Chryseobacterium luteum sp. nov., associated with the phyllosphere of grasses

Undine Behrendt,¹ Andreas Ulrich,¹ Cathrin Spröer² and Peter Schumann²

Correspondence

Undine Behrendt
ubehrendt@zalf.de

¹Leibniz-Centre for Agricultural Landscape Research (ZALF), Institute of Landscape Matter Dynamics, Eberswalder Straße 84, D-15374 Müncheberg, Germany

²DSMZ – German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, D-38124 Braunschweig, Germany

Three isolates obtained from grass samples were investigated by means of a polyphasic taxonomic study and were shown to represent a novel species within the genus *Chryseobacterium*. Comparison of 16S rRNA gene sequences and phenotypic features indicated that the three isolates belonged to a single species. On the basis of 16S rRNA gene sequence analysis, the closest phylogenetic neighbours were *Chryseobacterium shigense* and *Chryseobacterium vrystaatense*, which formed a stable cluster with the isolates; this phylogeny was supported by a high bootstrap value and was obtained using different treeing methods. A DNA–DNA hybridization study with the closest neighbour, *C. shigense* DSM 17126^T (98.3 % 16S rRNA gene sequence similarity), clearly demonstrated a separate species status for the grass isolate strain P 456/04^T. Comparisons involving physiological properties and whole-cell fatty acid profiles confirmed this result at the phenotypic level. On the basis of these results, strain P 456/04^T represents a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium luteum* sp. nov. is proposed. The type strain is P 456/04^T (=DSM 18605^T =LMG 23785^T).

The genus *Chryseobacterium* was proposed by Vandamme *et al.* (1994) and has a rapidly growing membership, as demonstrated by the multitude of novel species that have been proposed in recent years (de Beer *et al.*, 2005, 2006; Kim *et al.*, 2005; Shen *et al.*, 2005; Shimomura *et al.*, 2005; Young *et al.*, 2005; Gallego *et al.*, 2006; Park *et al.*, 2006; Tai *et al.*, 2006; Weon *et al.*, 2006). Strains belonging to this genus occur in a variety of ecological niches. They have been recovered from soil, sewage, fresh water, marine sediments, clinical samples and various food sources. Furthermore, *Chryseobacterium* strains have been found associated with plants, either living epiphytically in plant phyllospheres and rhizospheres or endophytically inside several plant species (Bacon & Hinton, 2006; Beattie, 2006; Park *et al.*, 2006).

In the context of a study on bacterial diversity associated with the phyllosphere of grasses, a culture-dependent approach (Behrendt, 2001) was used to isolate several bacterial strains, which were tentatively identified as *Chryseobacterium* sp. The aim of this study was to investigate three of these isolates by using a polyphasic

classification strategy to determine their taxonomic position(s).

To investigate the structure of the community of heterotrophic bacteria, grass samples were taken from plots characterized by different levels of management intensity, as described by Behrendt (2001). Grass material was homogenized in distilled water, using a Stomacher laboratory blender, and serial dilutions were plated on nutrient agar (SIFIN) supplemented with cycloheximide (0.4 g l⁻¹), to ensure selectivity for bacterial growth. After incubation of the plates at 21 °C for 7 days, a number of representative strains were isolated to determine the community structure of the culturable heterotrophic bacteria. Strains P 456/04^T, P 538/13 and P 528/18 were selected from a group of isolates displaying characteristics of the genus *Chryseobacterium* and were subjected to taxonomic investigations.

Sequencing of amplified 16S rRNA genes of the grass isolates was performed as described previously (Behrendt *et al.*, 2003). For phylogenetic analysis, sequences of the grass isolates and of species of the genus *Chryseobacterium* (including those with validly published names and those whose names have not been validly published; Fig. 1) were aligned using the CLUSTAL_X program (Thompson *et al.*, 1997). The phylogenetic trees were based on a 1396 nt alignment (*Escherichia coli* position 39–1445) and

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains P 456/04^T (=DSM 18605^T), P 528/18 and P 538/13 are AM489609, AM489610 and AM489611, respectively.

Fig. 1. Neighbour-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships of the studied grass isolates with respect to known species of the genus *Chryseobacterium*. Filled circles indicate branches of the tree that were also produced using the maximum-likelihood method (Felsenstein, 1981). To estimate the root position of the tree, *Flavobacterium hydatis* DSM 2063^T (GenBank accession no. AM230487) was used as an outgroup (not shown). Numbers at nodes indicate levels of bootstrap support (>50%), based on 1000 resampled datasets. Bar, 0.01 changes per nucleotide position.

constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms (PHYMLP, version 3.6; Felsenstein, 1993). As shown in Fig. 1, the grass isolates formed a separate branch and clustered tightly together, demonstrating a very close relationship. 16S rRNA gene sequence similarity values of 99.7% between P 456/04^T and P 528/18 and of 100% between P 456/04^T and P 538/13 supported their affiliation to the same species. The type strains of *Chryseobacterium shigense* and *Chryseobacterium vrystaatense* were phylogenetic neighbours of the grass isolates, showing sequence similarities of 98.3 and 97.3%, respectively, with respect to strain P 456/04^T. The clustering of the grass isolates with these two species was supported by a relatively high bootstrap value (97%). Furthermore, this branching was also found with the maximum-likelihood algorithm, which demonstrated the relatively high stability of the cluster. Recent recommendations by Stackebrandt & Ebers (2006) have suggested a 16S rRNA gene sequence similarity threshold of 98.7–99.0%, above which DNA–DNA reassociation experiments would be mandatory for confirming separate species status for a novel isolate. On the basis of this recommendation, the grass isolates could be clearly distinguished from their phylogenetic neighbours. Nevertheless, strain P 456/04^T and its closest neighbour, *C. shigense* DSM 17126^T, were subjected to a DNA–DNA hybridization study performed in 2 × SSC plus 10% DMSO at 61 °C according to Martin *et al.* (1997). A reassociation value of 14% (repetition, 10.6%) was found. According to the recommendation of Wayne *et al.* (1987) for species delineation, separate species status for the grass isolate was clearly supported at the genomic level.

Morphological and physiological characterization of the grass isolates was performed as described by Behrendt *et al.*

(1999). All strains were cultivated on nutrient agar or in the corresponding broth at 21 °C. The presence of flexirubin-type pigments was tested as described by Bernardet *et al.* (2002). An extensive investigation of oxidative acid production from carbohydrates was carried out using API 50 CH strips inoculated with a bacterial suspension in CHB/E Medium (bioMérieux). Reading of the results was not performed until 72 h, as the strains tested showed delayed reactions. In addition, physiological characteristics and enzyme activities were determined using API 20NE and API ZYM strips (bioMérieux). Contrary to the manufacturer's recommendation, all strips were incubated at 25 °C.

The morphological and physiological characteristics of the grass isolates are given in the species description. The characteristics of the isolates tested were compared and were found to be relatively consistent. Strain P 528/18 differed from the other two strains only in its inability to produce acid oxidatively from starch and to produce cystine arylamidase and α -chymotrypsin. These results supported the assumption, based on the results of the phylogenetic analysis, that the grass isolates belonged to one species.

A comparison of selected phenotypic features of the grass isolates with those of *Chryseobacterium* species (Table 1) revealed that the former differed in several respects. These differences, in combination, served to differentiate the isolates from recognized members of the genus *Chryseobacterium*.

The determination of whole-cell fatty acid compositions also provides valuable taxonomic information at the genus and species level for the taxon *Chryseobacterium* (Bernardet

Table 1. Differential phenotypic characteristics of strains P 456/04^T, P 528/18 and P 538/13 and *Chryseobacterium* species

Taxa: 1, strains P 456/04^T, P 528/18 and P 538/13 (*C. luteum* sp. nov.); 2, *C. balustinum* (n=1); 3, *C. caeni* (n=1); 4, *C. daecheongense* (n=1); 5, *C. defluvii* (n=1); 6, *C. formosense* (n=1); 7, *C. gleum* (n=2); 8, *C. hispanicum* (n=1); 9, *C. indologenes* (n=7); 10, *C. indoltheticum* (n=1); 11, *C. joostei* (n=11); 12, *C. piscium* (n=4); 13, '*C. proteolyticum*' (n=2); 14, *C. scopthalmum* (n=2); 15, *C. shigense* (n=1); 16, *C. soldanellicola* (n=1); 17, *C. taeanense* (n=1); 18, *C. taichungense* (n=1); 19, *C. taiwanense* (n=1); 20, *C. vrystaatense* (n=36); 21, *C. wanjuese* (n=1). Data are from Mudarris *et al.* (1994), Yamaguchi & Yokoe (2000), Hugo *et al.* (2003), de Beer *et al.* (2005), Shen *et al.* (2005), Shimomura *et al.* (2005), de Beer *et al.* (2006), Gallego *et al.* (2006), Park *et al.* (2006), Tai *et al.* (2006), Weon *et al.* (2006), Quan *et al.* (2007) and this study. +, Positive; -, negative; w, weak reaction; d, different reactions; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
Growth at:																					
5 °C	+	+	+	-	d	-	-	+	-	+	+	+	ND	d	+	+	+	-	+	+	+
37 °C	-	d	+	+	+	-	+	-	d	d	-	-	+	-	-	+	+	+	+	-	+
Growth on MacConkey agar	-	+	-	-	-	-	+	-	d	+	+	-	-	-	-	-	-	-	ND	-	w
Urease activity	-	-	+	-	-	-	d	-	-	-	d	+	-	+	-	-	-	-	-	+	-
Production of indole	+	+	-	-	+	+	+	w	+	+	+	+	+	-	+	-	-	w	+	+	-
Nitrate reduction	-	+	-	+	-	-	d	+	d	-	-	+	-	-	-	-	-	-	-	-	-
Acid production from:																					
L-Arabinose	+	-	-	-	-	-	d	ND	-	-	-	ND	+	+	-	-	-	-	+	ND	-
D-Cellobiose	-	+	ND	+	d	-	d	ND	-	-	-	ND	-	+	ND	ND	ND	-	+	ND	-
D-Fructose	+	+	-	+	+	ND	+	+	d	-	+	ND	ND	-	+	-	-	ND	d	ND	-
D-Glucose	+	+	-	-	+	w	+	+	+	+	+	ND	+	+	+	ND	ND	w	ND	ND	+
Glycerol	+	-	-	+	+	-	+	+	d	-	d	ND	w	-	-	-	-	ND	-	ND	-
Maltose	+	-	-	-	+	-	+	+	+	+	+	ND	+	-	-	-	-	+	ND	ND	-
D-Mannitol	-	-	-	-	-	-	-	-	d	-	d	ND	+	+	-	-	-	-	-	ND	-
Salicin	+	-	ND	-	-	ND	-	ND	-	-	-	ND	-	+	ND	ND	ND	-	ND	ND	-
Sucrose	+	d	-	-	-	-	-	ND	-	-	-	ND	+	+	ND	ND	ND	-	-	ND	-
Trehalose	+	-	-	+	+	+	+	-	+	-	+	ND	+	d	-	-	-	+	-	ND	+
D-Xylose	-	-	-	+	-	w	d	+	-	-	-	ND	ND	+	-	-	-	+	ND	ND	-
Hydrolysis of:																					
2-Naphthyl butyrate	-	-	+	-	w	+	-	+	-	-	-	ND	ND	+	ND	+	+	+	-	ND	w
N-Benzoyl-DL-arginine	+	-	-	-	+	+	-	+	-	-	+	ND	ND	+	ND	-	-	+	-	ND	-
2-naphthylamide																					
2-Naphthyl	+	-	-	-	-	-	-	-	-	-	-	ND	ND	-	ND	-	-	w	-	ND	-
β-D-galactopyranoside																					
2-Naphthyl α-D-glucopyranoside	+	-	-	-	+	-	+	+	+	+	+	ND	ND	-	ND	+	+	+	+	ND	w
6-Bromo-2-naphthyl	+	-	+	-	-	+	+	+	-	-	-	ND	ND	+	ND	-	+	+	-	ND	-
β-D-glucopyranoside																					
DNA	-	+	ND	ND	ND	ND	+	-	+	+	+	+	+	+	ND	-	-	ND	ND	+	w
Starch	+	-	-	+	+	ND	+	+	+	-	+	-	+	-	+	ND	ND	ND	+	-	+

et al., 2002). Fatty acid methyl esters were extracted from cells cultivated on tryptic soy agar for 1 day at 28 °C and analysed with the 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 grass isolates were relatively similar, supporting the suggestion that they are affiliated to one species. Furthermore, the presence of large amounts of iso-C_{15:0}, iso-C_{17:1ω9c} and iso-C_{17:0} 3-OH was typical of members of the genus *Chryseobacterium*. On the other hand, the presence of more than 1.0% C_{17:0} 2-OH served to distinguish the grass isolates from known species in the genus (with the exception of *Chryseobacterium indoltheticum*) (de Beer *et al.*, 2006; Gallego *et al.*, 2006; Park *et al.*, 2006; Tai *et al.*, 2006; Weon *et al.*, 2006).

The results of the phylogenetic and phenotypic analyses clearly demonstrated that the grass isolates represent a novel species of the genus *Chryseobacterium*, for which the name *Chryseobacterium luteum* sp. nov. is proposed.

Description of *Chryseobacterium luteum* sp. nov.

Chryseobacterium luteum (lu'te.um. L. neut. adj. *luteum* orange-coloured).

Cells are non-spore-forming, non-motile single rods. Gram-negative with classical Gram-staining but produces a false Gram-positive reaction with the fast KOH test. Strictly aerobic. On nutrient agar, produces orange colonies that are smooth with regular margins. Flexirubin-type pigments are present. Optimal growth

Table 2. Cellular fatty acid compositions (%) of the grass isolates

ND, Not detected; tr, traces (<1 %). ECL, Equivalent chain-length (i.e. unknown fatty acid).

Fatty acid	P 456/04 ^T	P 538/13	P 528/18
iso-C _{13:0}	1.45	1.20	2.14
ECL 13.565	1.78	1.44	1.70
C _{14:0}	ND	ND	tr
iso-C _{15:0}	40.51	34.85	37.81
C _{15:0} 2-OH	tr	tr	tr
iso-C _{15:0} 2-OH	6.84	9.26	7.93
iso-C _{15:0} 3-OH	2.83	2.89	2.77
anteiso-C _{15:0}	3.7	4.98	5.45
C _{16:0}	tr	tr	tr
iso-C _{16:0}	tr	tr	ND
C _{16:0} 3-OH	tr	tr	tr
iso-C _{16:0} 3-OH	tr	tr	tr
ECL 16.582	1.52	1.38	1.50
C _{17:0} 2-OH	1.36	2.53	2.33
iso-C _{17:0}	tr	tr	tr
iso-C _{17:0} 3-OH	16.15	17.05	17.29
iso-C _{17:1}	tr	tr	tr
iso-C _{17:1} ω9c	19.22	18.93	15.56
C _{18:1} ω5c	tr	tr	tr

temperature is 21 °C. At 4 °C, slow growth can be observed, but known isolates are unable to grow at 37 °C. Grows on nutrient and trypticase soy agars but not on MacConkey agar. Catalase and oxidase activities are present. Arginine dihydrolase activity is absent. Gelatin, starch and aesculin are hydrolysed. Hydrolysis of casein is strain-dependent. Chitin, urea and DNA are not hydrolysed. D-Glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate and phenylacetic acid are not assimilated in API 20NE test strips. With API 50 CH strips, acid is produced oxidatively from glycerol, L-arabinose, D-glucose, D-fructose, D-mannose, amygdalin, arbutin, salicin, maltose, sucrose, trehalose, glycogen and gentiobiose. Acid is not produced from erythritol, D-arabinose, D- or L-xylose, D-adonitol, methyl β-D-xylopyranoside, D-galactose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α-D-mannopyranoside, methyl α-D-glucopyranoside, N-acetylglucosamine, D-cellobiose, D-lactose (bovine origin), D-melibiose, inulin, D-melezitose, D-raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D- or L-fucose, D- or L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. Acid production from starch is strain-dependent. With API ZYM strips, alkaline phosphatase, esterase lipase (C8), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase and N-acetyl-β-glucosaminidase activities are present. Esterase (C4), lipase (C14), α-galactosidase,

β-glucuronidase, α-mannosidase and α-fucosidase activities are absent. Cystine arylamidase and α-chymotrypsin activities are strain-dependent. The fatty acids largely comprise iso-C_{15:0} (37.7 % ± 2.8), iso-C_{17:1} ω9c (17.9 % ± 2.0), iso-C_{17:0} 3-OH (16.8 % ± 0.6), iso-C_{15:0} 2-OH (8.0 % ± 1.2) and anteiso-C_{15:0} (4.7 % ± 0.9).

The type strain, P 456/04^T (=DSM 18605^T =LMG 23785^T), was isolated from the phyllosphere of grasses in Paulinenaue, Germany.

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