Agrococcus versicolor sp. nov., an actinobacterium associated with the phyllosphere of potato plants

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The taxonomic position of a group of actinobacterial strains isolated from the phyllosphere of potato plants was investigated by using a polyphasic approach. Although the similarity values for their 16S rRNA gene sequences suggested an intermediate position between *Microbacterium* and *Agrococcus*, the phylogenetic tree demonstrated a clear clustering of the representative strain, K 114/01^T, within the genus *Agrococcus*. The presence of 2,4-diaminobutyric acid as the diagnostic diamino acid in the cell-wall peptidoglycan of strain K 114/01^T substantiated the affiliation to the genus *Agrococcus*. An analysis performed using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry revealed highly similar spectral patterns for the isolated strains, which, together with their conformity regarding a multitude of phenotypic features, supported their affiliation to the same species. Differences in several physiological features, peptidoglycan and menaquinone composition and whole-cell fatty acid profiles enabled discrimination of the phyllosphere isolates with respect to recognized *Agrococcus* species. As the 16S rRNA gene sequence similarity values were below 97 %, the strains isolated from the phyllosphere of potato plants represent a novel species of the genus *Agrococcus*, for which the name *Agrococcus versicolor* sp. nov. is proposed. The type strain is K 114/01^T (=DSM 19812^T =LMG 24386^T).

The plant phyllosphere is known to be a typical habitat for several genera of the family *Microbacteriaceae*. The species of the genera *Microbacterium*, *Leifsonia*, *Curtobacterium*, *Clavibacter*, *Frigoribacterium*, *Agreia* and *Plantibacter*, in particular, colonize the surfaces of various plant species (Evtushenko & Takeuchi, 2006). In contrast, *Agrococcus* species have not been isolated from plant surfaces. Strains of this genus have been found in the air, in soil samples of different origins, on a medieval wall painting and on the surfaces of some cheeses (Groth *et al.*, 1996; Wieser *et al.*, 1999; Zlamala *et al.*, 2002; Mayilraj *et al.*, 2006; Bora *et al.*, 2007). Furthermore, the members of the genus are able to thrive under extreme living conditions, such as in permafrost environments (Katayama *et al.*, 2007; Zhang *et al.*, 2007), in extreme alkaline groundwater (Tiago *et al.*, 2004) or in limiting environments (clean rooms; La Duc *et al.*, 2007).

In the context of studying the effects of transgenic fructan-producing potatoes on the diversity of the microbial

communities in the phyllosphere, actinobacterial species were found to constitute a significant proportion of the colonizing bacteria. A relatively large number of bacterial taxa belonging to the family *Microbacteriaceae* were found on an isogenic potato line in comparison with the transgenic lines. Within this group, several strains were isolated that displayed characteristics of the genus *Agrococcus*. A comparative analysis of the 16S rRNA gene sequences indicated that these strains represent a novel species within this genus. The aim of this study was to describe the novel species on the basis of data collected by using a polyphasic approach.

To study the diversity of the bacterial community, samples from the phyllosphere of the investigated potato lines were processed as described by Heuer & Smalla (1999). Estimation of population densities and screening for bacterial isolates were performed using different culture media. Heterotrophic bacteria were determined on tryptic soy agar (Merck) and nutrient agar (SIFIN) supplemented with cycloheximide (0.4 g l⁻¹) and incubated at 21 °C for 7 days. To determine the abundance of bacterial taxa, some representative colonies were isolated from each medium, purified and then stored at –80 °C in nutrient broth (SIFIN) containing 40 % glycerol until further processing.

Abbreviations: DAB, 2,4-diaminobutyric acid; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K 114/01^T is AM940157.

The isolates were grouped on the basis of morphological and physiological tests, as described previously (Behrendt *et al.*, 1997, 1999). Representative strains selected from groups with uncertain affiliation at the genus level were investigated using partial sequence analysis of the 16S rRNA gene, as described by Ulrich *et al.* (2008). Strains K 114/01^T and K 35/01 (isolated from nutrient agar) and strain K 42/01 (isolated from tryptic soy agar) were tentatively assigned to the genus *Agrococcus* and subjected to further taxonomic investigation.

Phylogenetic analysis of a representative strain, K 114/01^T, on the basis of the 16S rRNA gene sequence was performed as described by Behrendt *et al.* (2003). Phylogenetic trees were based on a 1420 nt alignment (*Escherichia coli* positions 50–1458) and constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms (PHYLIP, version 3.6; Felsenstein, 1993). The closest phylogenetic neighbours of strain K 114/01^T, as determined from a pairwise sequence comparison, were the type strains of *Microbacterium deminutum* and *Agrococcus jenensis*, with similarities of 97.1 and 96.9 %, respectively. Although the 16S rRNA gene sequence similarity for strain K 114/01^T was slightly greater with respect to the species *M. deminutum*, it clustered with the established species of the genus *Agrococcus* (Fig. 1). This

phylogenetic position was confirmed by the maximum-likelihood tree and was supported by a bootstrap value that was greater than 50 %. Correspondingly, *M. deminutum* KV-483^T formed a cluster with species of the genus *Microbacterium* with both treeing methods (Fig. 1). This position was supported by high bootstrap percentages (86 and 92 %). As reviewed by Stackebrandt *et al.* (2007), the topology of phylogenetic trees in the family *Microbacteriaceae* changes depending on the strain set selected for analysis. For this reason, another tree comprising all type strains of the family was constructed in addition to the tree shown in Fig. 1, which is based on the established species of *Agrococcus* and representative species for the other genera. In the extended tree, the results were similar to those obtained in the tree shown in Fig. 1. Strain K 114/01^T clearly clustered with the species of the genus *Agrococcus*, and all species of the genus *Microbacterium* formed a monophyletic branch that clustered next to the genera *Okibacterium* and *Plantibacter* (data not shown). These results indicated the affiliation of strain K 114/01^T to the genus *Agrococcus*. Furthermore, similarity values below 97 % were obtained with respect to the 16S rRNA gene sequences of established species of the genus *Agrococcus*, suggesting a separate species position for the isolate.

Fig. 1. Neighbour-joining tree, based on almost-complete 16S rRNA gene sequences, showing the relationships between strain K 114/01^T and established species of *Agrococcus* and representative strains from all genera of the family *Microbacteriaceae*. Filled circles indicate branches of the tree that were also obtained using the maximum-likelihood method (Felsenstein, 1981). The sequence of *Arthrobacter globiformis* DSM 20124^T (GenBank accession no. M23411) was used as an outgroup (not shown). Numbers at nodes indicate bootstrap percentages >50 % (based on 1000 resampled datasets). Bar, 0.01 changes per nucleotide position.

A significant chemotaxonomic characteristic for genus affiliation in the family *Microbacteriaceae* is the amino acid composition of the cell wall. The amino acids and peptides in cell-wall hydrolysates of the peptidoglycan of strain K 114/01^T were analysed using two-dimensional ascending TLC on cellulose plates with the solvent system described by Schleifer & Kandler (1972). The characteristic cell-wall diamino acid was 2,4-diaminobutyric acid (DAB), which is typical for the genus *Agrococcus* (Evtushenko & Takeuchi, 2006). In contrast, species of the genus *Microbacterium* are characterized by the presence of ornithine or lysine as the diagnostic diamino acid (Evtushenko & Takeuchi, 2006). Thus, the cell-wall composition substantiates the affiliation of strain K 114/01^T to the genus *Agrococcus*. In addition to DAB, the amino acids alanine, glycine, threonine, aspartic acid and glutamic acid were detected. The cell-wall Ala/Gly/Thr/DAB/Asp/Glu ratio was 2.3:2.1:2.5:0.8:0.9:1.0. This composition is very similar to that of *A. jenensis* (Groth *et al.*, 1996), its closest phylogenetic neighbour in the genus. The same qualitative amino acid composition was reported for the peptidoglycan of *Agrococcus lahauensis* (Mayilraj *et al.*, 2006), while *Agrococcus baldri* (Zlamala *et al.*, 2002) and *Agrococcus citreus* (Wieser *et al.*, 1999) lacked threonine and *Agrococcus casei* (Bora *et al.*, 2007) lacked both threonine and aspartic acid.

Since the phylogenetic analysis and the investigation of the cell-wall composition were only performed for strain K 114/01^T, it was necessary to determine the similarity at the species level for isolates K 35/01 and K 42/01. Recent studies demonstrated the ability of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF MS) to permit the identification of bacteria at the species level in pure culture under carefully controlled culture and analysis conditions (Conway *et al.*, 2001; Fenselau & Demirev, 2001; Valentine *et al.*, 2005). Furthermore, spectra from MALDI-TOF MS reflect the phylogenetic relationship of micro-organisms, corresponding with the results of sequence analyses of conservative genes (Stackebrandt *et al.*, 2005). MALDI-TOF MS analysis was performed according to the method described by Tóth *et al.* (2008). As shown in Fig. 2, the isolates formed a tight cluster that was separable on the basis of clear spectral dissimilarities with respect to all type strains of the genus *Agrococcus*. The high level of spectral similarity between the phyllosphere isolates indicated their affiliation to the same species.

The phyllosphere isolates were also subjected to a multitude of phenotypic tests to determine their similarities. Standard tests for morphological and physiological characterization were performed as described by Behrendt *et al.* (1999). All strains were cultivated on nutrient agar or in the respective broth at 21 °C. Production of L-phenylalanine deaminase was investigated according to the method described by Richard & Kiredjian (1995). Hydrogen sulphide production was tested on Kligler iron agar (Oxoid) and the hydrolysis of L-tyrosine was determined on nutrient agar supplemented with 0.5 %

Fig. 2. Dendrogram, generated by the BioTyper program (version 1.1; Bruker Daltonics), showing the similarities of MALDI-TOF mass spectra of cell extracts from strains K 114/01^T, K 35/01, K 42/01 and type strains of the genus *Agrococcus*. The mass spectrum of *Leifsonia ginsengi* DSM 19088^T served as an outgroup.

(w/v) L-tyrosine. Oxidation of carbon sources was tested using Biolog GP2 microplates. The instructions of the manufacturer were modified in that the strains were cultivated on tryptic soy agar (Mayilraj *et al.*, 2006). Results were read visually after 24 and 48 h incubation at 30 °C. Oxidative acid production from carbohydrates was investigated using API 50 CH strips (bioMérieux) inoculated with a bacterial suspension in CHB/E medium (bioMérieux). The results were not read until after 72 h, as the strains tested showed delayed reactions. API 20NE (bioMérieux) test strips were applied to determine additional physiological and biochemical features. The phyllosphere isolates displayed high levels of conformity with regard to phenotypic features. On the GP2 microplates, in particular, identical reactions were obtained for all 95 carbohydrates. Furthermore, the API 50 CH results for acid production were very similar. Different reactions were found only for oxidative acid production from D-mannitol, N-acetylglucosamine, cellobiose and maltose. Whilst strain K 114/01^T did not show a reaction for these carbohydrates, acid production for strains K 35/01 and K 42/01 was weak for D-mannitol and N-acetylglucosamine but positive for cellobiose and maltose. These results supported those of the MALDI-TOF MS analysis, i.e. that all three isolates represented one species. Details of the morphological and physiological features of the strains are given in the species description. Characteristics that serve to differentiate the phyllosphere isolates from established species of the genus *Agrococcus* are presented in Table 1. The isolates were clearly distinguishable from these *Agrococcus* species on the basis of their ability to use several carbohydrates on the GP2 microplates.

A further prominent taxonomic feature that characterized the isolates and served to differentiate them from related species was their whole-cell fatty acid profiles. Fatty acid methyl esters were extracted from cells cultivated on tryptic soy agar for 1 day at 28 °C and were then analysed with the

Table 1. Differential phenotypic characteristics of *Agrococcus* species

Taxa: 1, *A. versicolor* sp. nov.; 2, *A. baldri*; 3, *A. casei*; 4, *A. citreus*; 5, *A. lahaulensis*; 6, *A. jenensis*. Data are from Groth *et al.* (1996), Wieser *et al.* (1999), Zlamala *et al.* (2002), Mayilraj *et al.* (2006), Bora *et al.* (2007) and this study. +, Positive; –, negative; w, weak reaction; –/+, different reactions obtained by the authors; D, delayed reaction, ND, no data available.

Characteristic	1	2	3*	4	5	6
Amino acids of the peptidoglycan	Ala, Gly, Thr, DAB, Asp, Glu	Ala, Gly, DAB, Asp, Glu	Ala, Gly, DAB, Glu	Ala, Gly, DAB, Asp, Glu	Ala, Gly, Thr, DAB, Asp, Glu†	Ala, Gly, Thr, DAB, Asp, Glu
Growth with 6.5 % NaCl	–	–/+	ND	+	+	+
Hydrolysis of:						
Aesculin	+	–/+	–	+	–	+
Tyrosine	–	–	–	+	–	+
Casein	+	–	–	–	+	–/+
Gelatin	D	–	ND	–	+	–
Starch	–	+	+	+	+	+
Utilization on Biolog GP2 microplates						
Dextrin	+	–	–	–	+	–
Tweens 40 and 80	–	–	+	–	–	–
<i>N</i> -Acetyl-D-glucosamine	+	–	–	–	–	–
L-Arabinose	+	+	–	+	+	+
D-Fructose	+	+	–	+	+	+
D-Gluconic acid	+	+	–	+	–	+
α -D-Glucose	+	+	–	–	–	+
Maltotriose	+	–	–	–	+	–
D-Mannose	+	+	–	+	–	+
D-Psicose	+	+	+	+	–	+
L-Rhamnose	–	–	–	–	+	–
D-Sorbitol	–	+	w	–	–	–
Sucrose	+	–	–	–	–	–
D-Tagatose	w	+	–	–	–	–
Trehalose	+	–	w	–	–	–
Turanose	+	–	–	+	–	–
D-Xylose	+	+	–	+	+	+
α -Ketovaleic acid	w	–	w	–	–	–
Methyl pyruvate	–	–	+	–	–	–
Propionic acid	w	–	w	–	–	–
<i>N</i> -Acetyl L-glutamic acid	–	–	+	–	–	–
L-Alaninamide	+	–	–	–	–	+
Glycerol	+	+	+	–	+	–
Adenosine	+	+	+	–	+	–
2'-Deoxyadenosine	–	–	+	–	+	+
Inosine	–	–	–	–	+	–
Thymidine	–	+	+	–	+	–
D-Fructose 6-phosphate	–	–	–	+	–	–

*Hydrolysis of tyrosine and casein as well as reactions on Biolog GP2 were determined in this study for *A. casei* DSM 18061^T.

†Determined in this study for *A. lahaulensis* K22-21^T.

Sherlock Microbial Identification system (MIDI, version 4.5) as described by Behrendt *et al.* (1999). As shown in Table 2, the cellular fatty acid profiles of the potato isolates were relatively similar, supporting the suggestion that they represent one species. The major fatty acids were anteiso-C_{15:0} (39.8 ± 3.3 %), iso-C_{15:0} (17.1 ± 1.6 %), C_{16:0} (13.9 ± 1.1 %) and anteiso-C_{17:1} (7.6 ± 4.3 %) and minor fatty acids were anteiso-C_{17:0} (5.7 ± 0.6 %), iso-C_{16:0}

(5.6 ± 1.2 %), C_{14:0} (3.0 ± 0.2 %), iso-C_{17:0} (1.9 ± 0.4 %) and iso-C_{14:0} (1.6 ± 0.8 %). Fatty acids typical of the genus, i.e. anteiso-C_{15:0}, iso-C_{15:0}, anteiso-C_{17:1} and iso-C_{16:0}, were present in the isolates. Nevertheless, significant quantitative and qualitative differences were found in comparison with *Agrococcus* species. Notable differences were demonstrated in the quantities of the fatty acids C_{16:0}, iso-C_{15:0} and anteiso-C_{17:0} (Zlamala *et al.*, 2002;

Table 2. Cellular fatty acid compositions (%) of the phyllosphere strains

ND, Not detected; tr, traces (<1 %).

Fatty acid	K 114/01 ^T	K 35/01	K 42/01
iso-C _{13:0}	1.13	tr	tr
anteiso-C _{13:0}	tr	tr	tr
C _{14:0}	3.21	2.81	3.14
iso-C _{14:0}	2.55	1.00	1.17
C _{15:0}	tr	tr	tr
iso-C _{15:0}	15.29	18.37	17.70
anteiso-C _{15:0}	43.56	37.57	38.24
iso-C _{15:1}	tr	1.03	1.02
anteiso-C _{15:1}	tr	tr	tr
C _{16:0}	15.22	13.33	13.26
iso-C _{16:0}	7.06	4.89	4.99
iso-C _{17:0}	1.54	2.34	1.94
anteiso-C _{17:0}	5.07	6.29	5.69
anteiso-C _{17:1}	2.69	9.19	10.78
iso-C _{20:0}	ND	1.25	ND

Mayilraj *et al.*, 2006; Bora *et al.*, 2007). The fatty acid anteiso-C_{17:1} was found only in the potato isolates, whereas C_{14:0} occurred also in the species *A. jenensis*, but only in trace amounts (Groth *et al.*, 1996). Thus, a clear demarcation between the potato isolates and established *Agrococcus* species was possible on the basis of fatty acid profiles. The isoprenoid quinone composition was determined as described by Groth *et al.* (1996). The quinone system of strain K 114/01^T consisted of the menaquinones MK-10, MK-11, MK-8 and MK-9 (ratio of peak areas 16:16:4:2). MK-12, which is typical of all established species of the genus *Agrococcus*, could not be detected. MK-10 was found as an additional major component (as in *A. lahaulensis*; Mayilraj *et al.*, 2006).

In summary, the results of the phenotypic and phylogenetic analyses demonstrated that the isolates of the potato phyllosphere represent a novel species of the genus *Agrococcus*, for which the name *Agrococcus versicolor* sp. nov. is proposed.

Description of *Agrococcus versicolor* sp. nov.

Agrococcus versicolor (ver.si'co.lor. L. adj. *versicolor* colour changing).

Cells are asporogenous, non-motile, short to ovoid rods (0.5–0.6 × 0.8–1.2 µm) and occur singly or in pairs, sometimes forming small irregular clusters. Gram-positive and strictly aerobic. Colonies on nutrient agar are dark pink to red in colour and are smooth with regular margins. Young, solitary, growing colonies are yellow, turning dark pink with age. The optimal growth temperature is 21 °C; no growth at 4 or 37 °C. Nitrate and nitrite are not reduced. Growth occurs at 1 and 2 % NaCl but not at higher concentrations. Catalase activity is present, but

oxidase, urease, L-phenylalanine deaminase, lysine and ornithine decarboxylase, arginine dihydrolase and tryptophan deaminase activities are absent. β-Galactosidase activity is weak. Gelatin, casein and aesculin are hydrolysed, but starch and tyrosine are not. Hydrogen sulphide is not produced. Indole production from L-tryptophan and the reaction in the Voges–Proskauer test are negative. The following are utilized on Biolog microplates GP2: dextrin, N-acetyl-D-glucosamine, L-arabinose, cellobiose, D-fructose, D-gluconic acid, α-D-glucose, maltose, D-mannitol, D-mannose, palatinose, D-psicose, D-ribose, sucrose, D-tagatose, turanose, D-xylose, propionic acid and glycerol. Maltotriose, trehalose, α-hydroxybutyric acid, α-ketovaleric acid, L-alaninamide and adenosine are utilized with prolonged incubation. The following are not utilized: α- and β-cyclodextrin, glycogen, inulin, mannan, Tweens 40 and 80, N-acetyl-D-mannosamine, amygdalin, D-arabitol, arbutin, D-galactose, L-fucose, D-galacturonic acid, gentiobiose, myo-inositol, α-D-lactose, lactulose, melezitose, melibiose, methyl α-D-galactoside, methyl β-D-galactoside, methyl α-D-glucoside, methyl β-D-glucoside, methyl α-D-mannoside, raffinose, L-rhamnose, salicin, sedoheptulosan, D-sorbitol, stachyose, xylitol, acetic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketoglutaric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D- and L-malic acid, monomethyl succinate, succinamic acid, succinic acid, N-acetyl L-glutamic acid, D- and L-alanine, L-alanyl glycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyroglutamic acid, L-serine, putrescine, 2,3-butanediol, 2'-deoxyadenosine, inosine, thymidine, uridine, adenosine 5'-monophosphate, thymidine 5'-monophosphate, uridine 5'-monophosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. Oxidative acid production with API 50 CH is positive for ribose and L-arabinose but weak for D-glucose, D-fructose, D-mannose, sucrose and turanose. Acid production is negative for glycerol, erythritol, D-arabinose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, amygdalin, arbutin, salicin, D-lactose (bovine origin), melibiose, trehalose, inulin, melezitose, raffinose, starch, glycogen, xylitol, gentiobiose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Strain-specific reactions are shown for D-mannitol, N-acetylglucosamine, D-cellobiose and maltose. The diagnostic cell-wall diamino acid is DAB; alanine, glycine, threonine, aspartic acid and glutamic acid are also present. The major fatty acids are anteiso-C_{15:0}, iso-C_{15:0}, C_{16:0} and anteiso-C_{17:1} and minor fatty acids are anteiso-C_{17:0}, iso-C_{16:0}, C_{14:0}, iso-C_{17:0} and iso-C_{14:0}. The major menaquinones are MK-10 and MK-11.

The type strain, K 114/01^T (=DSM 19812^T =LMG 24386^T), was isolated from the phyllosphere of potato plants in Dahnsdorf, Germany.

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