Chryseobacterium gregarium sp. nov., isolated from decaying plant material

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In this study, strain P 461/12^T, isolated from decaying plant material after mulching a pasture, was shown to represent a novel species of the genus *Chryseobacterium* by means of a polyphasic approach. The closest phylogenetic neighbours to the novel strain, as determined by 16S rRNA gene sequence analysis, were *Chryseobacterium daeguense* K105^T and *Chryseobacterium soldanellicola* PSD1-4^T with gene sequence similarities of 97.4 % and 97.2 %, respectively. Strain P 461/12^T could be differentiated by means of its RiboPrint pattern from the type strains of all recognized *Chryseobacterium* species belonging to the same cluster as determined by 16S rRNA gene sequence comparisons. The nearest phylogenetic neighbours, and in particular the closest relatives *C. daeguense* and *C. soldanellicola*, could be distinguished from the novel isolate by means of several physiological features and also by the remarkably lower proportion of anteiso-C_{15:0} in the whole-cell fatty acid profile. Based on these findings, the new isolate represents a novel species, for which the name *Chryseobacterium gregarium* sp. nov. is proposed. The type strain is P 461/12^T (=DSM 19109^T=LMG 24052^T).

Studies of the composition of the bacterial community in the soil, rhizosphere, phyllosphere and endosphere of plants have shown that strains of the genus *Chryseobacterium* are indigenous to these habitats (Mahaffee & Kloepper, 1997; Bacon & Hinton, 2006; Beattie, 2006). *Chryseobacterium indologenes* and *Chryseobacterium balustinum* were the most common species found in several studies (Krechel *et al.*, 2002; Beattie, 2006). In recent years, several novel species isolated from these habitats have been described (Young *et al.*, 2005; Park *et al.*, 2006; Tai *et al.*, 2006; Weon *et al.*, 2006; Behrendt *et al.*, 2007b). Furthermore, studies using newly developed primers have shown that members of the genus *Chryseobacterium* form a high proportion of the bacterial community of several composts (Green *et al.*, 2006). Investigation of seed and root surfaces of plants growing in soil amended with these composts has revealed a surprising prevalence and diversity of chryseobacteria, indicating that they are major contributors to nutrient cycling in plant environments (Green *et al.*, 2006).

In the context of studying low-intensity forms of grassland use, the effect of mulching treatments on the microflora colonizing the regrowth was investigated in comparison with the mowing of pastures for forage production (Behrendt, 2001). From the decaying grass material produced after mulching, a novel strain, designated

P 461/12^T, was isolated that displayed general characteristics of members of the genus *Chryseobacterium* (Bernardet *et al.*, 2006). A comparative analysis of the 16S rRNA gene sequences indicated that the new strain represented a novel species within this genus. The aim of this study was to determine the precise taxonomic position of the novel strain by using a polyphasic approach.

To study the community structure of culturable heterotrophic bacteria colonizing decaying grass, mulched material was taken from grassland plots as described by Behrendt (2001). The material was cut and homogenized in distilled water by using a Stomacher lab blender and serial dilutions were plated on nutrient agar (SIFIN), supplemented by cycloheximide (0.4 g l⁻¹). After incubation at 21 °C for 7 days, a representative number of strains was isolated to determine the community structure. Characterization of a multitude of randomly selected isolates by phenotypic and genetic analysis revealed several strains of unclear taxonomic position. One of these strains, isolate P 461/12^T, was subjected to a detailed taxonomic investigation.

The phylogenetic analysis based on 16S rRNA gene sequences was performed as described by Behrendt *et al.* (2003). Phylogenetic trees were based on a 1411 nt alignment (*Escherichia coli* positions 39–1445) and constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain P 461/12^T is AM773820.

(PHYLIP version 3.6; Felsenstein, 1993). As shown in Fig. 1, strain P 461/12^T clustered clearly with the type strain of *Chryseobacterium daeguense* in both methods of treeing, this was supported by a relatively high bootstrap of 78 % compared with the other species of the genus *Chryseobacterium* clustering next to the novel isolate. The 16S rRNA gene sequence similarity between strain P 461/12^T and *C. daeguense* was 97.4 %, followed by 97.2 % similarity to *Chryseobacterium soldanellicola*. Species that were found in the cluster grouping strain P 461/12^T and *C. soldanellicola* (Fig. 1) showed gene sequence similarity values of 94.5–96.8 %. A comparison between the neighbour-joining and the maximum-likelihood tree showed that the positions of several species were not stable in this cluster. However, the 16S rRNA gene sequence similarity of the novel isolate to its closest phylogenetic neighbours fell far below the range of 98.7–99.0 % recommended by Stackebrandt & Ebers (2006) as the threshold above which DNA–DNA reassociation experiments should be performed in order to confirm the status of a novel species. Thus, the analysis of the 16S rRNA gene sequences demonstrated the separate position of the novel isolate compared with other species of the genus *Chryseobacterium*.

Ribotyping was also performed to differentiate between the closest phylogenetic neighbours. This fingerprint technique

Fig. 1. Neighbour-joining tree showing the relationship of strain P 461/12^T with other species of the genus *Chryseobacterium*. Filled circles indicate branches of the tree that were also formed by using the maximum-likelihood method (Felsenstein, 1981). *Flavobacterium hydatidis* (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.

is based on the analysis of fragments of genomic DNA generated by digestion with restriction endonucleases (e.g. *EcoRI*, *PvuII*). The DNA fragments are separated by electrophoresis according to their size, plotted on a nylon membrane and subsequently hybridized with a chemically labelled probe derived from the rRNA operon of *Escherichia coli*. The bands are detected by using a chemiluminescent substrate and the image of the bands is recorded by a customized charge-coupled device camera (Allerberger & Fritschel, 1999). It is a sensitive, highly discriminatory technique that is effective for the differentiation of strains. As shown for *Elizabethkingia meningoseptica* (formerly *Chryseobacterium meningosepticum*), the use of more than one restriction enzyme has enabled the technique to be used for epidemiological studies (Bernardet *et al.*, 2006). On the other hand, the potential of this technique to differentiate between species with the use of one restriction enzyme has been demonstrated for members of the genus *Pseudomonas* (Sikorski *et al.*, 2001; Behrendt *et al.*, 2003, 2007a). Strain P 461/12^T and the type strains of related species that formed the internal cluster in the 16S rRNA gene sequence analysis (Fig. 1) were studied by ribotyping with the restriction enzyme *EcoRI*. The analysis was performed with an automated RiboPrinter microbial characterization system (Qualicon Du Pont). Band patterns were compared using the BioNumerics software (Applied Maths) and clustering was carried out by UPGMA based on Pearson's correlation coefficient (optimization coefficient, 1.2 %). As shown in Fig. 2, the novel isolate was clearly differentiated from the type strains of its nearest phylogenetic neighbours *C. daeguense* and *C. soldanellicola*. Both type strains were grouped in a separate cluster showing a low similarity to strain P 461/12^T. The most similar ribopattern was found for *Chryseobacterium daecheongense*, which shared 96.1 % 16S rRNA gene sequence similarity with the isolate. However, the dendrogram displayed a higher distance between these strains compared with the distance between the established species *Chryseobacterium wanjuae* and *C. soldanellicola* (Fig. 2). Thus, the results of RiboPrinting substantiated the separate species position of the novel isolate as determined by 16S rRNA gene sequence analysis.

Morphological and physiological characterization of the novel strain isolated from decaying plant material was performed as described by Behrendt *et al.* (2007b). Incubation of the API 20NE, API 50 CH and API ZYM test strips (bioMérieux) was performed at 25 °C. The reading of the results of the API 50 CH test strip was postponed to 72 h as the novel strain showed a delayed reaction. Production of L-phenylalanine deaminase was investigated according to the method of Richard & Kiredjian (1995). Hydrogen sulphide production was tested on Kligler iron agar (Oxoid) and hydrolysis of L-tyrosine was examined on nutrient agar (SIFIN) supplemented with 0.5 % (w/v) L-tyrosine. Growth on cetrimide agar was examined on a *Pseudomonas* agar base with CFC Selective Supplement (Oxoid). All morphological and

Fig. 2. RiboPrint patterns of *Eco*RI-restricted DNA of strain P 461/12^T and the type strains of related *Chryseobacterium* species as determined by 16S rRNA gene sequence comparisons.

physiological characteristics are given in the species description. As shown in Table 1, selected phenotypic features of strain P 461/12^T were compared with those of species of the genus *Chryseobacterium*. Several characteristics for each species were different, which allowed the novel isolate to be distinguished from the recognized species of the genus *Chryseobacterium*.

The DNA base composition was determined after disruption of bacterial cells by using a French pressure cell. After purification on hydroxyapatite according to the procedure of Cashion *et al.* (1977), the DNA was degraded to nucleosides by using P1 nuclease and bovine intestinal mucosa alkaline phosphatase as described by Mesbah *et al.* (1989). The nucleosides were separated by reversed-phase HPLC as described by Tamaoka & Komagata (1984). The G+C content of the DNA of strain P 461/12^T was 38.4 mol%. This value is at the upper end of the G+C content range previously determined for the genus *Chryseobacterium* as shown in Table 1.

The cellular fatty acid content of cells grown on trypticase soy agar (Merck) for 24 h at 28 °C was determined with the Sherlock Microbial Identification system (MIDI version 4.5) as described by Behrendt *et al.* (1999). The fatty acid profile of strain P 461/12^T consisted of branched fatty acids iso-C_{15:0} (35.1 %), iso-C_{17:1ω9c} (16.9 %), anteiso-C_{15:0} (9.1 %) and iso-C_{13:0} (1.3 %), the hydroxy fatty acids iso-C_{15:0} 2-OH (10.6 %), iso-C_{17:0} 3-OH (10.0 %), iso-C_{15:0} 3-OH (2.8 %) and C_{16:0} 3-OH (1.2 %), the unsaturated fatty acid C_{18:1ω5c} (1.2 %) and two unknown fatty acids with equivalent chain lengths (ECL) of 13.565 (1.2 %) and 16.582 (1.1 %). Furthermore, traces (<1.0 % of total fatty acids) were found for the straight chain fatty acids C_{16:0} and C_{14:0}, the branched fatty acids iso-C_{17:0} and iso-C_{16:0}, the hydroxy fatty acids C_{17:0} 2-OH, iso-C_{16:0} 3-OH and C_{15:0} 2-OH, and the unsaturated fatty acid iso-C_{17:1}. This fatty acid profile was similar to those of other *Chryseobacterium* species. The presence of large amounts of iso-C_{15:0}, iso-C_{17:1ω9c} and iso-C_{17:0} 3-OH in particular is typical for the genus *Chryseobacterium*. On the other hand, a relatively high proportion of the fatty acid anteiso-C_{15:0} (9.1 %) was found for isolate P 461/12^T. This

differentiated the novel strain from the nearest phylogenetic neighbours *C. daeguense* and *C. soldanellicola*, which contain traces and 1.9 % of this fatty acid, respectively (Park *et al.*, 2006; Yoon *et al.*, 2007). Furthermore, all other recognized species of the genus *Chryseobacterium* contain <4.5 % of anteiso-C_{15:0} (Park *et al.*, 2006; Tai *et al.*, 2006; Behrendt *et al.*, 2007b; Quan *et al.*, 2007; Zhou *et al.*, 2007), except *Chryseobacterium indoltheticum* for which levels of 4.9 % (Quan *et al.*, 2007) and 6.2 % (Tai *et al.*, 2006) of this fatty acid have been reported. Thus, the relatively high proportion of this fatty acid is a further prominent phenotypic feature for the characterization of the novel isolate.

The results of the phylogenetic and phenotypic analyses demonstrate that the new isolate from the decaying plant material does indeed represent a novel species within the genus *Chryseobacterium*, for which the name *Chryseobacterium gregarium* sp. nov. is proposed.

Description of *Chryseobacterium gregarium* sp. nov.

Chryseobacterium gregarium (gre.ga'ri.um. L. neut. adj. *gregarium* belonging to the flock/herd; an ordinary species of the genus).

Cells are non-spore-forming, non-motile rods which are 0.6–0.8 µm in diameter and 1.5–2.2 µm in length and occur singly. They are Gram-negative by classical Gram-staining but the fast KOH test shows a false Gram-positive reaction. Strictly aerobic. The orange colonies on nutrient agar are smooth with regular margins. Flexirubin type pigments are produced. Slow growth occurs at 4 °C, active growth occurs at 20–30 °C and no growth occurs at 37 °C. No growth occurs on MacConkey or cetrimide agars. Catalase, oxidase and L-phenylalanine deaminase activities are present, but urease, arginine dihydrolase and DNase activities are absent. Casein, gelatin, starch, Tween 80 and aesculin are hydrolysed, but chitin and L-tyrosine are not. Indole is produced from L-tryptophan. Nitrate and nitrite are not reduced. Hydrogen sulphide is not produced. D-Glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, maltose, potassium gluconate, capric acid,

Table 1. Differential phenotypic characteristics of species of the genus *Chryseobacterium*

Species: 1, *C. gregarium* sp. nov. ($n=1$); 2, *C. balustinum* ($n=1$); 3, *C. caeni* ($n=1$); 4, *C. daecheongense* ($n=1$); 5, *C. daeguense* ($n=1$); 6, *C. defluvii* ($n=1$); 7, *C. flavum* ($n=1$); 8, *C. formosense* ($n=1$); 9, *C. gleum* ($n=2$); 10, *C. hispanicum* ($n=1$); 11, *C. indologenes* ($n=7$); 12, *C. indoltheticum* ($n=1$); 13, *C. joostei* ($n=11$); 14, *C. luteum* ($n=3$); 15, *C. piscium* ($n=4$); 16, '*C. proteolyticum*' ($n=2$); 17, *C. scopthalmum* ($n=2$); 18, *C. shigense* ($n=1$); 19, *C. soldanellicola* ($n=1$); 20, *C. taeanense* ($n=1$); 21, *C. taichungense* ($n=1$); 22, *C. taiwanense* ($n=1$); 23, *C. vrystaatense* ($n=36$); 24, *C. wanjuense* ($n=1$). Data from Mudarris *et al.* (1994), Yamaguchi & Yokoe (2000), Hugo *et al.* (2003), Kämpfer *et al.* (2003), de Beer *et al.* (2005), Kim *et al.* (2005), Shen *et al.* (2005), Shimomura *et al.* (2005), Young *et al.* (2005), de Beer *et al.* (2006), Gallego *et al.* (2006), Park *et al.* (2006), Tai *et al.* (2006), Weon *et al.* (2006), Behrendt *et al.* (2007b), Quan *et al.* (2007), Yoon *et al.* (2007), Zhou *et al.* (2007) and this study. +, Positive; –, negative; w, weak reaction; D, delayed reaction; v, variable; 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	22	23	24
Growth at:																								
5 °C	D	D	+	–	–	–	+	–	–	D	–	+	+	D	+	ND	D	+	+	+	–	+	+	+
37 °C	–	+	+	+	+	+	+	–	+	–	v*	v*	–	–	–	+	–	–	+	+	+	+	–	+
Growth on:																								
MacConkey agar	–	+	–	–	–	–	+	–	+	–	v	+	+	–	–	–	–	–	–	–	–	ND	–	w
Cetrimide agar	–	–	ND	ND	ND	–	+	ND	–	ND	+	–	+	–	–	ND	–	ND	–	–	ND	–	+	ND
Activity of:																								
Urease	–	–	+	–	–	–	–	–	v	–	–	–	v	–	+	–	+	–	–	–	–	–	+	–
Phenylalanine deaminase	+	–	ND	ND	ND	–	ND	ND	–	ND	–	+	–	–	+	–	–	ND	–	+	ND	+	–	ND
Production of:																								
H ₂ S	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	w	–	–	–	–	–	–	v	ND
Indole	–	+	–	–	–	+	–	+	+	w	+	+	+	–	+	+	–	+	–	–	w	–	+	–
Reduction of:																								
Nitrate	–	+	–	+	–	–	–	–	v	+	v	–	–	–	+	–	–	–	–	–	–	+	–	–
Nitrite	–	–	–	–	–	ND	–	ND	+	+	–	–	–	–	–	–	–	ND	–	–	ND	–	+	ND
Acid production from:																								
L-Arabinose	+	–	–	–	w	–	ND	–	v	+	–	–	–	+	ND	+	–	–	–	–	–	+	ND	–
D-Cellobiose	–	+	ND	+	–	w	–	–	–	ND	–	–	–	–	ND	–	+	ND	ND	ND	–	+	ND	–
D-Fructose	–	+	–	+	+	+	+	ND	+	+	D	–	+	+	ND	ND	–	+	–	–	ND	v	ND	–
D-Glucose	+	+	–	–	+	+	+	w	+	+	+	+	+	+	ND	+	+	+	ND	ND	w	ND	ND	+
Glycerol	–	–	–	+	–	+	ND	–	+	+	D	–	v	+	ND	w	–	–	–	–	ND	–	ND	–
Maltose	+	–	–	–	+	+	+	–	+	+	+	+	+	+	ND	+	–	–	–	–	+	ND	ND	–
Salicin	+	–	ND	–	–	–	–	ND	–	ND	–	–	–	+	ND	–	+	ND	ND	ND	–	ND	ND	–
Sucrose	+	–	–	–	–	–	–	–	–	ND	–	–	–	+	ND	+	+	ND	ND	ND	–	–	ND	–
Trehalose	+	–	–	+	+	+	+	+	+	–	+	–	+	+	ND	+	+	–	–	–	+	–	ND	+
D-Xylose	–	–	–	+	w	–	+	w	v	+	–	–	–	–	ND	ND	–	–	–	–	+	ND	ND	–
Hydrolysis of:																								
2-Naphthyl butyrate	+	–	+	–	+	w	ND	+	–	+	–	–	–	–	ND	ND	+	ND	+	+	+	–	ND	w
N-Benzoyl-DL-arginine 2-naphthylamide	–	–	–	–	–	+	ND	+	–	+	–	–	+	+	ND	ND	+	ND	–	–	+	–	ND	–
2-Naphthyl β-D-galactopyranoside	–	–	–	–	–	–	ND	–	–	–	–	–	–	+	ND	ND	–	ND	–	–	w	–	ND	–
2-Naphthyl α-D-glucopyranoside	w	–	–	–	+	+	ND	–	+	+	+	+	+	+	ND	ND	–	ND	+	+	+	+	ND	w
6-Bromo-2-naphthyl β-D-glucopyranoside	+	–	+	–	–	–	ND	+	+	+	–	–	–	+	ND	ND	+	ND	–	+	+	–	ND	–
DNA	–	+	ND	ND	+	ND	–	ND	+	–	+	+	+	–	+	+	+	ND	–	–	ND	ND	+	w
Starch	+	–	–	+	+	+	ND	ND	+	+	+	–	+	+	–	+	–	+	ND	ND	ND	+	–	+
Tween 80	+	+	–	–	+	ND	+	–	+	–	+	+	+	v	v	+	+	ND	–	+	ND	+	–	ND
Tyrosine	–	–	ND	ND	+	+	+	ND	+	ND	–	D	+	–	–	+	+	ND	–	–	ND	–	–	+
DNA G + C (mol%)	38.4	33.1	38.2	36.6	36.8	38.8	37.2	ND	38.0	34.3	38.5	33.8	36.8	38.7†	33.6	37.1	34.2	36.6	28.8	32.1	ND	36.8	37.1	37.8

*Different reactions given by the authors.

†Determined for the type strain DSM 18605^T.

adipic acid, malic acid, trisodium citrate and phenylacetic acid are not assimilated in the API 20NE strip. Oxidative acid production occurs in the API 50 CH strip for L-arabinose, D-ribose, D-glucose, D-mannose, amygdalin, arbutin, salicin, maltose, D-lactose, sucrose, trehalose and gentiobiose, while oxidative acid production does not occur for glycerol, erythritol, D-arabinose, D- and L-xylose, D-adonitol, methyl β -D-xylopyranoside, D-galactose, D-fructose, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl α -D-mannopyranoside, methyl α -D-glucopyranoside, N-acetylglucosamine, D-cellobiose, melibiose, inulin, melezitose, raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. Activity on the API ZYM strip is observed for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -glucosidase and N-acetyl glucosamidase. No activity is found for esterase (C4), lipase (C14), cystine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -galactosidase, β -glucuronidase, α -mannosidase or α -fucosidase. The reaction for esterase lipase (C8), valine arylamidase and α -glucosidase is weak. The main fatty acids (>9%) are iso-C_{15:0}, iso-C_{17:1}ω9c, iso-C_{15:0} 2-OH, iso-C_{17:0} 3-OH and anteiso-C_{15:0}. The DNA G+C content of the type strain is 38.4 mol%.

The type strain, P 461/12^T (=DSM 19109^T=LMG 24052^T), was isolated from decaying plant material in Paulinenaue (Germany).

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