Leucobacter tardus sp. nov., isolated from the phyllosphere of *Solanum tuberosum* L.

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In the course of studying the composition of the microbial community in the phyllosphere of potato plants, a Gram-positive bacterium, strain K 70/01^T, was isolated. On the basis of 16S rRNA gene sequence analysis, it was shown that the investigated isolate represents a novel species in the genus *Leucobacter*; the closest phylogenetic neighbour was *Leucobacter chromiireducens* subsp. *chromiireducens*, with a sequence similarity of 96.8 % to the type strain. Chemotaxonomic characteristics supported affiliation to the genus *Leucobacter*. Strain K 70/01^T displayed the diagnostic diamino acid 2,4-diaminobutyric acid in the cell-wall peptidoglycan. The major fatty acids were anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}, which are typical of the genus. The quinone system consisted of the menaquinones MK-10, MK-11 and MK-9. Differences in several physiological features and the absence of the cell-wall amino acids γ -aminobutyric acid and threonine enabled the isolate to be differentiated from all recognized *Leucobacter* species. Based on these phylogenetic and phenotypic results, it can be concluded that the isolate from the phyllosphere of potato plants represents a novel species, for which the name *Leucobacter tardus* sp. nov. is proposed. The type strain is K 70/01^T (=DSM 19811^T =LMG 24388^T).

The genus *Leucobacter* was proposed by Takeuchi *et al.* (1996) to accommodate a species representing a distinct phylogenetic lineage in the family *Microbacteriaceae* that was characterized by the presence of γ -aminobutyric acid (GABA), an unusual amino acid, in the cell wall. The number of members of the genus *Leucobacter* has increased recently through the description of several novel species (Lin *et al.*, 2004; Morais *et al.*, 2004, 2006; Muir & Tan, 2007; Somvanshi *et al.*, 2007). Although all species are characterized by the diagnostic diamino acid 2,4-diaminobutyric acid (DAB) in the peptidoglycan, the cell-wall type of the genus is not consistent, as differences in the amino acid composition have been detected in several species. The unusual GABA has been found only in *Leucobacter albus* and *Leucobacter komagatae*. Nevertheless, phylogenetic studies on the basis of 16S rRNA gene sequences have shown that the species of the genus form a monophyletic assemblage.

Strains belonging to this genus have been recovered from diverse ecological niches, such as activated sludge from chromium-contaminated wastewater (Morais *et al.*, 2004, 2006), soil (Lin *et al.*, 2004), river sediments (Morais *et al.*,

2006), the water phase of tropospheric clouds (Amato *et al.*, 2007) and nematodes (Muir & Tan, 2007; Somvanshi *et al.*, 2007). In the course of studying the composition of microbial communities in the phyllosphere of potato plants, a strain was isolated that was tentatively affiliated to the genus *Leucobacter* by partial 16S rRNA gene sequence comparison. As a consequence, an extensive phenotypic and phylogenetic investigation was performed to reveal the unambiguous taxonomic position of this phyllosphere isolate.

To study the bacterial community composition, samples from the pinnate leaves of potato plants were processed as described by Heuer & Smalla (1999). Culturable bacteria were investigated on various media after incubation at 21 °C for 7 days. The strain under study, K 70/01^T, was isolated from Baird-Parker agar (Oxoid), used as a semi-selective medium particularly to favour the isolation of bacteria of the family *Micrococcaceae*.

Phylogenetic analysis of strain K 70/01^T on the basis of 16S rRNA gene sequences was performed as described by Behrendt *et al.* (2003). Phylogenetic trees were based on a 1445 nt alignment (*Escherichia coli* positions 38–1469) and constructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms (PHYLIP version 3.6; Felsenstein, 1993). As shown in Fig. 1, the isolate clustered clearly with *Leucobacter* species

Abbreviation: DAB, 2,4-diaminobutyric acid; GABA, γ -aminobutyric acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain K 70/01^T (=DSM 19811^T=LMG 24388^T) is AM940158.

Fig. 1. Neighbour-joining tree based on nearly complete 16S rRNA gene sequences showing the relationship between strain K 70/01^T, established species of the genus *Leucobacter* and type strains from all genera of the family *Microbacteriaceae*. Dots indicate branches of the tree that were also formed by 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 levels of bootstrap support >50 % based on 1000 resampled datasets. Bar, 0.01 changes per nucleotide position.

using both treeing methods. This branch was supported by a 100 % bootstrap confidence level. Nevertheless, strain K 70/01^T was separate from the other *Leucobacter* species within this branch, reflecting the higher sequence similarities among almost all of the established species in comparison with the novel isolate. Pairwise comparisons of 16S rRNA gene sequences between the investigated isolate and the type strains of *Leucobacter* species revealed similarity values in the range 95.8–96.8 %; the shortest evolutionary distance was from *Leucobacter chromiireducens* subsp. *chromiireducens*. The sequence divergence value of >3 % for the phyllosphere isolate was higher than the recommended threshold value that is accepted as a criterion for the delineation of different species (Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). Strain K 70/01^T can thus be considered to be a representative of a separate species in the genus *Leucobacter* on the basis of phylogenetic analysis.

In the family *Microbacteriaceae*, several chemotaxonomic features are important for genus identification and species affiliation (Evtushenko & Takeuchi, 2006). The composition of the cell-wall peptidoglycan in particular is a prominent feature for characterizing novel species. The amino acids and peptides in cell-wall peptidoglycan

hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates using the solvent system described by Schleifer & Kandler (1972). The peptidoglycan of strain K 70/01^T consisted of the amino acids alanine, glycine, DAB and glutamic acid (molar ratio 3.2 : 2.1 : 0.5 : 1.0). This composition was most similar to that determined for *Leucobacter aridicollis* (Moraes *et al.*, 2004). As with *L. aridicollis*, no GABA or threonine was found in the isolate, whereas all other members of the genus have either GABA or threonine in their peptidoglycan (Table 1). Nevertheless, the presence of the diagnostic diamino acid DAB in the isolated strain corresponds with the description of members of the genus *Leucobacter* (Takeuchi *et al.*, 1996). A further chemotaxonomic feature, the isoprenoid quinone composition, was determined as described by Groth *et al.* (1996). The quinone system of strain K 70/01^T consisted of the menaquinones MK-10, MK-11 and MK-9 (molar ratio 9 : 7 : 1). As shown in Table 1, menaquinones of the established *Leucobacter* species are dominated by MK-11, whereas MK-10 and MK-11 were the predominant menaquinones in the investigated strain. Thus, strain K 70/01^T can be differentiated from other species of the genus by this chemotaxonomic feature. The cellular fatty acid composition of cells grown on tryptic soy agar (TSA; Merck) for 24 h at 28 °C was determined with the Sherlock

Table 1. Differential phenotypic characteristics of *Leucobacter* species

Species/subspecies: 1, *L. tardus* sp. nov. (strain K 70/01^T; data from this study); 2, *L. albus*; 3, *L. alluvii*; 4, *L. aridicollis*; 5, *L. chromiireducens* subsp. *chromiireducens*; 6, *L. chromiireducens* subsp. *solipictus*; 7, *L. iarius*; 8, *L. komagatae*; 9, *L. luti*. Except where indicated, data for reference taxa were taken from Takeuchi *et al.* (1996), Lin *et al.* (2004), Morais *et al.* (2004, 2006), Muir & Tan (2007) and Somvanshi *et al.* (2007). +, Positive; –, negative; w, weak reaction; +/–, different reactions obtained; ND, no data available.

Characteristic	1	2	3	4	5	6	7	8	9
Cell-wall amino acids (molar ratios)									
DAB	0.5	0.8	0.5	1.0	1.0	0.5	0.5	0.8	1.0
Alanine	3.2	1.8	2.4	4.0	2.1	1.8	1.5	1.9	1.0
Glycine	2.1	1.1	1.2	2.3	1.4	1.0	0.9	0.9	0.6
Glutamic acid	1.0	1.0	1.0	2.0	1.1	1.0	1.0	1.0	2.0
GABA	–	0.7	–	–	–	–	–	0.7	–
Threonine	–	–	0.7	–	0.7	0.6	0.7	–	0.6
Menaquinones (MK)									
Major components	10, 11	11	11	11	11	11	11	11	11
Minor components	9	12, 9	10	10	10	10	10, 9, 12*	10, 12	10
Utilization of (in Biolog GP2 MicroPlates):†									
<i>N</i> -Acetyl-D-glucosamine	–	–	–	–	–	–	+	–	–
<i>N</i> -Acetyl-L-glutamic acid	+	+	–	–	–	–	–	–	w
Adenosine	+	w	+	+	+	–	+	–	–
AMP	–	–	–	+	–	–	w	–	–
L-Alaninamide	+	+	+	+	+	+	+	+	–
L-Alanine	–	w	+	+	+	+	w	–	+
L-Alanyl glycine	–	+	+	+	+/–	+	–	–	–
L-Arabinose	+	–	–	–	–	–	–	–	–
L-Asparagine	–	+	–	+	–	–	–	–	–
2,3-Butanediol	+	–	–	–	–	–	–	–	–
2'-Deoxyadenosine	+	+	–	+	+	–	–	+	w
Dextrin	–	–	+	–	–	–	–	–	–
D-Fructose	w	–	+	–	–	–	+	–	–
D-Fructose 6-phosphate	–	+	+	+	+/–	–	+	+	+
D-Gluconic acid	+	–	–	–	–	–	–	–	–
α-D-Glucose	+	–	+	–	–	–	–	–	–
D-Glucose 6-phosphate	+	–	–	–	–	–	–	–	–
L-Glutamic acid	–	+	–	+	+	+	w	w	w
Glycerol	+	+	+	+	–	+	+	+	–
DL-α-Glycerol phosphate	+	–	–	–	–	–	–	–	–
Glycyl L-glutamic acid	–	+	–	+	+	+	+	w	w
α-Hydroxybutyric acid	+	–	–	–	–	–	–	–	–
β-Hydroxybutyric acid	–	–	–	+	–	–	–	–	–
<i>p</i> -Hydroxyphenylacetic acid	+	–	+	+	+	+	+	w	+
α-Ketovaleric acid	w	+	–	w	–	–	–	–	–
L-Lactic acid	+	–	+	–	–	–	–	–	–
Methyl α-D-galactoside	+	–	–	–	–	–	–	–	–
Methyl β-D-galactoside	+	–	–	–	–	ND	–	–	–
3-Methyl glucose	+	–	–	–	–	–	–	–	–
Methyl pyruvate	+	+	+	–	+	w	–	–	+
Putrescine	+	–	–	–	+/–	+	–	–	–
Pyruvic acid	+	–	–	–	–	–	–	–	–
D-Ribose	+	+	–	+	–	–	+	+	–
L-Serine	+	+	–	+	+/–	+	w	–	–
D-Sorbitol	+	–	–	–	–	–	–	–	–
Succinamic acid	+	–	–	–	–	w	–	–	–
Succinic acid	+	–	–	–	–	–	–	–	–

Table 1. cont.

Characteristic	1	2	3	4	5	6	7	8	9
Thymidine	+	+	+	+	+	–	+	–	–
TMP	–	+	–	+	–	–	+	–	–
Trehalose	–	+	–	–	–	–	–	–	–
Xylitol	–	–	+	–	–	–	–	–	–
D-Xylose	+	–	–	–	–	–	–	–	–
Uridine	+	+	–	+	+/-	–	+	–	–
UMP	–	w	–	+	–	–	+	–	–

*Trace amounts.

†Apart from strain K 70/01^T, data are from Muir & Tan (2007) and Somvanshi *et al.* (2007).

Microbial Identification system (MIDI version 4.5) as described by Behrendt *et al.* (1999). The fatty acid profile of strain K 70/01^T consisted of the branched fatty acids anteiso-C_{15:0} (53.0 %), anteiso-C_{17:0} (25.8 %), iso-C_{16:0} (14.3 %), iso-C_{15:0} (1.5 %) and iso-C_{14:0} (1.1 %) and the straight-chain fatty acid C_{16:0} (3.0 %). Furthermore, traces (<1.0 % of total fatty acids) of the branched fatty acids iso-C_{17:0} and anteiso-C_{13:0}, the unsaturated fatty acid anteiso-C_{15:1} and the straight-chain fatty acids C_{14:0} and C_{15:0} were found. This fatty acid profile is similar to those of other *Leucobacter* species. The presence of large amounts of anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0} is typical of members of the genus (Muir & Tan, 2007; Somvanshi *et al.*, 2007).

Morphological and physiological characterization of the phyllosphere isolate was performed as described by Behrendt *et al.* (1999). Unless stated otherwise, the strain was cultivated on nutrient agar (Sifin) or in the respective broth at 21 °C. The optimal growth temperature was determined by turbidity measurements at 10, 15, 21, 25, 30 and 35 °C after 24 h incubation in liquid medium. Growth at 4 and 37 °C was tested on agar plates after 7 days of incubation. Aerobic acid production from carbohydrates was assessed using an API 50CH strip inoculated with a bacterial suspension (from a 24 h-old culture grown at 25 °C on TSA) in CHB/E medium (bioMérieux). Results were determined for up to 5 days incubation at 30 °C as indicated by Morais *et al.* (2004) and Somvanshi *et al.* (2007). Additional physiological and enzymic characteristics were determined using the API 20E test strip (bioMérieux) according to the manufacturer's instructions. Acid production from carbohydrates was tested under aerobic conditions. An extensive investigation of carbon substrate utilization was conducted using Biolog GP2 MicroPlates (MicroLog System) as recommended by the manufacturer. Results were scored visually after 24 and 48 h incubation at 30 °C. Detailed characteristics of strain K 70/01^T are given in the species description. The investigated strain reacted negatively towards most of the substrates provided on the API 50CH test strips, which was similar to the results for *Leucobacter* species obtained by Somvanshi *et al.* (2007). Only a few carbohydrates could be assimilated following prolonged incubation. Similar results

were obtained for reactions on the API 20E substrates. In contrast, several carbohydrates provided on the Biolog GP2 MicroPlates were utilized within 48 h. This result supported the observation by Somvanshi *et al.* (2007) that GP2 test plates are more suitable for distinguishing *Leucobacter* species than the other methods used. As shown in Table 1, the investigated strain differed from other *Leucobacter* species in its utilization of a number of carbohydrates. Thus, the substrate utilization pattern on GP2 MicroPlates was an effective feature for distinguishing strain K 70/01^T from other species of the genus *Leucobacter*.

As a result of the phylogenetic and phenotypic analyses, it was clearly demonstrated that strain K 70/01^T, isolated from the phyllosphere of potato plants, represents a novel species of the genus *Leucobacter*, for which the name *Leucobacter tardus* sp. nov. is proposed.

Description of *Leucobacter tardus* sp. nov.

Leucobacter tardus (tar'dus. L. masc. adj. *tardus* slow, pertaining to the slow reactions in several physiological tests).

Cells are asporogenous, non-motile, short rods (0.4–0.6 × 0.8–1.6 µm), which occur singly or in pairs, sometimes forming small irregular clusters. Gram-positive and strictly aerobic. The lemon-yellow colonies found on nutrient agar are smooth with regular margins. Optimal temperature for growth is 21–30 °C; no growth at 4 or 37 °C. Catalase activity is present, but urease, β-galactosidase, arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase activities are absent. Citrate utilization, H₂S production from sodium thiosulfate, tryptophan deaminase, indole production from L-tryptophan and reduction of nitrate are negative. Does not produce acetoin from sodium pyruvate. No growth on MacConkey agar. Weak oxidase reaction. Hydrolyses aesculin, but not starch, casein or gelatin. Oxidative acid production occurs on API 50CH and API 20E substrates for D-fructose, melezitose, raffinose and L-rhamnose, but not for N-acetylglucosamine, D-adonitol, amygdalin, D- or L-arabinose, D- or L-arabitol, arbutin, D-cellobiose, dulcitol, erythritol, D- or L-fucose, D-galactose, gentiobiose, D-glucose, glycerol, glycogen, inositol, inulin, D-lactose, D-

lyxose, maltose, D-mannitol, D-mannose, melibiose, methyl α -D-glucopyranoside, methyl α -D-mannopyranoside, methyl β -D-xylopyranoside, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate, D-ribose, sucrose, salicin, D-sorbitol, L-sorbose, starch, D-tagatose, trehalose, turanose, xylitol or D- or L-xylose. Utilizes *N*-acetyl-L-glutamic acid, adenosine, L-alaninamide, L-arabinose, 2,3-butanediol, 2'-deoxyadenosine, D-gluconic acid, α -D-glucose, D-glucose 6-phosphate, glycerol, DL- α -glycerol phosphate, α -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, L-lactic acid, methyl α -D-galactoside, methyl β -D-galactoside, 3-methyl glucose, methyl α -D-glucoside, methyl pyruvate, putrescine, pyruvic acid, D-ribose, L-serine, D-sorbitol, succinamic acid, succinic acid, thymidine, Tweens 40 and 80, uridine and D-xylose on Biolog GP2 MicroPlates. Weak utilization is observed for D-fructose, *myo*-inositol, α -ketovaleric acid, D-mannitol, palatinose, D-psicose and D-tagatose. Acetic acid, *N*-acetyl-D-glucosamine, *N*-acetyl- β -D-mannosamine, AMP, D- and L-alanine, L-alanyl glycine, amygdalin, D-arabitol, arbutin, L-asparagine, D-cellobiose, α - and β -cyclodextrin, dextrin, D-fructose 6-phosphate, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, α -D-glucose 1-phosphate, L-glutamic acid, glycogen, glycyl L-glutamic acid, β - and γ -hydroxybutyric acids, inosine, inulin, α -ketoglutaric acid, lactamide, D-lactic acid methyl ester, α -D-lactose, lactulose, D- and L-malic acid, maltose, maltotriose, mannan, D-mannose, melezitose, melibiose, methyl β -D-glucoside, methyl α -D-mannoside, monomethyl succinate, propionic acid, L-pyrogutamic acid, raffinose, L-rhamnose, salicin, sedoheptulosan, stachyose, sucrose, TMP, trehalose, turanose, UMP and xylitol are not assimilated. Cell-wall amino acids are DAB, alanine, glycine and glutamic acid. The main menaquinones are MK-10, MK-11 and MK-9. The fatty acid profile consists predominantly of anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}; C_{16:0}, iso-C_{15:0} and iso-C_{14:0} are present in minor amounts.

The type strain is K 70/01^T (=DSM 19811^T =LMG 24388^T), isolated from the phyllosphere of potato plants in Dahnsdorf (Germany).

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