Leucobacter iarius sp. nov., in the family Microbacteriaceae

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A novel Gram-positive bacterium, strain 40^T, was isolated in the course of identifying bacteria from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum*. Based on 16S rRNA gene analysis, strain 40^T was found to be related to the type strains of recognized species of the genus *Leucobacter*, family Microbacteriaceae. The 16S rRNA gene sequence similarity values of strain 40^T and *Leucobacter albus* IAM 14851^T, *Leucobacter luti* LMG 23118^T, *Leucobacter alluvii* LMG 23117^T, *Leucobacter komagatae* DSM 8803^T, *Leucobacter chromiireducens* CIP 108389^T and *Leucobacter aridicollis* CIP 108388^T, respectively, were 97.3, 97.5, 97.6, 97.6, 97.6 and 98.5 %. Chemotaxonomic analysis also supported the affiliation of strain 40^T to the genus *Leucobacter*: the major menaquinone was MK-11, the peptidoglycan cross-linkage was of the B-type, the cell wall diamino acid was L-diaminobutyric acid and the major fatty acids were anteiso-C_{15:0} (42 %), anteiso-C_{17:0} (34 %) and iso-C_{16:0} (16 %). Based upon the biochemical and genomic analyses, strain 40^T is sufficiently distinct from the type strains of recognized *Leucobacter* species to warrant the description of a novel species, for which the name *Leucobacter iarius* sp. nov. is proposed. The type strain is strain 40^T (= DSM 17402^T = CIP 108831^T).

Members of those genera of the family Microbacteriaceae that contain 2,4-diaminobutyric acid in their B-type cross-linked peptidoglycan (Schleifer & Kandler, 1972) differ from each other mainly in menaquinone type, cell wall sugars, G+C content of the DNA and physiochemical features (Morais *et al.*, 2004). The genus *Leucobacter* was proposed to accommodate Gram-positive rods with a distinct peptidoglycan with an unusual amino acid, γ -aminobutyric acid, a high percentage of menaquinone 11 (MK-11) and moderate 16S rRNA gene sequence similarities with other members of the family. Diagnostic sugars were absent from the cell wall of *Leucobacter komagatae*, the only species available at the time of the study (Takeuchi *et al.*, 1996). In 2004, three new members, *Leucobacter chromiireducens*, *Leucobacter aridicollis* (Morais *et al.*, 2004) and *Leucobacter albus* (Lin *et al.*, 2004), and in 2006 two new members, *Leucobacter alluvii* and *Leucobacter luti* (Morais *et al.*, 2006), were added to the genus on the basis of the aforementioned properties.

Members of the genus *Leucobacter* have been isolated from diverse environments, such as activated sludge from a treatment plant containing chromium-contaminated wastewater (*L. chromiireducens*, *L. aridicollis* and *L. luti*) (Morais

et al., 2004, 2006) and from soil in Thailand (*L. albus*) (Lin *et al.*, 2004), while *L. komagatae* was isolated as a contaminant from an ampoule containing '*Pseudomonas riboflavina*' (= *Devosia riboflavina*) (Takeuchi *et al.*, 1996) and *L. alluvii* from sediment of the Alviela river, Portugal. Here we report the isolation and characterization of a novel *Leucobacter* species from the bacterial flora associated with the entomopathogenic nematode *Steinernema thermophilum* (Ganguly & Singh, 2000), recovered from soil in India.

Strain 40^T was one of the isolates found in the course of identifying bacteria from infective juveniles of the entomopathogenic nematode *Steinernema thermophilum* by using a procedure established by Akhurst (1980) with the modifications described by Somvanshi *et al.* (2006). The type strains of recognized *Leucobacter* species were obtained from the DSMZ and CRBIP, Paris. Cultural characteristics of strain 40^T are indicated in the species description.

Genomic DNA was extracted using a DNeasy Tissue kit (Qiagen) following the manufacturer's instructions. The 16S rRNA gene was amplified as described by Rainey *et al.* (1996). The PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced directly by using a CEQ Dye Terminator Cycle sequencing kit. The products were separated on a CEQ 8000 Genetic Analysis

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain 40^T is AM040493.

system. The 16S rRNA gene sequences were aligned with corresponding sequences from the database of DSMZ using the ae2 editor (Maidak *et al.*, 1997). Evolutionary distances were calculated using the method of Jukes & Cantor (1969). A phylogenetic tree based on distance analysis, reconstructed using the neighbour-joining algorithm (Saitou & Nei, 1987), and bootstrap values were determined by analysing 1000 resamplings (Felsenstein, 1993).

The 16S rRNA gene sequence of strain 40^T (1508 nt) showed 97.3–98.5 % similarity to those of type strains of recognized species of the genus *Leucobacter*, the closest relative being *L. aridicollis* (Fig. 1). However, these values were lower than those determined for certain strain pairs, such as *L. albus* and *L. aridicollis* (98.9 %), *L. albus* and *L. komagatae* (98.8 %) and *L. komagatae* and *L. aridicollis* (98.7 %). As previous studies have confirmed DNA–DNA reassociation values of less than 70 % for the latter two strain pairs with high 16S rRNA gene similarities [40 % (Lin *et al.*, 2004) and 38 % (Morais *et al.*, 2004, 2006), respectively], we refrained from testing this parameter and consider strain 40^T to represent a distinct genospecies (see also Stackebrandt & Ebers, 2006).

Physiological and biochemical tests on strain 40^T and the type strains of other *Leucobacter* species were performed at 28 °C using API 50 CH strips (bioMérieux), according to the manufacturer's instructions, with bioMérieux medium E [2 g ammonium sulphate, 0.5 g yeast extract, 1 g tryptone (bovine/porcine origin), 3.22 g disodium phosphate, 0.12 g monosodium phosphate, 10 ml trace elements, 0.17 g phenol red, 1000 ml demineralized water]. All tests were

inoculated with 24 h-old cultures grown on trypticase soy agar. The reactions were scored up to 5 days, as indicated by Morais *et al.* (2004). Strain 40^T was also characterized by using an API 20NE strip. Biolog GP plates (Oxoid) were incubated for 24 h before being read. Wells showing a photometric value above 20 or 30 % of the highest value of an individual strain were scored as weak or positive, respectively. Conventional biochemical tests were performed according to standard methods (Smibert & Krieg, 1994). Cultural characteristics such as colony size, shape and colour were determined after 24 h incubation at 28 °C on CASO agar plates (Merck). The temperature range for growth was determined by incubating inoculated slant cultures (trypticase soy agar, pH 7.0) at 4, 25, 30, 37 and 45 °C. Growth was also assessed in trypticase soy broth (Difco) at pH 5–9. pH values of the test medium of less than 7.5 were obtained by adding HCl and pH values greater than 7.5 by addition of sterilized sodium sesquicarbonate buffer (1 M). The ability to grow in the presence of NaCl was determined using trypticase soy broth containing NaCl (5, 7 or 10 %). Growth characteristics are given in the species description.

On the basis of the results of the physiological tests, it was concluded that strains of the genus *Leucobacter* react negatively towards most of the substrates provided on the API 50 CH panel. There was no common substrate on the API 50 CH test panels that was acidified by all members of the genus (Table 1). According to Morais *et al.* (2004), many

Fig. 1. 16S rRNA gene-based phylogenetic tree (Felsenstein, 1993) showing the position of strain 40^T among *Leucobacter* species and some other members of the family Microbacteriaceae. Bar, 1 % sequence divergence. Bootstrap percentages greater than 70 %, based on 1000 resamplings, are shown.

Table 1. Results of substrate acidification as determined using an API 50 CH test panel with medium E, scored after 2 days

Strains: 1, strain 40^T (*Leucobacter iarius* sp. nov.); 2, *L. albus* DSM 17379^T; 3, *L. chromiireducens* DSM 17381^T; 4, *L. aridicollis* DSM 17380^T; 5, *L. komagatae* DSM 8803^T; 6, *L. luti* CIP 108818^T; 7, *L. alluvii* CIP 108819^T. *L. luti* was weakly positive for production of acid from rhamnose. All strains were negative for all other substrates provided on the API 50 CH test panels. +, Positive; –, negative; w, weakly positive.

Acidification reaction	1	2	3	4	5*	6	7
Glycerol	+	w	–	–	+	–	–
D-Arabinose	–	w	–	–	–	–	–
L-Arabinose	–	–	–	–	w	–	–
D-Adonitol	–	–	–	–	w	–	–
Ribose	+	+	–	–	+	+	–
Fructose	+	–	–	–	–	–	–
N-Acetylglucosamine	+	–	–	–	–	–	–
Trehalose	–	+	–	–	–	–	–
L-Fucose	–	+	–	–	–	–	–
5-Ketogluconate	w	–	–	–	–	–	–
Inositol	w	–	–	–	–	w	–
Salicin	w	–	–	–	–	–	–

*Data from Morais *et al.* (2004).

Table 2. Results of substrate utilization as determined by using a Biolog GP2 test panel, scored after 24 h

Strains: 1, strain 40^T (*Leucobacter iarius* sp. nov.); 2, *L. albus* DSM 17379^T; 3, *L. chromiireducens* DSM 17381^T; 4, *L. aridicollis* DSM 17380^T; 5, *L. komagatae* DSM 8803^T; 6, *L. luti* CIP 108818^T; 7, *L. alluvii* CIP 108819^T. All strains tested negative for all other substrates on the Biolog GP2 test plates. ++, Very strong reaction (>70% of highest reading); +, strong reaction (30–70%); w, 20–30%; – <20%.

Carbon source	1	2	3	4	5	6	7
Dextrin	–	–	–	–	–	–	+
Tween 40	++	++	++	+	+	++	++
Tween 80	+	++	+	+	+	++	+
Glucose	–	–	–	–	–	–	++
N-Acetylglucosamine	+	–	–	–	–	–	–
D-Fructose	++	–	–	–	–	–	+
D-Psicose	w	–	–	–	–	–	w
D-Ribose	+	++	–	+	+	–	–
Xylitol	–	–	–	–	–	–	+
D-Trehalose	–	+	–	–	–	–	–
α-Hydroxybutyric acid	–	–	–	–	–	–	–
β-Hydroxybutyric acid	–	–	–	+	–	–	–
p-Hydroxyphenylacetic acid	+	–	+	+	w	+	+
D-Lactic acid methyl ester	–	–	–	–	–	–	w
L-Lactic acid	–	–	–	–	–	–	+
α-Ketovaleric acid	–	+	–	w	–	–	–
Pyruvic acid methyl ester	–	+	+	–	–	+	+
Pyruvic acid	–	++	+	+	–	–	w
N-Acetyl-L-glutamic acid	–	+	–	–	–	w	–
L-Alaninamide	+	+	++	+	+	–	++
L-Alanine	w	w	+	+	–	+	+
L-Alanyl glycine	–	+	–	+	–	–	+
L-Asparagine	–	+	–	+	–	–	–
L-Glutamic acid	w	+	+	+	w	w	–
Glycyl-L-glutamic acid	+	+	+	+	w	w	–
L-Serine	w	+	–	+	–	–	–
Glycerol	++	+	–	+	+	–	++
Adenosine	+	w	w	++	–	–	+
2'-Deoxyadenosine	–	+	w	++	+	w	–
Inosine	–	w	–	+	–	–	–
Thymidine	++	++	w	++	–	–	+
Uridine	+	+	+	+	–	–	–
Adenosine	w	–	–	++	–	–	–
5'-monophosphate	+	+	–	++	–	–	–
Thymidine	+	+	–	++	–	–	–
5'-monophosphate	+	w	–	+	–	–	–
Uridine	+	w	–	+	–	–	–
5'-monophosphate	+	w	–	+	–	–	–
Putrescine	++	++	++	++	++	++	++

tests were scored 'weak' for *L. chromiireducens* and *L. aridicollis* in the original description whereas, according to our results, they were negative for all the respective substrates even when read after 5 days. The Biolog GP test plates were more suitable for distinguishing *Leucobacter* strains (Table 2). These data reveal a clear preference of the strains for utilization of amino acids over carbohydrates. Strain 40^T differed from the other strains in utilization of N-acetylglucosamine and the weak acidification of 5-ketoglucuronate and salicin within the API 50 CH substrate panels. In addition, a change of the indicator dye was observed for fructose (Table 1). The reactions of some strains towards certain substrates differed between the Biolog and API substrate panels (Tables 1 and 2).

Cultures for use for determination of the fatty acid composition were grown on trypticase soy broth agar for 24 h at 28 °C. Processing and analysis of fatty acids as methyl esters were done using the protocol of the MIDI Sherlock Microbial Identification System (MIDI, 1999).

Cellular fatty acid analysis was carried out for all the type strains except *L. komagatae* IAM 1093^T, *L. luti* CIP 108818^T and *L. alluvii* CIP 108819^T, for which data were taken from Takeuchi *et al.* (1996) and Morais *et al.* (2006) (Table 3). The major fatty acids of strain 40^T were anteiso-C_{15:0} (41.9%), anteiso-C_{17:0} (33.6%) and iso-C_{16:0} (15.5%). These results are in accord with the fatty acid compositions of other members of the genus, confirming the affiliation of strain 40^T with the genus.

Purified cell wall preparations were obtained by using the method of Schleifer & Kandler (1972). Amino acids and peptides in cell wall hydrolysates were analysed by two-dimensional ascending thin layer chromatography on cellulose plates using previously described systems (Schleifer & Kandler, 1972). The N-terminal amino acid of the interpeptide bridge was determined by dinitrophenylation as

Table 3. Major fatty acid compositions (%) of *Leucobacter* species

Strains: 1, strain 40^T (*Leucobacter iarius* sp. nov.); 2, *L. albus* DSM 17379^T; 3, *L. chromiireducens* DSM 17381^T; 4, *L. aridicollis* DSM 17380^T; 5, *L. komagatae* IAM 1093^T; 6, *L. luti* CIP 108818^T; 7, *L. alluvii* CIP 108819^T. The fatty acid content differs slightly from that reported by Morais *et al.* (2004). ND, No data available.

Fatty acid	1	2	3	4	5*	6†	7†
anteiso-C _{15:0}	41.86	54.20	55.28	50.86	65	61	51
anteiso-C _{17:0}	33.58	19.08	23.73	26.27	22	14	25
iso-C _{16:0}	15.50	17.04	11.53	11.37	13	12	9
iso-C _{15:0}	2.83	3.49	0.91	3.43	–	ND	ND
C _{16:0}	4.16	4.06	5.80	6.15	–	7	12

*From Takeuchi *et al.* (1996).

†From Morais *et al.* (2006).

described by Schleifer (1985). Strain 40^T possessed glutamic acid (1.0), L-diaminobutyric acid (0.5), alanine (1.5), glycine (0.9) and threonine (0.7), a composition that confirmed the presence of a B-type cross-linked peptidoglycan. As revealed by denitrophenylation, alanine was the N-terminal amino acid. The dipeptide Gly–Glu was detected after partial hydrolysis. 4-Aminobutyric acid, as detected in some other *Leucobacter* strains (*L. komagatae*, *L. albus*), was not observed. The N-terminal alanine, the presence of threonine and the Gly–Glu dipeptide corresponds to the information reported for *L. chromiireducens*, *L. luti* and *L. alluvii* (Morais *et al.*, 2006). Lipiquinone and polar lipid analyses were done by using previously described methods (Groth *et al.*, 1996). The dominant menaquinone was MK-11, consistent with the results obtained with the type strains of other *Leucobacter* species. MK-10 and MK-9 were present in minor amounts, while MK-12 occurred in trace amounts only. The polar lipids were phosphatidylglycerol and diphosphoglycerol. Several unidentified components occurred, i.e. a phospholipid, a glycolipid and an aminophospholipid.

In conclusion, based upon the phylogenetic and chemotaxonomic evidence, strain 40^T represents a novel member of the genus *Leucobacter*, family *Microbacteriaceae*, for which the name *Leucobacter iarius* sp. nov. is proposed.

Description of *Leucobacter iarius* sp. nov.

Leucobacter iarius [i.a'ri.us. N.L. masc. adj. *iarius* (arbitrary name) formed from the acronym IARI (Indian Agricultural Research Institute, New Delhi, India) to commemorate its centenary year].

Cells are Gram-positive, straight, non-motile rods (1.66–2.91 × 0.37–0.49 µm). Neither mycelium nor spores are detected. Colonies are round, 0.4–1 mm in diameter with smooth edges, low-convex, opaque, whitish in colour, finely granular and odourless. Optimum temperature for growth is 30 °C; growth does not occur at temperatures higher than 37 °C or at 4 °C. Growth occurs at pH 5.0–9.0. Growth is reduced in the presence of 5–7 % NaCl; no growth occurs with 10 % NaCl. Urease-negative, nitrate is not reduced, D-glucose is not fermented, and aesculin and PNPG (β-galactosidase) are positive on API 20NE strips. Gelatinase-negative. Utilizes *N*-acetylglucosamine and weakly utilizes 5-ketogluconate, inositol and salicin. Additional biochemical and physiological characteristics are given in Tables 1 and 2. MK-11 is the major menaquinone; MK-10 and MK-9 are present in minor amounts while MK-12 occurs in trace amounts only. Polar lipids are phosphatidylglycerol and diphosphoglycerol. Several unidentified components occur, i.e. a phospholipid, a glycolipid and an aminophospholipid. Cross-linkage of peptidoglycan is of the B-type; cell wall amino acids are L-diaminobutyric acid, alanine, glycine, threonine and glutamic acid. 4-Aminobutyric acid is absent. Major fatty acids are anteiso-C_{15:0}, anteiso-C_{17:0} and iso-C_{16:0}; iso-C_{15:0} and C_{16:0} are present in minor amounts.

The type strain is strain 40^T (= DSM 17402^T = CIP 108831^T), which was isolated from crushed infective juveniles of *Steinernema thermophilum* collected from soil of the Indian Agricultural Research Institute, New Delhi, India.

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