microbacterium yannicii* sp. nov. is proposed. The type strain is G72^T (=DSM 23203^T =LMG 25521^T).

Plants are generally colonized by many microbes. Apart from pathogenic bacteria, there are also bacteria that improve the growth and health of their host plants (Lugtenberg & Kamilova, 2009). To date, the beneficial effects of bacteria on plant growth have been mainly investigated in crop plants, but little is known about the molecular and physiological mechanisms that ultimately result in growth promotion. In our study, we isolated bacteria from roots of *Arabidopsis thaliana*, which is a model organism in plant research. Therefore, a wide array of molecular and genetic techniques is available to perform further research, which should lead to a better understanding of the mechanism leading to bacterially induced plant growth promotion. Here we describe the isolation and characterization of a bacterial strain, designated G72^T, isolated from surface-sterilized roots of *A. thaliana* growing in the field, with the subsequent aim to investigate its effect on plant growth. As a result of pheno- and genotypic characterization, we propose that strain G72^T represents a novel species of the genus *Microbacterium*, for which we propose the name *Microbacterium yannicii* sp. nov.

Strain G72^T was isolated from roots of *A. thaliana* (thale cress) collected from a field in Golm (52° 24' 57" N 12° 58'

18" E, Potsdam, Germany; soil texture: loamy sand). Roots were surface-sterilized with 70 % ethanol for 1 min and 2.5 % sodium hypochlorite for 3 min. After rinsing three times in sterile distilled water, roots were ground using a sterile mortar and pestle. The ruptured root tissue was incubated in 1/4 strength Ringer solution (Merck) for 45 min with shaking at room temperature. After 5 min of sedimentation, dilutions of the suspension were spread on modified tryptic soy agar (TSA) medium adapted from the method of Jacxsens *et al.* (2003). To simulate nutrient availability in *Arabidopsis*, this medium contained 50 % *Arabidopsis* leaf juice, 50 % tryptic soy broth (TSB; SIFIN) and 1 % agar (w/v) in total. Leaf juice was obtained from greenhouse grown *A. thaliana* plants and heated for 2 h at 80 °C to denature enzymes. The filtrate was mixed with an equal volume of TSB. After addition of the required amount of agar, the solution was autoclaved for 15 min at 121 °C. Single colonies were selected after 3 days of growth at 16 °C. Further cultivation was performed on TSA or TSB at 16 °C unless otherwise stated.

To determine the 16S rRNA gene sequence, chromosomal DNA was extracted from the bacterial isolate and purified using the PureLink genomic DNA mini kit (Invitrogen) according to the manufacturer's instructions. The gene was amplified by PCR with the universal primers P0 and P6 as described by Ventura *et al.* (2001) and the Advantage HF2 PCR kit (Clontech). Purified PCR products were sequenced by AGOWA (Berlin, Germany). The resulting sequence of 1466 base pairs of the 16S rRNA gene of strain G72^T was

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain G72^T is FN547412.

A supplementary figure is available with the online version of this paper.

used to perform a similarity search against entries in the EzTaxon database of type strains of prokaryotic species with validly published names (www.eztaxon.org; Chun *et al.*, 2007) by means of the BLAST (Altschul *et al.*, 1997) and megaBLAST (Zhang *et al.*, 2000) programs. Strain G72^T showed highest pairwise sequence similarity to taxa of the genus *Microbacterium*. A similarity of 98.427 % was obtained with *Microbacterium hominis* (IFO 15708^T; AB004727), 98.339 % with *Microbacterium insulae* (DS-66^T; EU239498) and 98.292 % with *Microbacterium thalassium* (IFO 16060^T; AB004713). Phylogenetic and molecular evolutionary analyses were carried out using the MEGA4 software package (Kumar *et al.*, 1994; Tamura *et al.*, 2007). Multiple sequence alignment was performed with CLUSTAL X, version 2.0.11 (Larkin *et al.*, 2007). A phylogenetic tree (Supplementary Fig. S1, available in IJSEM Online) based on 16S rRNA gene sequences of accepted type strains of the genus *Microbacterium* was generated by applying the neighbour-joining algorithm (Saitou & Nei, 1987). The length of the sequences used to reconstruct the phylogenetic tree was 1166 nt. Evolutionary distances were computed using the maximum composite likelihood method (Tamura *et al.*, 2004) and bootstrap analysis (Felsenstein, 1985) with 1000 resamples was used to evaluate the tree topology. The 16S rRNA gene sequence of *Arthrobacter globiformis* was used as an out group. A detailed representation of the cluster comprising *M. yannicii* sp. nov. and its closest phylogenetic neighbours is shown in Fig. 1. On the basis of this phylogenetic analysis, the type strains of the species *Microbacterium trichothecenolyticum*, *M. insulae* and *M. hominis* were selected for comparative analyses.

Analysis of biochemical and morphological traits of strain G72^T was carried out by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

The cell-wall peptidoglycan structure was analysed by means of TLC, 2D-TLC, GS and GC/MS (Schleifer, 1985; MacKenzie, 1987; Groth *et al.*, 1996). The total hydrolysate (4 M HCl, 100 °C, 16 h) contained ornithine, alanine, glycine, homoserine and small amounts of glutamic acid. Using GC/MS analysis, high amounts of 3-hydroxyglutamic

acid (Hyg) were detected. According to Schleifer & Kandler (1972), these data suggested that the peptidoglycan structure of the isolate was of type B2 β with partial substitution of glutamic acid by 3-hydroxyglutamic acid (Glu/Hyg–Gly–D–Orn).

For cellular fatty acid analysis, cells were grown on TSA for 2 days at 30 °C. Fatty acid methyl esters were obtained from 40 mg of cells, scraped from Petri dishes, by saponification, methylation and extraction using minor modifications of the methods of Miller (1982) and Kuykendall *et al.* (1988). Extracts were separated and analysed using the Microbial Identification System (MIS; MIDI), which consisted of a gas chromatograph (6890N; Agilent Technologies) equipped with a 5 % phenyl-methyl silicone capillary column, a flame-ionization detector (17683A; Agilent Technologies) and the MIDI database TSBA 4.10. Peaks were automatically integrated and fatty acid names and percentages calculated by the Sherlock MIS 6.1 software. As major compounds of the fatty acid profile, anteiso-C_{15:0} (44.76 %), anteiso-C_{17:0} (29.45 %) and iso-C_{16:0} (13.50 %) were identified. This analysis was done as a comparative study for which the fatty acid profiles of strain G72^T and its closest phylogenetic neighbours were determined under the same conditions (Table 1).

Respiratory lipoquinones and polar lipids were extracted from 100 mg freeze-dried cell material using the two-stage method described by Tindall (1990a, b). Respiratory quinones were extracted using methanol/hexane (Tindall, 1990a, b), followed by phase separation into hexane. Polar lipids were extracted by adjusting the remaining methanol/0.3 % aqueous NaCl phase (containing the cell debris) to give a chloroform/methanol/0.3 % aqueous NaCl mixture of 1:2:0.8 (by vol.). The extraction solvent was stirred overnight and the cell debris pelleted by centrifugation. Polar lipids were recovered into the chloroform phase by adjusting the chloroform/methanol/0.3 % aqueous NaCl mixture to a ratio of 1:1:0.9 (by vol.). Respiratory lipoquinones were separated into their different classes (menaquinones, ubiquinones, etc.) by thin layer chromatography on silica gel (Macherey-Nagel art. no. 805 023), using hexane/*tert*-butylmethylether (9:1, v/v) as

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the position of strain G72^T among closely related type strains of the genus *Microbacterium*. The tree was reconstructed by the neighbour-joining method. Bootstrap values >50 % based on 1000 replications are shown at branching points. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the maximum composite likelihood method. Bar, 2 substitutions per 1000 nt positions. GenBank accession numbers for the 16S rRNA gene sequence of each reference species are shown in parentheses. The sequence length used to reconstruct the phylogenetic tree was 1166 nt. An extended version of this tree is available as Supplementary Figure S1.

Table 1. Fatty acid profiles of strain G72^T and the three most closely related type strains

Strains: 1, *Microbacterium yannicii* sp. nov. G72^T; 2, *M. trichothecenolyticum* DSM 8608^T; 3, *M. insulae* DSM 23024^T; 4, *M. hominis* DSM 12509^T. Values are percentages of total fatty acids. –, not detected or <1 %.

Fatty acid	1	2	3	4
iso-C _{14:0}	0.83	0.34	0.79	0.61
iso-C _{15:0}	4.88	2.10	3.69	3.12
anteiso-C _{15:0}	44.76	45.76	47.57	41.51
iso-C _{16:0}	13.50	14.40	13.08	18.55
C _{16:0}	0.81	0.87	1.34	5.31
iso-C _{17:0}	3.57	6.85	7.91	1.27
anteiso-C _{17:0}	29.45	22.38	17.62	28.63
C _{17:0}	0.10	0.19	0.22	0.75
iso-C _{18:0}	1.11	3.11	5.60	–
C _{18:0}	0.73	2.60	0.92	–
anteiso-C _{19:0}	0.18	1.40	0.77	–

solvent. UV absorbing bands corresponding to the different quinone classes (e.g. menaquinones or ubiquinones) were removed from the plate and further analysed by HPLC. This step was carried out on a LDC Analytical (Thermo Separation Products) HPLC fitted with a reverse phase column (Macherey-Nagel; 2 mm × 125 mm, 3 µm, RP18) using methanol/heptane 9:1 (v/v) as the eluent. Respiratory lipoquinones were detected at 269 nm. The predominant quinones detected in strain G72^T were MK-13 (major component), MK-12 and MK-11. Polar lipids were separated by two-dimensional silica gel thin layer chromatography (Macherey-Nagel art. no. 818 135). The first direction was developed in chloroform/methanol/water (65:25:4, by vol.), and the second in chloroform/methanol/acetic acid/water (80:12:15:4, by vol.). Total lipid material and specific functional groups were detected using dodecamolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate–Schiff (α-glycols), Dragendorff (quaternary nitrogen) and α-naphthol/sulphuric acid (glycolipids). Full details are given by Tindall *et al.* (2007). The major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and three unidentified glycolipids.

Carbon-source utilization, urease and arginine dihydrolase activity, and hydrolysis of gelatin were determined by using API 20 NE and API Coryne galleries according to the manufacturer's instructions (bioMérieux). Hydrolysis of starch and Tween 80 and the assimilation of acetate, fumarate and DL-lactate were assayed as described by Stanier *et al.* (1966). Aerotolerance was determined as described by Brewer (1940). All other analyses were performed according to Reddy *et al.* (2007). Cell morphology and motility were observed under a phase-contrast microscope (Axioskop; Zeiss) at ×1000 magnification. Results are summarized in the species description and were compared with biochemical traits of phylogenetically related

type strains of species of the genus *Microbacterium* grown and analysed under the same conditions (Table 2). Strain G72^T differs in several biochemical properties from the most closely related species.

To determine the G + C content, genomic DNA was isolated and purified according to the method of Cashion *et al.* (1977). After subsequent enzymic degradation, deoxyribonucleotides were analysed by reversed-phase HPLC. The G + C content was calculated as described by Mesbah *et al.* (1989) and was 70.1 mol%.

For DNA–DNA hybridization experiments, the most closely related type strains, *M. trichothecenolyticum*, *M. insulae* and *M. hominis*, were chosen according to the phylogenetic analysis of 16S rRNA gene sequences (Fig. 1). 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

Table 2. Biochemical characteristics that differentiate strain G72^T from its nearest phylogenetic neighbours

Strains: 1, *Microbacterium yannicii* sp. nov. G72^T; 2, *M. trichothecenolyticum* DSM 8608^T; 3, *M. insulae* DSM 23024^T; 4, *M. hominis* DSM 12509^T. +, Positive; –, negative; w, weakly positive. All strains were positive for Gram reaction, α- and β-glucosidase, N-acetyl-β-glucosaminidase, H₂S formation, hydrolysis of gelatin and starch, and assimilation of sucrose. All strains were negative for urease, arginine dihydrolase, and assimilation of phenylacetate, ribose, lactose and glycogen.

Characteristic	1	2	3	4
Growth in 2 % NaCl	–	+	+	+
Growth at 37 °C	w	+	+	+
Enzymic activity				
Pyrroline oxidase	+	–	–	+
Pyrroline oxidase	–	+	–	–
Alkaline phosphatase	–	+	–	–
β-Glucuronidase	+	–	–	–
β-Galactosidase	+	+	–	+
Catalase	+	+	w	+
Voges–Proskauer test	–	–	–	+
Nitrate reduction	–	+	–	–
Hydrolysis of:				
Tween 80	+	+	–	–
Assimilation of:				
Sodium acetate	+	+	+	–
DL-Lactate	+	+	–	–
DL-Malate	–	+	–	–
Fumarate	–	+	+	–
L-Arabinose	+	+	+	–
Glucose	w	+	+	+
Mannitol	–	–	–	+
Maltose	w	w	+	+

100 Bio UV/VIS spectrometer equipped with a Peltier-thermostat 6×6 multicell changer and a temperature controller with *in-situ* temperature probe (Varian). This analysis showed a genetic relatedness of $45.1 \pm 4.2\%$ (mean $\pm$ standard deviation) between strain G72^T and *M. trichothecenolyticum* DSM 8608^T, $31.2 \pm 6.3\%$ with *M. insulae* DSM 23024^T and $15.9 \pm 3.4\%$ with *M. hominis* DSM 12509^T. These degrees of genomic relatedness were well below the limit of 70% for species definition recommended by Wayne *et al.* (1987).

The data presented are consistent with the classification of strain G72^T in the genus *Microbacterium*. However, on the basis of biochemical and genetic analyses this isolate is clearly capable of being differentiated from the closest related species. Therefore, strain G72^T is considered to represent an independent species of the genus *Microbacterium*, for which we propose the name *Microbacterium yannicii* sp. nov.

Description of *Microbacterium yannicii* sp. nov.

Microbacterium yannicii (yan.ni'ci.i. N.L. gen. masc. n. *yannicii* of Yannic, in honour of the inspirational contribution of Yannic Karojet to the research project that led to the isolation and identification of the bacterial species).

Cells are Gram-positive, the KOH string test is negative and aminopeptidase activity is not detected. Colonies are 1–2 mm in diameter after 72 h of incubation at 16 °C on TSA, circular, low convex with entire margins, moist and with a transparent milky colour. Colony colour changes to an opaque, intense yellow after 10 days. Cells are non-motile, irregular short rods, $0.5\text{--}0.6 \times 1.5\text{--}3.0\ \mu\text{m}$ in size. Exhibits micro-aerophilic and aerobic growth. Temperature range for growth is from 4 to 37 °C. Growth does not occur in the presence of 2% (w/v) NaCl. Cells demonstrate enzymic activity of pyrazinamidase, β -glucuronidase, β -galactosidase, α - and β -glucosidase, *N*-acetyl- β -glucosaminidase and catalase, but no activity of pyrrolidonyl arylamidase, alkaline phosphatase, urease or arginine dihydrolase. Voges-Proskauer test result is negative. H₂S is produced. Nitrate is not reduced. Gelatin, starch and Tween 80 are hydrolysed. Sodium acetate, DL-lactate, L-arabinose and sucrose are utilized as substrates, but not DL-malate, fumarate, sodium propionate, citrate, phenylacetate, ribose, mannitol, lactose or glycogen. Glucose and maltose are poor substrates. The peptidoglycan is of type B2 β with partial substitution of glutamic acid by 3-hydroxyglutamic acid (Glu/Hyg-Gly-D-Orn). The major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} and the polar lipid profile consists of the major lipids diphosphatidylglycerol, phosphatidylglycerol and three unidentified glycolipids. Predominant menaquinones are MK-13, MK-12 and MK-11. The G + C content of the genomic DNA is 70.1 mol%.

The type strain, G72^T (=DSM 23203^T =LMG 25521^T), was isolated from roots of *Arabidopsis thaliana* plants that were growing in the fields of Golm, in the area around Potsdam, Germany.

Acknowledgements

Dr Susanne Verbarg of the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) is kindly acknowledged for her help with chemotaxonomic and DNA–DNA hybridization analysis and the discussion of the results. We thank Professor J.P. Euzéby for his help with the Latin construction of the species name.

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